Engineering a pH-Responsive Protein Nanocage for Controlled Cargo Release

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1. Introduction

Protein nanocages are self-assembling hollow structures formed from multiple copies of protein subunits, creating shells of defined size and geometry that can encapsulate molecular cargo. Encapsulins are a class of protein nano compartments found in bacteria that naturally package specific cargo proteins. Cargo proteins can also be engineered for encapsulation through fusing to short targeting peptides, making encapsulins attractive platforms for applications including enzyme encapsulation, drug delivery, and bio nanotechnology [1, 2].

The T=4 *Quasibacillus thermotolerans* encapsulin (QtEnc) is a large bacterial encapsulin, formed from 240 identical subunits into a nanocage of approximately 42 nm in diameter [3]. Its substantial internal volume makes QtEnc particularly attractive as a cargo-delivery platform [3, 4]. However, the high stability that enables effective cargo protection can also limit controlled cargo release.

Controlled cargo release is essential for therapeutic applications. Drug delivery systems such as liposomes can protect therapeutic cargo [6], but achieving site-specific and controlled release remains a challenge, leading to the development of stimuli-responsive delivery systems. This is particularly relevant in cancer therapy, where non-specific drug exposure can damage healthy tissues. The tumour microenvironment is often mildly acidic compared with healthy tissues, providing a potential trigger for pH-responsive release systems [7].

To introduce pH-responsive disassembly into the system, the GALA peptide was introduced. GALA is a short synthetic peptide that adopts an unstructured coil at neutral pH, but folds into an α-helix under acidic conditions [3,5].By inserting GALA into a flexible surface loop of the encapsulin subunit, it was hypothesised that acid-induced GALA folding would disrupt inter-subunit interactions and promote cage disassembly [3]. This engineered variant, T4GALA, was previously shown to disassemble and reassemble reversibly in response to pH changes.

Building on this, this project aimed to express and purify wild type EncStrep and T4GALA EncStrep in *Escherichia coli*, characterise the pH-dependent assembly behaviour of T4GALA EncStrep across a range of pH values using native polyacrylamide gel electrophoresis (native PAGE), and investigate GFP cargo loading and pH-triggered release using a T4GALA EncStrep construct co-expressing GFP fused to a cargo loading peptide (CLP).

2. Methodology

2.1 Bacterial Strains and Plasmids

Three encapsulin constructs were used in this project: wild type EncStrep encapsulin carrying a C-terminal Strep-tag II (WT EncStrep), an engineered variant containing a 16-residue GALA peptide insertion in between triple glycine linkers between residues Glu61 and Ala62 of the E-loop region (T4GALA EncStrep) [3], and T4GALA EncStrep co-expressing GFP fused to a cargo-loading peptide (GFP-CLP). WT EncStrep and T4GALA EncStrep were provided by the Frank laboratory (Department of Biochemical Engineering). The T4GALA EncStrep + GFP-CLP construct was generated during this project as described in Section 2.8.

2.2 Protein Expression

Plasmids encoding the respective encapsulin constructs were transformed into *E. coli* BL21 Star (DE3) cells and plated on LB agar supplemented with chloramphenicol. Single colonies were selected to prepare starter culture, which were subsequently used to inoculate 200 mL of autoinduction medium supplemented with chloramphenicol at an initial OD_{600} of 0.05. Cultures were incubated for 24 hr at 25°C with shaking at 200 rpm. OD_{600} was measured after expression to assess culture growth before harvesting.

2.3 Cell Lysis and Clarification

Cells were harvested by centrifugation at 5,000 × g for 15 minutes. The supernatant was discarded and the cell pellet was resuspended in a 50 mM Tris-HCl buffer. Cells were lysed by sonication, after which Benzonase nuclease (0.3 µL) was added, and the lysate was incubated at 37°C at 1 hr to degrade nucleic acids and reduce sample viscosity.

The lysate was then centrifuged to remove the cell debris at 18,000 × g for 45 minutes at 4°C. The clarified lysate was filtered using a filter unit before being used for Bradford analysis and subsequent protein purification.

2.4 Bradford Assay

Total protein concentration was determined using the Bradford assay with Bovine serum albumin standards. Standards of concentration 0-1000 µg/mL were prepared and were used as a reference to determine the protein concentration. Protein samples were diluted at a 20-fold before analysis, absorbance was measured at 595 nm, and protein concentrations were interpolated from BSA calibration curve.

2.5 Protein Purification – StrepTactin Affinity Purification

Strep-tag II tagged proteins were purified using StrepTactin XT gravity flow columns (IBA Lifesciences). The column was equilibrated with two column volumes of Buffer W (100 mM Tris/HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA). The clarified lysate was applied to the column and allowed to flow through by gravity. To remove the non-specifically bound proteins, the column was washed with five column volumes of Buffer W, with each fraction being collected separately. The target protein was eluted with three fractions of Buffer BXT (100 mM Tris/HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA; 50 mM biotin). All fractions were retained for Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.6 Size Exclusion Chromatography (SEC)

Following affinity purification, encapsulin-containing fractions were further purified by SEC to separate assembled nanocages from smaller molecular species. Elution fractions from the previous step were concentrated and loaded onto the SEC column equilibrated in Tris buffer. Protein elution was monitored by absorbance at 280 nm (UV_{280}). The elution fractions were collected using a fraction collector and the chromatogram was exported for analysis. Fractions corresponding to the peak fractions were retained for downstream analysis.

2.7 SDS-PAGE

SDS-PAGE was performed to assess protein expression and purity and to identify bands corresponding to the expected molecular masses of the target proteins. Samples were mixed 1:1 with 2x Laemmli loading buffer and heating at 95°C for 7 minutes. Samples loaded included non-induced (NA) and induced (IND), whole cell lysate (LY), flow-through (FT), wash fractions W1 to W5, elution fractions E1, E2, E3, SEC fraction (SEC), and a positive control (PC). Following electrophoresis, gels were stained with Coomassie Blue and imaged using a gel documentation system.

2.8 Construction of the T4GALA EncStrep + GFP-CLP System

A plasmid encoding WT EncStrep and GFP fused to a cargo-loading peptide (GFP-CLP) was used as the starting construct. The GALA sequence was introduced into the encapsulin E-loop by site-directed mutagenesis. Following PCR amplification, the product was treated with KLD enzyme mix for 5 min at room temperature to circularise the amplified plasmid and digest parental template DNA. The product was transformed into competent DH5α *E. coli* and plated on chloramphenicol selective LB agar. Seven colonies were screened by colony PCR, with PCR products analysed by 1% agarose gel electrophoresis.

Three positive colonies (2, 3, and 7) were selected for overnight culture and plasmid extraction by miniprep. DNA concentration and purity was assessed by NanoDrop spectrophotometry (Table 1.1), and all three plasmids were submitted for Sanger sequencing to verify GALA insertion. Following sequence analysis, colony 7 was selected for subsequent expression in BL21 Star (DE3).

2.9 Buffer Preparation

Buffers spanning pH 3-9 were prepared to investigate pH-dependent encapsulin assembly.

Citrate buffers (20 mM, 150 mM NaCl) were prepared at pH 3,4 and 5 by combining citric acid monohydrate and adjusting to the target pH. MES buffer (20 mM, 150 mM NaCl) was prepared at pH 6 by dissolving MES in water and adjusting pH with NaOH. Tris buffers (20 mM, 150 mM NaCl) were prepared at pH 7, 8, and 9 by diluting 1M Tris buffer at pH 8 and then adjusting pH with HCl/NaOH. All buffers were prepared to final volume of 50 mL, pH-adjusted to the target value using a calibrated pH meter.

2.10 Native PAGE pH screen

To minimise carry-over of the purification buffer, purified WT EncStrep and T4GALA EncStrep were precipitated using 40% PEG and NaCl, pelleted by centrifugation and resuspended in the respective pH 3-9 buffers. Samples were incubated overnight at 4°C before analysis by native PAGE under non-denaturing conditions. WT EncStrep was analysed in parallel as a control.

For the GFP cargo experiment, WT EncStrep + GFP-CLP was analysed alongside T4GALA EncStrep + GFP-CLP. Following native PAGE, gels were imaged using a gel documentation system with laser excitation to detect GFP fluorescence before staining with Coomassie Blue for protein detection.

3. Results and Discussion

3.1 Protein Expression and Quantification

Both WT EncStrep and T4GALA EncStrep were successfully expressed in *E. coli* BL21 Star (DE3) under autoinduction conditions. The clarified lysates had total protein concentrations of 6.4 mg/ml for the WT EncStrep sample and 8.1 mg/ml for the T4GALA EncStrep sample, as determined by Bradford assay. These concentrations were used to calculate the sample volume onto the column for affinity purification.

3.2 Purification and SEC

StrepTactin XT affinity purification was performed on all three clarified lysates: WT EncStrep, T4GALA EncStrep, and T4GALA EncStrep + GFP-CLP. Elution fractions were collected and analysed by SEC to confirm the assembly state of the purified protein.

SEC analysis of the WT EncStrep produced a dominant early-eluting peak (Figure 1), consistent with the presence of a high-molecular-weight assembled nanocage species.

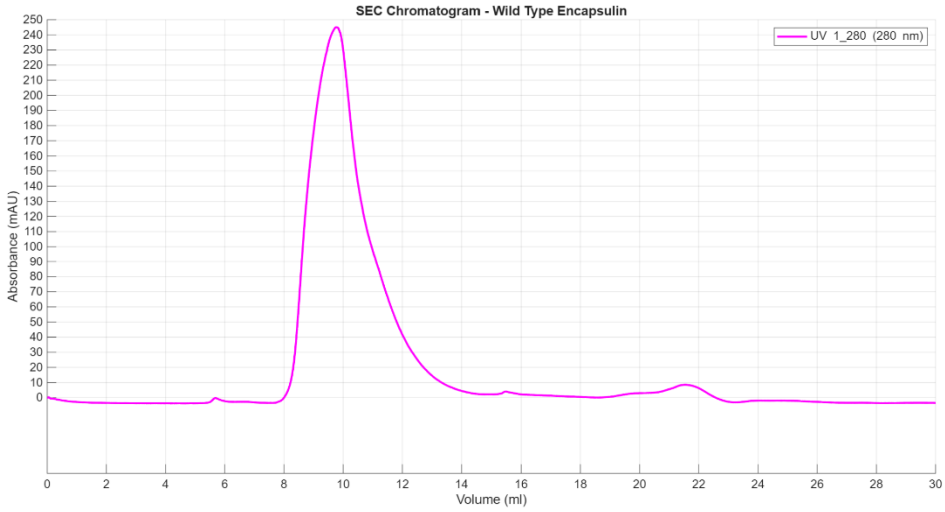

Figure 1 ***SEC chromatogram of WT EncStrep****. A dominant early-eluting peak was observed at approximately 9.8 mL, consistent with a high-molecular-weight assembled encapsulin population. Protein elution was monitored by absorbance at 280 nm.*

SEC analysis of the T4GALA EncStrep revealed two peaks, Peak A at 9.40 ml and Peak B at 15.76 ml (Figure 2). Peak A is consistent with assembled T4GALA EncStrep nanocages. The presence of Peak B suggests a small partially assembled nanocage co-eluting with the assembled cage fraction. This two-peak profile is consistent with the sensitivity of T4GALA, SEC was performed in Tris buffer, which has been shown to promote T4GALA disassembly at physiological pH [3]. It is therefore likely that a proportion of the T4GALA EncStrep cages partially disassembled during the SEC run under these buffer conditions, which corresponds to the smaller species observed at Peak B. This observation highlights the importance of the buffer identity contributing to T4GALA assembly, as previously reported. However, its identity cannot be established from SEC alone.

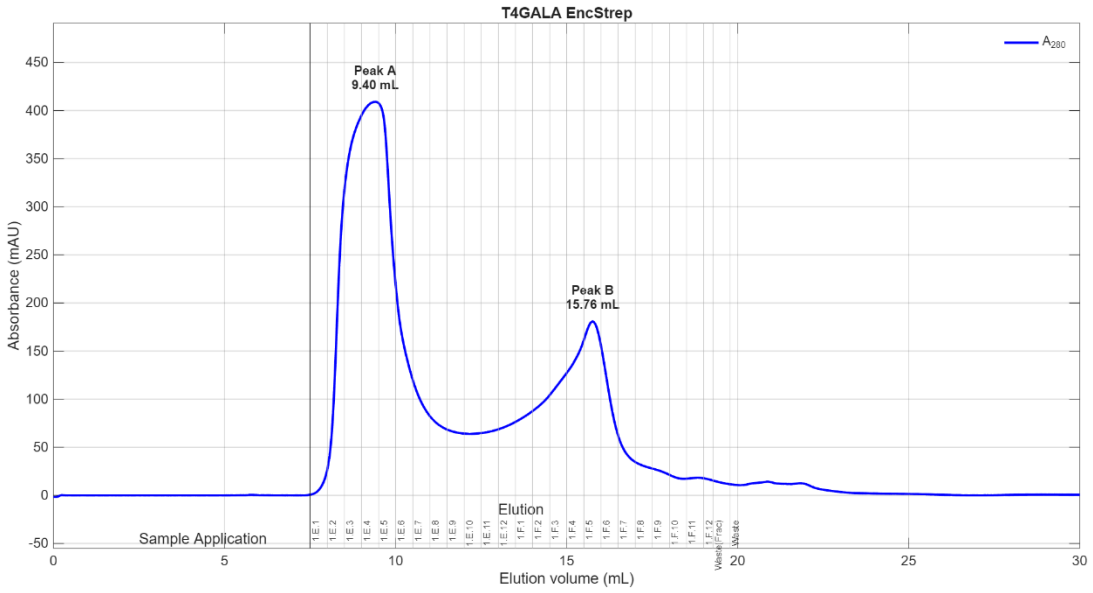

Figure 2 ***SEC chromatogram of T4GALA*** *EncStrep. Absorbance at 280 nm shows an early peak at 9.40 mL and a second peak at 15.76 mL, indicating high- and lower-molecular-weight species, respectively.*

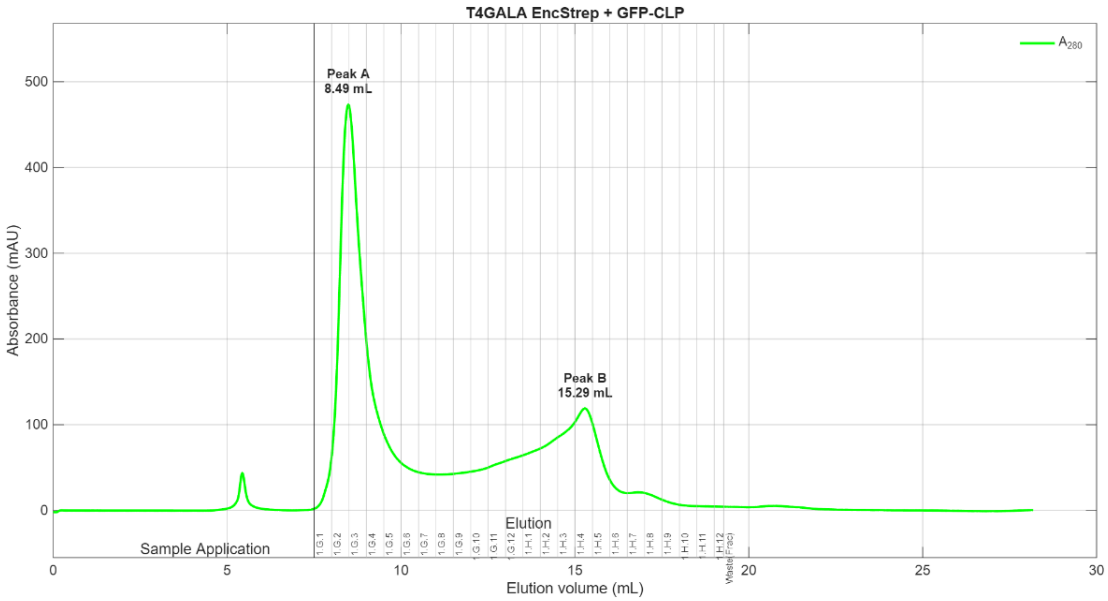

Figure 3 ***SEC chromatogram of T4GALA EncStrep +GFP****. Two major A280 peaks were observed at 8.49 mL and 15.29 mL, corresponding to high- and lower-molecular-weight populations, respectively.*

SEC analysis of T4GALA EncStrep + GFP similarly revealed two peaks, Peak A at 8.49 mL and Peak B at 15.29 mL (Figure 3), indicating the presence of both high and lower molecular weight species. Further analysis would be required to establish the composition of the later peak.

Bradford Assay after SEC purification confirmed protein concentrations of 0.5 mg/mL for WT EncStrep and 0.43 mg/mL for T4GALA EncStrep following purification, indicating successful recovery of protein across the purification steps.

3.3 SDS-PAGE Analysis

SDS-PAGE was performed on all purification fractions to assess protein purity and confirm protein identity across three constructs.

WT EncStrep showed a dominant band at approximately 36 kDa in the elution and SEC fractions (Figure 4), consistent with the expected encapsulin subunit size [1]. The purified fractions showed a much cleaner banding pattern compared with the cell lysate, indicating successful purification of WT EncStrep.

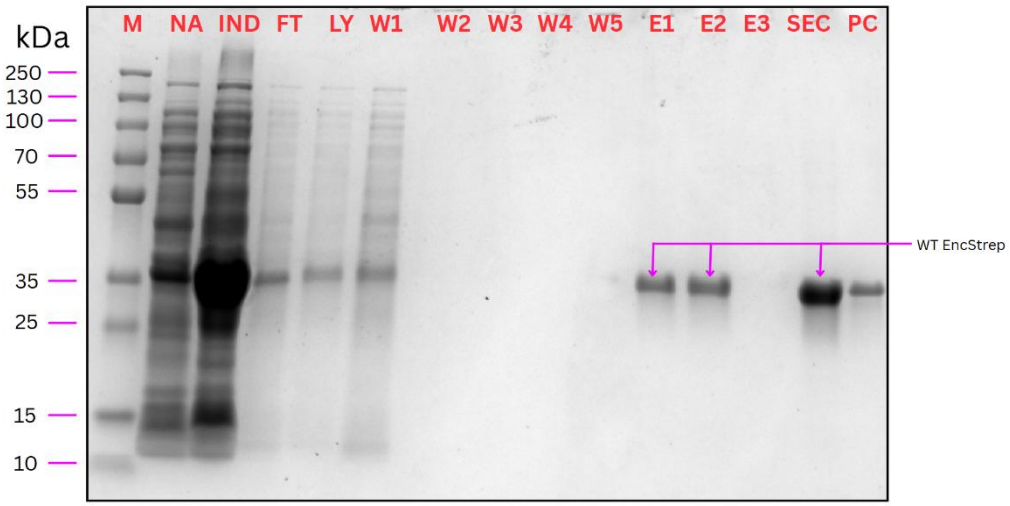

Figure 4 ***SDS-PAGE analysis of WT EncStrep expression and purification****. A dominant band at approximately 36 kDa was observed in the elution and SEC fractions, consistent with the expected WT encapsulin subunit size. M, molecular-weight marker; NA, non-induced; IND, induced; FT, flow-through; LY, lysate; W1–W5, wash fractions; E1–E3, elution fractions; SEC, SEC fraction; PC, positive control.*

For T4GALA EncStrep, initial purification attempts resulted in two prominent bands in the elution fractions, a dominant band at approximately 36-39 kDa and a secondary band at approximately 25 kDa (Figure 5A). Prolonged storage of the clarified lysate at 4°C prior to affinity purification was identified as a contributing factor, as extended exposure to residual proteases in the lysate is likely to have increased degradation of the T4GALA subunit during this period. A fresh preparation was therefore performed immediately following lysis to minimise this effect. The resulting SDS-PAGE showed a dominant band at approximately 36-39 kDa consistent with the full length of T4GALA subunit, with only a faint lower band remaining (Figure 5B). This improvement suggests that rapid processing reduces but does not eliminate the lower band.

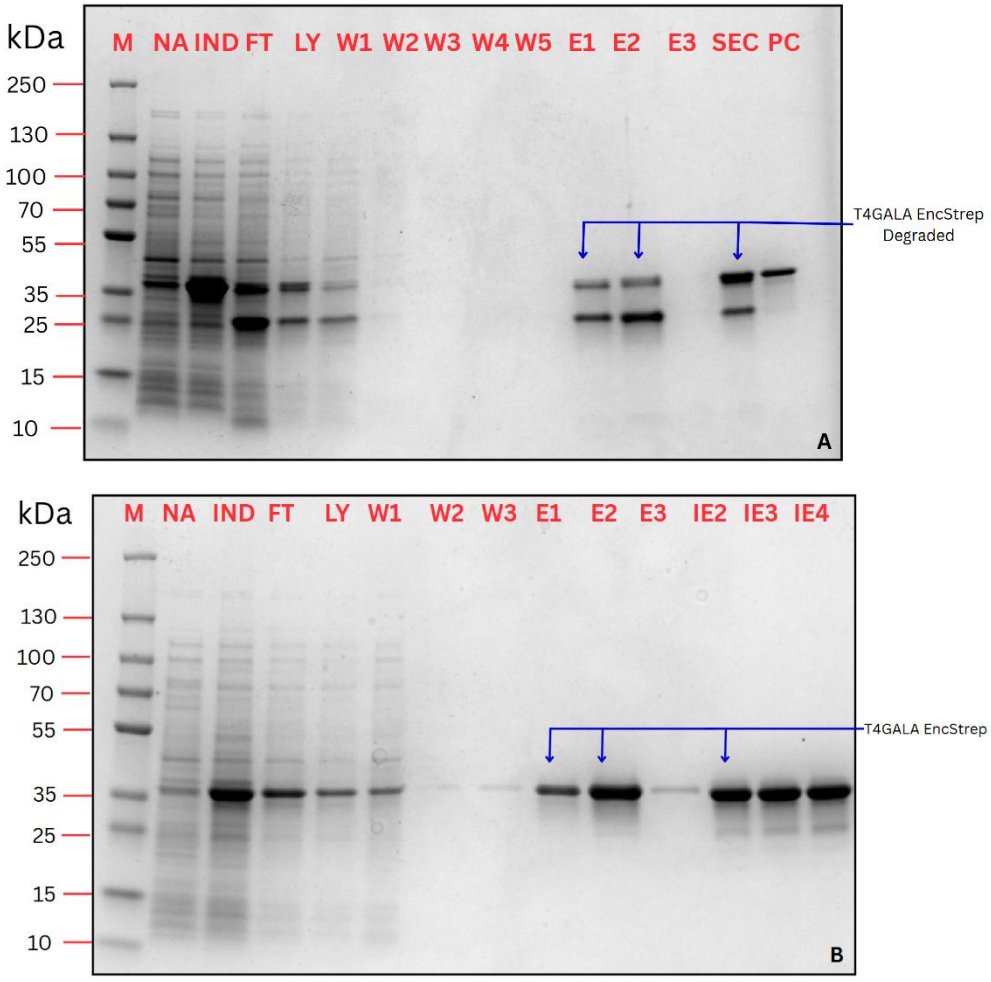

Figure 5 ***SDS-PAGE analysis of T4GALA EncStrep purification****. (A) Initial preparation showing bands at approximately 36–39 kDa and ~25 kDa. (B) Fresh preparation processed immediately after lysis, showing a dominant ~36–39 kDa band and reduced lower-molecular-weight species. Lane abbreviations are as defined in Figure 4, including IE2, IE4, and IE5 are SEC fractions From Figure 2.*

The lower-molecular-weight species was observed in T4GALA lysate but was not clearly detectable in the induced whole-cell sample, suggesting that degradation occurred predominantly after cell lysis rather than during protein expression. Although fresh preparation reduced the intensity of this lower band, it did not eliminate it completely. One possible explanation is that the GALA modification increases the susceptibility of T4GALA EncStrep to proteolysis following cell lysis. This interpretation is supported by the absence of comparable degradation in WT EncStrep processed and stored under similar conditions, suggesting that the GALA-modified encapsulin may be more susceptible to degradation. The lower-molecular-weight species continued to be observed following affinity purification, although its identity was not determined in this study. Further characterisation, such as mass spectrometry, would be required to determine its identity and origin.

For T4GALA EncStrep + GFP-CLP, SDS-PAGE of the elution fractions showed bands at approximately 36-40 kDa and 26-27 kDa, consistent with T4GALA EncStrep and GFP-CLP, respectively (Figure 6). GFP-CLP does not carry a Strep-tag but it co-purified with the Strep-tagged encapsulin during StrepTactin affinity purification. This supports successful association of GFP-CLP with the encapsulin and is consistent with cargo loading through the CLP, which directs cargo proteins into assembling encapsulin cages [2].

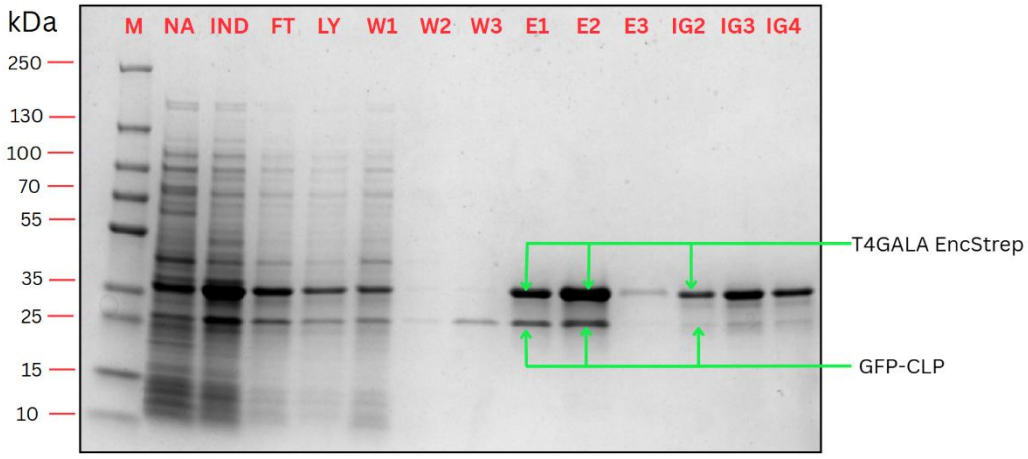

Figure 6 ***SDS-PAGE analysis of T4GALA EncStrep+GFP purification****. Bands at approximately 36–40 kDa and 26–27 kDa are consistent with the T4GALA encapsulin subunit and GFP-CLP, respectively. Lane abbreviations are as defined in Figure 4, with IG2, IG3, IG4 being SEC fractions from Figure 3.*

3.4 Native PAGE pH Screen

Native PAGE was used to compare WT EncStrep and T4GALA EncStrep assembly across pH 3-9 (Figure 7). WT EncStrep was included as a control in all conditions to allow direct comparison of pH sensitivity between two constructs.

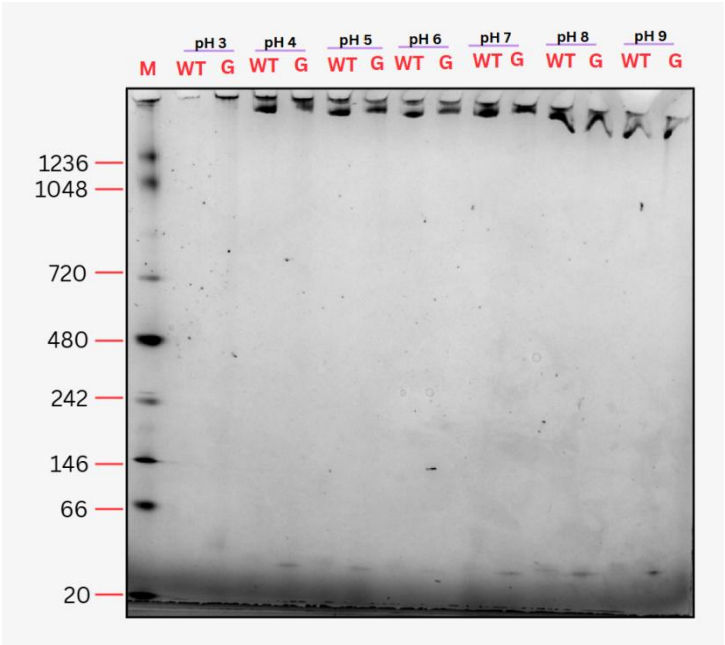

Figure 7 ***Native PAGE analysis of WT EncStrep and T4GALA EncStrep across pH 3–9****. WT and T4GALA (G) were incubated in citrate (pH 3–5), MES (pH 6) or Tris (pH 7–9) buffers before native PAGE.*

The results demonstrated clear pH-dependent assembly behaviour for T4GALA EncStrep (Figure 7). At pH 3, no band was detected for either WT or T4GALA, indicating loss of assembled cages. The extreme acidity of pH 3 likely causes generalised protein denaturation rather than specific GALA-mediated disassembly.

At pH 4, 5 and 6, cage associated bands remained visible but showed an apparent trend towards reduced intensity compared with the neutral and basic conditions. As band intensity was not quantified, this observation was interpreted qualitatively.

At pH 7, 8, and 9, strong bands were observed for both constructs, indicating stable assembly under near neutral and basic conditions. Interestingly, the T4GALA band at pH7 in Tris buffer appeared slightly weaker than the equivalent WT band. This is consistent with the previously reported sensitivity of T4GALA to Tris buffer at physiological pH, where charged primary amine group of Tris is hypothesized to interact with glutamate residues at the GALA insertion site, promoting helix formation and partial disassembly even at neutral pH [3]. However, this mechanism was not directly investigated in the present study.

The reduced assembly observed at pH 6 suggests that T4GALA may respond as conditions become acidic, however, the tumour relevant range around pH 6.5-6.8 was not directly tested. Future experiments should therefore examine narrower pH intervals around physiological, and tumour associated conditions using a consistent buffer system.

3.5 GFP Cargo Loading and Fluorescence

Fluorescence signal was observed at both the top and bottom of the gel across multiple pH conditions. Signal at the top of the gel is consistent with GFP retained within intact assembled cages. However, the interpretation of the bottom signal is less straightforward. Fluorescence at the bottom of the gel was observed across both WT EncStrep + GFP-CLP and T4GALA EncStrep + GFP-CLP lanes, suggesting that this signal does not arise from GFP released upon T4GALA disassembly. As purified samples were used for native PAGE freely soluble GFP present after expression would be expected to have been largely removed.

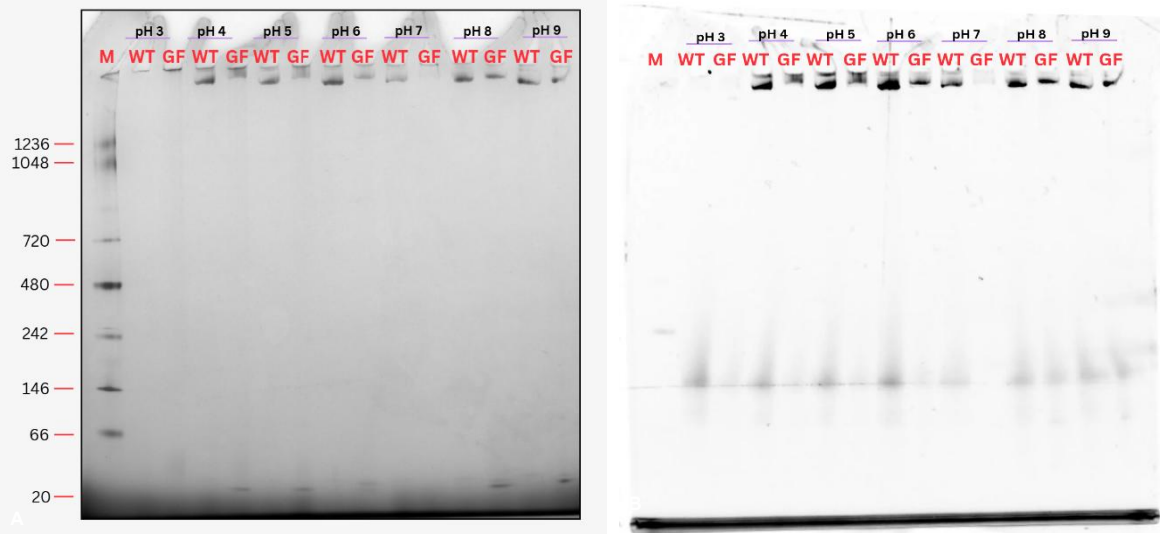

Figure 8 ***Native PAGE analysis of the T4GALA EncStrep+GFP system across pH 3–9.*** *(A) Coomassie-stained native PAGE comparing WT EncStrep + GFP-CLP (WT) and T4GALA EncStrep +GFP (GF). (B) Fluorescence image acquired before Coomassie staining to visualise GFP. Fluorescence associated with slowly migrating cage species and lower-migrating material was observed across multiple pH conditions.*

Therefore, the origin of the lower-migrating fluorescence could not be determined from these experiments. Further analysis would be required to distinguish encapsulated, associated and released GFP.

4. Conclusion

This project demonstrated that T4GALA EncStrep responds to acidic conditions, with loss of the assembled-cage population observed under strongly acidic conditions. Assembly was retained under near-neutral conditions. Further investigation at narrower, physiologically relevant pH intervals is therefore required to determine its potential for controlled cargo release in tumour-associated conditions.

An unexpected lower-molecular weight species consistently co-purified with T4GALA, indicating possible degradation or truncation that may influence cage stability and cargo protection. Its identity and significance require further investigation.

Overall, this work establishes T4GALA as a promising pH-responsive encapsulin system while identifying key limitations that must be addressed. Future work should quantify GFP release under defined pH conditions, characterise the unexpected protein species using mass spectrometry, and optimise expression. Testing narrower, biologically relevant pH ranges while controlling for buffer identity will be essential to determine whether T4GALA can provide reliable, controlled cargo release.

5. References

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