Programmed spatial organization of biomacromolecules into discrete, coacervate-based protocells

Wiggert J. Altenburg, N. Amy Yewdall, Daan F. M. Vervoort, Marleen H. M. E. van Stevendaal, Alexander F. Mason & Jan C. M. van Hest

The cell cytosol is crowded with high concentrations of many different biomacromolecules, which is difficult to mimic in bottom-up synthetic cell research and limits the functionality of existing protocellular platforms. There is thus a clear need for a general, biocompatible, and accessible tool to more accurately emulate this environment. Herein, we describe the development of a discrete, membrane-bound coacervate-based protocellular platform that utilizes the well-known binding motif between Ni²⁺-nitrilotriacetic acid and His-tagged proteins to exercise a high level of control over the loading of biologically relevant macromolecules. This platform can accrete proteins in a controlled, efficient, and benign manner, culminating in the enhancement of an encapsulated two-enzyme cascade and protease-mediated cargo secretion, highlighting the potency of this methodology. This versatile approach for programmed spatial organization of biologically relevant proteins expands the protocellular toolbox, and paves the way for the development of the next generation of complex yet well-regulated synthetic cells.
The bottom-up recreation of cellular processes into synthetic compartments has in recent years emerged as an exciting line of research to study biological processes in a controlled environment. Historically, liposomes have been favored in the creation of photocellular compartments, encapsulating communication networks, and complex biochemical reactions, such as DNA-mediated self-replication, signaling cascades, and in vitro transcription and translation (IVTT). Other membrane-bound protocols, such as polymersomes, colloidosomes, and proteinsomes, are also able to encapsulate functional biomolecular systems within cell-mimetic capsules. However, these membrane-bound protocols share intrinsic weaknesses, such as low and heterogeneous encapsulation efficiencies, lack of control over stoichiometry of multimeric cargoes, and rudimentary release mechanisms, limiting their development into truly cell-mimetic platforms. Microrheological techniques can mitigate this to a degree, but are often difficult to implement compared to self-assembled systems, which rely on molecular interactions to form hierarchical structures.

Protocell models based on condensed aqueous droplets, such as complex coacervates, aqueous two-phase systems (ATPS), and liquid–liquid phase separation of intrinsically disordered proteins, could provide unique opportunities for the incorporation of additional functionalities. In these systems, attractive or segregative mechanisms drive the formation of polymer-rich, crowded cell-sized droplets, which typically exhibit strong incorporation of cargo into their core due to charge complementarity and/or hydrophobicity. Various examples have been reported in which these condensed droplets have been used as protocells capable of controlled growth, as tools to study synthetic organelles, as well as compartments in which the activity for ribozyme was enhanced or IVTT could be performed. In addition, the inherently crowded environment within membrane-free protocells resembles the interior of a living cell more closely than membrane-bound protocells, as ~30% of the cytoplasmic volume is occupied by biomacromolecules. This is an important parameter to take into consideration, as crowding is reported to influence macromolecular association, protein conformation, and diffusional processes. However, while cargo loading within membrane-free protocells is improved compared to membrane-bound systems, the loading mechanism is typically discriminate: there is only selective uptake of components in the coacervate phase when they are modified with a complementary charge or low complexity, intrinsically unstructured regions, such as LAF-1 or elastin, which limits the general applicability of these platforms.

For example, in our recently published work describing discrete terpolymer-stabilized complex coacervate protocols, the formation of stable coacervate protocells was demonstrated while protein uptake remained a challenge due to the coacervates’ (positively) charged nature. As a result, highly negatively charged proteins, made by either amino acid mutations on the protein surface or via succinylation of surface accessible lysine residues into carboxylates, were readily taken up inside the coacervate phase, whereas neutral or positively charged proteins were excluded.

Succinylation is an effective way to overcome the limitation of charged cargo, the programmed uptake of macromolecular cargo into the coacervate core during the formation process required a negative charge. In order to overcome the limitation of charged cargo, the programmed uptake of recombinant proteins was devised by functionalizing amylose with an NTA group, which coordinates Ni2+ and binds His-tagged proteins. NT-Amylose (NT-Am) was synthesized from Cm-Am in a two-step reaction (Supplementary Fig. 2). The carboxylic acid moiety was first activated via EDC/NHS chemistry, followed by the addition of N,N,N′,N′-bis(carboxymethyl)-1-lysine hydrate under basic conditions to yield NT-Am. In order to assess protein sequestration via this methodology, recombinant superfolder Green Fluorescent Protein with a cleavable histidine-tag (sfGFP-His) was chosen as a model protein, as this fluorescent protein is not sufficiently negatively charged to be taken up in the coacervate core via electrostatic interactions. sfGFP-His was mixed during protocell formation with the amylose polymers (20:8:0.2 mass ratio of Q:Cm:NTA) in a Ni2+ containing buffer (PBS, pH 7.4, 7.5 μM NiSO4). The uptake of protein was immediately noticeable inside the discrete coacervate protocells (Fig. 2a). As expected, the uptake efficiency was found to be dependent on the presence of the His-tag as well as the Ni2+ ions, demonstrated by the absence of protein sequestration when either was omitted. In fact, adjusting the Ni2+ concentration results in control over the protein uptake, with the maximum of protein loading observed at 7.5 μM Ni2+, which corresponds well to the estimated 8.7 μM of NTA groups added (Supplementary Fig. 6).

Importantly, sfGFP uptake into the coacervate core is homogeneous and independent of protocell size. This was clearly seen in confocal micrographs (Fig. 2a and Supplementary Fig. 4), and also confirmed by flow cytometry analysis (Fig. 2b), represented by a linear correlation between protocell volume and fluorescence intensity of sfGFP-His. At larger size there does appear to be a deviation in the linear behavior, this likely due to the fact that these larger protocells measured exceed the recommended instrumental limits. The local sfGFP-His concentration inside the coacervate was assessed with the aid of an sfGFP-His fluorescence calibration curve. An initial bulk concentration of 250 nM.
droplets are stabilized. These stabilized coacervates can be loaded with functional cargo to allow for an enhanced enzymatic activity or protease-mediated degradation (blue), and Ni-NTA-Am (teal) are mixed to form a coacervate droplet in which His-tagged proteins are loaded. Upon addition of the terpolymer, the structures present inside the protocells containing His-tagged proteins are contained, demonstrating the ability of the controlled uptake mechanism to sequester proteins irrespective of their surface charge, two additional His-tagged GFP variants with different surface charges were explored. Both the negatively supercharged variants were recombiantly expressed and obtained in high purity (Fig. 3a and Supplementary Fig. 10). Additionally, for each variant, the His-tag was removed by TEV protease (Supplementary Fig. 11). Without the His-tag, uptake was dominated by electrostatics, which is consistent with previously reported results, but by utilizing our programmed uptake mechanism, an increase of fluorescence was observed for all His-tag variants inside the coacervate core (Fig. 3b). In the case of +36GFP and sfGFP, a clear distinction in loading was observed in the presence of the targeted Ni\(^{2+}\)-NTA/His interaction, while for −30GFP this effect was not as pronounced as its interactions were still dominated by favorable electrostatics. The punctate structures present inside the protocells containing +36GFP(-His) are protein aggregates resulting from its instability in a low salt buffer (Supplementary Fig. 13). Next, we investigated the diffusivity of encapsulated cargoes, to ensure that our programmed uptake strategy does not eliminate cargo mobility. This is an important parameter, because for many proteins function is dependent on their spatiotemporal organization, for example in living cells. The similarity in diffusion rates for −30GFP, −36GFP and −30GFP-His showed slower diffusion rates, respectively, while the sfGFP-His showed slower diffusion rates, and the sfGFP showed slower diffusion rates, respectively. The similarity in diffusion rates for both His-tagged proteins indicates that the diffusion is mainly dominated by the His-tag and Ni\(^{2+}\)/NTA interaction rather than by the charged nature of the coacervates. Despite the decreased diffusivity, the His-tagged proteins are still able to diffuse through the core and are not immobilized, a prerequisite for any sequestered enzymes to retain activity upon loading.

Enhanced enzymatic activity inside coacervates. An interesting phenomenon seen in living cells is the localization of cofactor-dependent molecules. This not only leads to high selectivity but also to high local concentrations, which can influence the rate of enzymatic reactions. To explore the capabilities of our controlled macromolecular uptake mechanism, the conversion of L-tryptophan (L-Trp) to indigo by a synthetic, two-enzyme cascade was selected. Tryptophan anhydrase (TnaA) is responsible for the conversion of L-Trp to indole with pyridoxal-5-phosphate (PLP) as a cofactor. The indole is then oxidized by flavin-containing monooxygenase (FMO), consuming nicotinamide adenine dinucleotide phosphate (NADPH) in the process. This cascade was selected for its simple readout and the fact that as a synthetic cascade, these enzymes do not have a

**Fig. 1** Schematic overview of chemically programmed loading of His-tagged proteins into Ni\(^{2+}\)-NTA-functionalized protocells. Q-Am (red), Cm-Am (blue), and Ni-NTA-Am (teal) are mixed to form a coacervate droplet in which His-tagged proteins are loaded. Upon addition of the terpolymer, the droplets are stabilized. These stabilized coacervates can be loaded with functional cargo to allow for an enhanced enzymatic activity or protease-mediated release.
was produced due to the appearance of a blue color. When either the substrate mix or the enzymes were omitted, no blue color was observed (Supplementary Fig. 21). To study the effect of programmed co-localization on this reaction, NADPH consumption and indoxyl production were followed over time via absorption and fluorescence spectroscopy respectively. Within ~3.5 h all the NADPH was consumed by the enzymes sequestered in the coacervates (orange), compared to >12 h for the same number of enzymes in solution (gray); a clear increase in reaction rate was observed (Fig. 4e). By decreasing the total amount of enzyme for the 0.5:1 ratio by half, the reaction also takes twice as long, from ~3.5 to ~7 h. Meanwhile, in solution, a much lower rate is observed (Fig. 4f). Moreover, for both concentrations of enzyme, the indoxyl fluorescence intensity did not reach the same level inside the coacervates as in solution, showcasing the faster conversion of the intermediate (Supplementary Fig. 22). The potential of programmed cargo uptake can be demonstrated by investigating different ratios of loaded enzymes. Over a wide range of TnaA:FMO ratios, the overall rate stays the same with a constant amount of FMO (Supplementary Fig. 23), confirming that FMO is the rate-limiting enzyme. In solution, however, a clear distinction between the different ratios can be made, where there is a clear dependency on the amount of TnaA present for the reaction rate (gray) (Supplementary Fig. 23). Only when the system is pushed by a large excess of TnaA (2:1 TnaA:FMO), the rate in solution becomes similar to that in the coacervate system. This type of analysis would not be possible without a robust programmable uptake strategy. Interestingly, in the absence of Ni²⁺, the time for complete NADPH consumption was similar to Fig. 4e (Supplementary Fig. 24). This was unexpected as TnaA has a similar theoretical isoelectric point to GFP (6.19 and 6.04, respectively) and thus should be excluded from the coacervate core. Confocal microscopy indicated non-specific TnaA adsorption on the periphery of the protocells, accomplishing co-localization in the same microenvironment but without the control over the enzyme stoichiometry (Supplementary Fig. 25). This illustrates the need for a generally applicable, bio-orthogonal uptake strategy, which delivers control over both the local concentration and co-localization of the enzymes involved. These data represent an important progression towards the study of complex enzymatic cascades in confined, discrete, cell-mimetic environments.

TEV protease-mediated release. As a final proof of the functional diversity that can be accomplished with this protocell platform, a unique cargo release mechanism was designed, which utilizes both electrostatic interactions and enzymatic control. As established earlier in Fig. 3b, sfGFP is not taken up into the coacervate core unless it is His-tagged. Thus, if the His-tag can be cleaved from the sfGFP-His cargo, excretion of the protein from the protocell system would be expected (Fig. 5a). To engineer this release, a commercially available, His-tagged TEV protease was selected, which specifically cleaves the amino acid sequence between sfGFP and the hexahistidine tag in our constructs. After loading both the sfGFP-His and TEV protease into the protocells, a clear drop in fluorescence intensity was observed inside the coacervate core after one hour of incubation (Fig. 5b). This release was not observed when TEV protease was omitted. Similarly, for +36GFP release was also observed for when incubated with the TEV protease (Supplementary Fig. 28), but again was not studied in detail due to protein aggregation. As an additional control, −30GFP-His was loaded, which resulted in no observable release, a consequence of the strong electrostatic interactions between the coacervate core and the negatively charged protein, negating the effect of the His-tag. In order to
confirm the in-situ removal of the His-tag, the diffusivity of the cargo in the protocell core was determined via FRAP experiments (Fig. 5c and Supplementary Fig. 29). For sfGFP-His, a slow recovery was observed with a $D_{\text{app}}$ of 0.092 $\mu$m$^2$/s, which is in line with the $D_{\text{app}}$ of sfGFP-His reported in Fig. 3d. This indicates that FRAP predominantly proceeds with the His-tagged protein, as the sfGFP-His cleaved by the TEV protease has been excluded from the coacervate core. In the case of $-30$GFP-His, a fast recovery was seen with a $D_{\text{app}}$ of 0.279 $\mu$m$^2$/s, which is similar to the observed diffusion speed of $-30$GFP without His-tag (Fig. 3d), confirming that TEV protease has cleaved between the protein and His-tag, and the $-30$GFP remains due to electrostatics. In order to gain an insight into the kinetics of protease-mediated cargo excretion, confocal microscopy was utilized to measure the amount of protein left inside the coacervate core over time (Fig. 5d). Once again a clear difference was observed when TEV protease was excluded, and the release of sfGFP proceeded in a gradual manner, as expected for an enzyme-mediated process. Moreover, by adding different amounts of TEV protease the rate of release can be tuned (Supplementary Fig. 31). However, after 60 min the rate of all concentrations is similar, indicating that the system is diffusion-limited. This can be explained by the low apparent diffusion coefficient for His-tag loaded proteins in this system. Despite the slower diffusivity, the system approximates full release after 2 h (Supplementary Fig. 32). The ability to achieve enzyme-mediated protein secretion opens up many possibilities for the release of biologically active proteins, such as cytokines and growth factors. Furthermore, the concept could be easily extended toward the light or chemically triggered release as well.

**Discussion**

In this work, we have designed a methodology for the introduction of biologically inspired function in protocells. In the field of synthetic cells, advances in functional capabilities are achieved by incorporating increasingly complex arrays of both synthetic and natural macromolecular species. However, using current encapsulation techniques, there are several challenges that have yet to be surmounted. The first of these, specific to statistical encapsulation, is a typically low encapsulation efficiency, which limits the amount of included cargo, impacting functional performance and necessitating purification procedures to remove non-encapsulated cargoes. Secondly, there is either poor control over the final composition of encapsulated species (statistical) or non-biased uptake (membrane-free protocells), which are particularly undesirable when attempting to efficiently encapsulate a large number of different species. These challenges clearly stand...
in the way of creating bottom-up protocells with a degree of cargo complexity on par with nature and have been overcome with the development of the system described herein.

The inclusion of the biocompatible Ni\(^{2+}\)-NTA/His-tag binding motif within discrete, stable protocells has resulted in control over the concentration and stoichiometry of incorporated cargo. Significantly, this method is not reliant on statistical encapsulation efficiencies, microfluidic methodologies, or the intrinsic interactions between cargo and the protocell platform. These benefits have been highlighted by the incorporation of the synthetic indigo producing cascade, where enhanced activity was obtained using His-tagged, aggregation-prone enzymes. However, such high levels of local enzyme concentration expose parameters that need to be accounted for in reconstituted synthetic cascades. There is still a limited understanding of substrate/cofactor (e.g., amino acids, nucleotides, NADPH, etc.) localization and availability in this system, which were until now not necessary to consider. This substrate bottleneck has also been observed in the way of creating bottom-up protocells with a degree of cargo complexity on par with nature and have been overcome with the development of the system described herein.

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delivery of growth factors in tissue engineering, targeted protein-drug release, and on a more fundamental level, for the modeling of protein-based signaling pathways in a controlled environment.

In summary, we have demonstrated an elegant, robust, and general method for the controlled loading and release of a broad range of His-tagged recombinant proteins into coacervate-based protocols via Ni²⁺-NTA/His interactions. The efficient and programmed uptake of near-native macromolecular cargo, modified only with a His-tag, into discrete protocells opens up an enormous range of possibilities for exploring protein-based biological processes, from enzymatic cascades to signaling pathways. This work represents a significant step forwards for the field of bottom-up synthetic cells and provides a robust, adaptable, and accessible foundation for the creation of increasingly complex cell-mimetic microenvironments.

**Methods**

**DNA molecular biology and cloning.** All DNA was ordered through Integrated DNA Technologies (IDT). The sequence of each protein was derived from either Lawrence et al. for the GFP’s, and Giesen et al. for the enzymes TnaA (Tryptophanase) and FMO (Flavin-containing monoxygenase). The constructs were optimized using the IDT Codon Optimization Tool for Escherichia coli (E. coli) based on the amino acid sequence. The vector pET-28a and the gBlocks were both digested using the restriction enzymes NcoI and XhoI (New England Biolabs). After ligation, the constructs were verified using Sanger sequencing (BaseClear). Constructs were transformed into BL21(DE3) bacterial cells (Novagen).

**Protein expression and purification.** For all proteins, 600 mL 2YT medium supplemented with kanamycin (100 µg mL⁻¹) was inoculated using an overnight culture grown at 37 °C, 200 rpm to an optical density (OD600) of 0.6, and then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1 mM) and lysed using an EmulsiFlexC3 High-Pressure homogenizer (Avestin) at 15,000 psi for three executive rounds. For +36GFP, 10 mg mL⁻¹ DNaseI (PanReac AppliChem) and 5 mg mL⁻¹ RNase A (ThermoFisher) were added 30 min before lysis. Cell debris and insoluble proteins were removed by centrifugation (15,000×g, 15 min, 4 °C) and the pellet was either lysed immediately or flash-frozen in liquid N₂ and stored at −20 °C.

For the GFP variants, the purification protocol was based on previous reports. The cell pellets were resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8, and for the +36GFP variant, 1 M NaCl was used in addition) and lysed using an EmulsiFlexC3 High-Pressure homogenizer (Avestin) at 15,000 psi for three executive rounds. For +36GFP, 10 mg mL⁻¹ RNase A (PanReac AppliChem) and 5 mg mL⁻¹ DNaseI (PanReac AppliChem) were added 30 min before lysis. Cell debris and insoluble proteins were removed by centrifugation (15,000×g, 10 min, 4 °C). The His-tagged proteins were purified from the soluble lysate using Ni-NTA affinity chromatography (His-Bind Resin, Novagen). The lysates were incubated with the His-bind resin for 1.5 h at 4 °C on a shaking table, prior to loading onto an empty gravity flow column (Bio-Rad).
resin was washed twice with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8; again for the +36GFP variant, 1 M NaCl was used). The His-tagged proteins were eluted from the resin with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8; for the +36GFP variant, 1 M NaCl was used).

In case of TnaA and FMO proteins, the purification was based on Giessen et al. The cell pellets were resuspended in Buffer A (50 mM Tris, pH 8, 300 mM NaCl, 10 mM imidazole, 1 mM glyceral, 250 mM imidazole). Extensive dialysis was performed to remove the imidazole using a 10 kDa molecular weight cut off (MWCO) membrane (Millipore), into phosphate-buffered saline (PBS), pH 7.4 (for the sGFP, ~36GFP, TnaA and FMO proteins) or 50 mM NaPi, 600 mM NaCl, pH 7.5 (storage buffer for the +36GFP protein). The samples were concentrated using 3 kDa MWCO Amicon Ultra Filters (Millipore).

For further purification, the samples were passed through a HiLoad Superdex 26/600 200 prep preparative column (GE Healthcare) connected to an AKTApurifier FPLC (GE Healthcare) at a flow rate of 1 mL min⁻¹, and the absorbances at 280 nm and 488 nm (GFP variants only) were monitored. The eluted fractions were analyzed using SDS-PAGE (4–20% Mini-PROTEAN TGX Precast Protein Gel, Bio-Rad) and the purest fractions were pooled and concentrated. For the FMO enzyme specifically, only the fractions of peaks 1 and 2 were pooled (Supplementary Fig. 1). The other two peaks correspond to the protein without FAD, visible by the absence of a yellowish color at high µM concentration, and are not functional.

Protein concentrations were calculated using the 280 nm absorbance determined by ccd of 20,400 M⁻¹ cm⁻¹, by thresholding the image and converting it into a binary image. To determine the Region of Interest (ROI’s), the coacervate droplets, watershed function was used following the intensity of the reference area, as described by Jia et al. Coacervate droplets were analyzed with an Aria III (BD Biosciences) FACS equipped with a 70 µm nozzle, 488 nm laser, and a 585 ± 7.5 nm bandpass filter. The fluorescence intensity of 1,000 individual droplets was collected. Any data point that was at the maximum value of the detector was removed from the data set.

Flow cytometry analysis. Coacervates loaded with 250 nM sGFP-His were analyzed with an Anari III (BD Biosciences) FACS equipped with a 70 µm nozzle, 488 nm laser, and a 585 ± 7.5 nm bandpass filter. Single coacervates were selected based on the forward scatter of the coacervate droplets, and the laser power was modified with a P-110E-5 × 10⁻⁵ laser power. The intensity of the detector unless stated otherwise. Images of 2048 × 2048 pixels were acquired with a pixel dwell of 1.6 µs.

Single-particle image analysis. All images were analyzed with Fiji (ImageJ). In order to determine the fluorescence intensity, a two-color image of the interior and the background of the coacervate droplets was required. Only standard ImageJ functions were used for the analysis. In the membrane image the polymer layer was selected by thresholding the image and converting it into a binary image. To determine the Region of Interest (ROI’s), the coacervate droplets, watershed function was used following the intensity of the reference area, as described by Jia et al. Coacervate droplets were analyzed with an Aria III (BD Biosciences) FACS equipped with a 70 µm nozzle, 488 nm laser, and a 585 ± 7.5 nm bandpass filter. Single coacervates were selected based on the forward scatter of the coacervate droplets, and the laser power was modified with a P-110E-5 × 10⁻⁵ laser power. The intensity of the detector unless stated otherwise. Images of 2048 × 2048 pixels were acquired with a pixel dwell of 1.6 µs.

Measuring local GFP concentration and calibration curve. Preparation and imaging of the coacervate droplets were performed as described above. The calibration slide was prepared by placing a Press-to-Scale™ Silicon Isolator with Adhesive, eight wells, 9 mm diameter, 0.5 mm deep (Invitrogen) on isopropanol cleaned super frost micro slide (VWR). Following, 20 µL of sGFP-His protein ranging from 1 to 50 µM was loaded onto the slide and sealed with a glass coverslip (VWR). The calibration slide was imaged with the exact same settings as the sGFP-His loaded coacervate sample. The fluorescence intensity of each concentration was determined using Fiji.

FRAP (fluorescence recovery after photobleaching). Coacervate droplets were prepared as described above and loaded with 250 nM of a GFP variant. 100 µL of each sample was transferred on a µ-slide 8 well glass bottom (Ibidi). FRAP experiments were performed with the bleaching interface available in Zeiss 2009 (Zeiss). For imaging, the same settings were used as described above. An initial image was acquired in order to define the region of interest (ROI), 5–10 µm in diameter, within a coacervate. Following, three images of 1024 × 1024 with a pixel dwell of 0.8 µs as acquired prior to the bleaching. Subsequently, the ROI was bleached for 10 iterations with a two-photon laser (Chameleon, Coherent) set at 810 nm, 50% laser power. The recovery was monitored with a 5 s interval, for a total of 33 images. The intensities of the bleached ROI, reference area, a nearby coacervate that was not bleached, and background were extracted from the images with Fiji. Data were normalized by removing the background intensity and dividing the intensity of the referred area with the intensity of the ROI. A first-order exponential function was fitted using Origin 2019 (OriginLab) from the half-life and D_app were calculated as reported by Poudyal et al.
Labeling of the enzymes with sulfo-Cy5-NHS. A few grains of sulfo-Cy5-NHS (Lumiprobe) were dissolved in ddH2O. The concentration was determined using the absorption at 460 nm, ε = 271,000 cm⁻¹ M⁻¹ using an ND-1000 spectrophotometer (ThermoScientific). The reagent was added in an equimolar ratio to 60 µM of TnaA or FMO protein (~1:30 ratio of NHS to lysine residues for both enzymes) in PBS and left for 1 h at 4 °C. Unreacted dye was removed using a PD-25 spin trap column (GE Healthcare). The average labeling per protein was measured using the absorption of the dye at 660 nm and at 280 nm for the protein. TnaA-Cy5 degree of labeling: 0.48, FMO-Cy5 degree of labeling: 0.56. For the experiments regarding the uptake without Ni²⁺ present, the degree of labeling was TnaA-Cy5: 0.52, FMO-Cy5: 0.32. The loading and imaging of these labeled proteins were performed as described above. For excitation of the Cy5 dye a 633 nm HeNe laser was used. The brightfield images were made with a Zeiss Axios Observer D1 equipped with an LD Plan-Neofluor 40×/0.6 Corr, Halogen lamp, and an AxioCamMR3.

Enzymatic activity inside coacervates. Coacervates were formed as described above. Experimental settings were based on Giessen et al.47. Experimental settings were based on Giessen et al.47.

Enzymatic activity inside coacervates. Coacervates were formed as described above. Experimental settings were based on Giessen et al.47. Enzymatic activity measurements. Technical triplicates of enzyme-loaded coacervates, containing different amounts of enzyme were made as described above. For each condition, an enzyme mastermix was created from which each sample was made, with the following final concentrations: 125:250, 125:500, 250:500, 1000:500 nM of TnaA:FMO, respectively. In all cases, TnaA and FMO were preincubated with PLP, 5× excess compared to the TnaA concentration, at room temperature for at least 15 min. After formation, the samples were left for 5–10 min on the bench. Following, the sample was transferred to a Non-Binding black microplate 384 well with a transparent bottom (Greiner Bio-One) and a substrate mastermix containing 1 mM L-Trp and 0.5 mM NADPH was added. NADPH consumption was monitored for over 12 h, and a measurement was taken every 10 min using the absorption of the dye at 340 ± 20 nm as well as the indoxyl fluorescence (ex. 375 ± 20 nm, em. 470 ± 20 nm) at 30 °C using a Spark 10 M plate reader (Tecan). To prevent evaporation the plates were sealed with EASYseal™ sealing plates. In the case that the endpoint was not yet reached at the end of the 12 h, the plate was moved to a 30 °C incubator and measured at least 3 data points again after another 6 h, 750 nM of the enzyme, or 24 h, 375 nM of the enzyme. All data were normalized to their endpoint, flat line in absorbance, indicating that all NADPH was consumed. Without PLP or at temperatures below RT, the TnaA enzyme is not able to form its active tetrameric assembly, which results in a large variation between samples inside the coacervates (Supplementary Fig. 26).

TEV protease-mediated release. 100 nM of sGFP-His – 30GFP-His or +3GFP, 5 U of TEV-His protease (Protein), and 13 mM TCEP were loaded into the coacervates as described above. After 1 h of incubation at room temperature, the samples were analyzed with confocal microscopy as described earlier. For sGFP and –30GFP, FRAP analysis was also performed on these samples.

Time traces. The samples for microscopy were prepared as described above containing 250 nM of sGFP-His, 6.25 (0.5×), 12.5 (1×), 25 U (2×) of TEV-His protease and 16.5, 33, 66 mM TCEP, respectively; as a reference, 250 nM of sGFP-His was used without any TCEP or TEV. The samples were analyzed with a Leica DMi8-ES equipped with an HC PL APO CS2 63×/1.20 water objective, 488/552 nm DsRed, HyD detector. For imaging of the GFP, an OPLS set at 488 nm and a BP filter of 500–560 nm were used. Nile red staining was visualized using an OPLS of 554 nm and a BP filter of 560–662 nm. For each wavelength, the pinhole was set to 1 airy unit. The transmission and detector gain were optimized for each different fluorescent protein/fluorophore to utilize the maximum amount of gray values of the detector unless stated otherwise. Images of 1024 × 1024 pixels were acquired with a pixel dwell of 0.4 µs with a line averaging of 4. For each sample, 4–5 positions were analyzed every 3 min.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**Author contributions**

W.I.A., A.F.M., and J.C.M.H. wrote the manuscript; W.I.A., A.F.M., N.A.Y., and J.C.M.H. designed the research; W.I.A., A.F.M., D.F.M.V., and M.H.M.E.v.S. performed the experiments. All authors reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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**Correspondence and requests for materials should be addressed to A.F.M. or J.C.M.H.**

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