

Mechanistic insights into the preparation and stabilisation of supersaturated pea protein isolate-curcumin nanoparticles prepared by a pH-driven method

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ARTICLE INFO

Keywords:

Plant proteins
Delivery system
Curcumin
Supersaturation
pH-driven method
Kinetic solubilisation

ABSTRACT

The water solubility of the polyphenol curcumin was enhanced by preparing pea protein isolate nanoparticles loaded with curcumin using a well-established pH-driven method. This process generated supersaturated nanoparticles, with 96% of the solubilised curcumin precipitating within 24 h. The supersaturated and stable mass concentrations were 1.39 ± 0.093 mg/mL and 0.0528 ± 0.0088 mg/mL, respectively. The study assessed the kinetics, stability, and adsorption and desorption behaviour of curcumin with these nanoparticles, comparing the supersaturated and stable states. A schematic mechanism encompassing all steps from nanoparticle preparation to curcumin precipitation is proposed. The phenomenon is explained by the aggregation or isomerisation of tightly packed curcumin molecules attached to the nanoparticle surface. The precipitation kinetics are influenced by stirring, temperature, and lyophilisation, and are slowed by dilution and the addition of bisdemethoxycurcumin. The studied system is also evaluated from a pharmaceutical perspective and is proposed as a supersaturated drug-delivery system (SDDS).

1. Introduction

Plant-derived proteins have recently gained significant interest for their ability to solubilise bioactive compounds. The food and pharmaceutical industries are particularly interested, as both require formulations that manage substantial amounts of water. Many compounds of interest, such as vitamins, carotenoids, and polyphenols, are difficult to solubilise in water due to low solubility or stability. Consequently, delivery systems are necessary to deliver their health benefits to the human body (Gomes & do Amaral Sobral, 2021; Tang, 2021a; Wan et al., 2015).

From an economic perspective, plant proteins are attractive carriers because they are widely available, affordable, and align with the demand for vegetarian and vegan products and formulations. From a scientific perspective, (plant) proteins are multifunctional macromolecules with the potential to form a variety of nanoarchitectures depending on how they are processed and how they interact with other compounds of interest (Hadidi et al., 2022; Hadidi et al., 2023; Tang, 2021b). Protein

functionality is given by the self-assembly of intact, native tertiary and quaternary structures, which provide access to binding sites and determine the interactions with the bound compound and the surrounding medium (Zhang, 2003). These qualities are significantly diminished in commercial protein powders. Due to extraction and drying processes, commercial powders are protein mixtures containing impurities such as fibers and minerals and are often denatured, resulting in a loss of functionality (Ge et al., 2020; Ma et al., 2022; Tanger et al., 2020). Nevertheless, various methods exist to partially restore their functionality. Chemical methods (i.e., pH shifting, acylation), physical methods (i.e., heating, extrusion), biological methods (i.e., enzymatic hydrolysis), and other modifications (i.e., complexation) can be used to modify the protein structure. Depending on the treatment, different functionalities can be improved or modified, enabling the creation of diverse delivery systems such as emulsions, complexes, nanoparticles, or gels (Nikbakht Nasrabadi et al., 2021).

Soy is the most widely studied plant protein source, and its

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<https://doi.org/10.1016/j.foodchem.2026.149156>

Received 23 September 2025; Received in revised form 13 January 2026; Accepted 4 April 2026

Available online 5 April 2026

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performance in solubilising bioactive molecules is frequently reported alongside various treatments (Chen, Li & Tang, 2015a; Liu & Tang, 2013; Tang, 2021a; Yang et al., 2020; Zhang et al., 2018). Its good functionality and well-balanced amino acid profile make it attractive in human nutrition; however, soy protein is allergenic and is often genetically modified. In contrast, pea protein is hypoallergenic, clean-labeled, and produced on a large scale. Pea protein consists of 55–85% globulin proteins. Native globulins are storage proteins soluble in dilute salt solutions, with the two main fractions being legumin (300–410 kDa) and vicilin (150–180 kDa). The isoelectric point of the protein mixture is around 4.8, and the water solubility at pH 7 of commercial products ranges from 1 mg/mL to 60 mg/mL, depending on the extraction process, the manufacturer, and the raw material source (Hadidi et al., 2022; Lam et al., 2018; Lu et al., 2020; Tanger et al., 2020). Its applications in the food industry go beyond food processing and milk and meat substitutes (Boye et al., 2010). Pea protein isolate (PPIp) is also being researched as a carrier for various nutraceuticals in developing functional foods. The term refers to food products enriched with nutraceuticals such as antioxidants and vitamins (Hadidi et al., 2023; Shanthakumar et al., 2022).

Curcumin (Cur) is a polyphenol extracted from turmeric that has a bis- α , β -unsaturated β -dicarbonyl structure with the possibility to undergo keto-enol tautomerisation. It is capable of H-bond donation and acceptance and reacts as a Michael acceptor. Due to its molecular structure, Cur is highly sensitive to environmental stresses such as pH and light, and its solubility and stability heavily depend on the surrounding medium (Heger et al., 2014). Its solubility in water or buffer systems at neutral pH ranges from 11 ng/mL to 10 μ g/mL, and without stabilisation, Cur degrades rapidly within a few minutes (Kharat et al., 2017; Nelson et al., 2017). Its main degradation products include bicyclopentadione, vanillin, vanillic acid, ferulic acid, feruloyl aldehyde, feruloyl methane, and hydroxybenzoic acid, depending on pH or photodegradation (Kharat et al., 2017; Peram et al., 2017). Cur is widely studied not only for its bright orange colour, but also for its antioxidant and anti-inflammatory properties (Kharat et al., 2017). Ongoing research explores its anti-diabetic (Hegde et al., 2023) and anti-cancer effects (Willenbacher et al., 2019), as well as potential against Alzheimer's disease (Chin et al., 2013). Due to its very low water solubility, and, thus, in a broader sense, low bioavailability and chemical stability under physiological conditions, it remains challenging to access these highly praised health-beneficial properties to a degree that proves a positive impact on human health (Kharat et al., 2017; Nelson et al., 2017). However, whether used as an anti-cancer agent, supplement, or food colouring, increasing Cur's water solubility while maintaining its stability is crucial to making these benefits accessible.

To enhance these properties of Cur, various strategies have been explored (Zheng & McClements, 2020). Plant protein-based carrier systems have been extensively studied over the past decade. As previously mentioned, soy protein isolate has been thoroughly examined to solubilise Cur and improve its galenic properties. However, zein (Dai et al., 2019; Patel et al., 2010; Wang et al., 2024; Yuan, Huang, et al., 2021; Yuan, Xiao, et al., 2021), pea protein isolate (Guo, Bayram et al., 2021; Qiao et al., 2023; Ren et al., 2024; Vijayan et al., 2021), sunflower protein (Mehryar et al., 2021), rice protein (Cheng et al., 2024), and others have also demonstrated promising results. To understand the underlying mechanisms by which plant proteins solubilise and stabilise Cur, simulations and molecular docking studies have been conducted. Li et al. examined the binding of Cur to soy protein isolate and utilised the major protein components β -conglycinin and soybean glycinin to determine the optimal binding conformation of Cur at different pH levels (Li et al., 2022). Ren et al. performed molecular docking and molecular dynamics simulations with the native proteins legumin, vicilin, and convicilin, which are the main fractions of pea protein, to investigate their interactions with Cur (Ren et al., 2024). Wang et al. predicted the native structure of zein to explore its interactions with Cur through molecular docking analysis and molecular dynamics (Wang

et al., 2024). These fundamental studies have demonstrated that Cur binds to the hydrophobic binding sites of proteins via non-covalent interactions, including hydrophobic interactions and hydrogen bonds, and that the protein structure also changes during this process (Li et al., 2022; Ren et al., 2024; Wang et al., 2024). This knowledge was mainly applied to the more common case of commercial protein isolates rather than native single-protein fractions. Zhan et al. proposed a mechanism for the pH-driven formation of whey protein isolate and zein composite nanoparticles to solubilise Cur (Zhan et al., 2020). Chen et al. presented a schematic mechanism of Cur complexation with soy protein isolate, discussing the effects of low or high Cur loading and ultrasonication of the solutions (Chen, Li, & Tang, 2015b). Teng et al. outlined a schematic mechanism for encapsulating Cur in soy protein isolate nanoparticles using a desolvation method (Teng et al., 2012). To fully understand Cur binding in these cases, the entire preparation process must be considered, as the protein structure undergoes several changes during preparation. Chuan-He Tang published two comprehensive reviews on encapsulating Cur (and other nutraceuticals) with food proteins, combining both theoretical and practical approaches and how to correlate the knowledge from both (Tang, 2020, 2021a). However, to the best of our knowledge, the kinetic stability of systems prepared by pH shifting and heating has not been thoroughly investigated.

To accurately assess the potential of plant proteins and evaluate their advantages and limitations, or to manipulate them, understanding the solubilisation mechanism is essential. We hypothesize that it is a complex combination of thermodynamic and kinetic stabilisation. We propose here a comprehensive overview of the individual steps in the binding mechanism of Cur with a commercial PPIp, incorporating knowledge from the literature and results from additional experiments. The observed supersaturation is discussed, and potential stabilisation approaches are evaluated. The subsequent release is also examined under various parameters.

2. Materials and methods

2.1. Materials

Pea protein isolate NUTRALYS® F85F ($\geq 93\%$, batch WE802) (PPIp) was gifted from Roquette, Lestrem, France. Curcumin (synthetic, $>97.0\%$) (Cur) was obtained from TCI Chemicals, Eschborn, Germany. Ethanol ($\geq 99.8\%$, p.a.) (EtOH) and sodium hydroxide solution (1 N) (NaOH) were purchased from Carl Roth, Karlsruhe, Germany. Hydrochloric acid (1 N) (HCl) was purchased from Merck KGaA, Darmstadt, Germany. Deionized Millipore water (18 M Ω ·cm) was obtained from an in-house Millipore purification system.

2.2. Methods

2.2.1. Preparation of pea protein nanoparticles loaded with Cur (PPICur)

The pea protein nanoparticles loaded with Cur (PPICur) were prepared using a pH shifting and heating method inspired by the protocols of Han et al. (Han et al., 2022) and Pan et al. (Pan et al., 2014): a dispersion of PPIp in Millipore water at a concentration of 1% (w/v) (10 mg/mL) was prepared in a volume of 10 mL and stirred for 30 mins at 22 °C. The pH was adjusted to pH 12.00 ± 0.05 with NaOH (1 N) and stirred for another 30 mins. The solution was heated to 85 °C and stirred for an additional 30 mins, maintaining the temperature. After cooling to 22 °C, the pH was readjusted to pH 12.00 ± 0.05 , and the solution was saturated with 80 mg of Cur. The saturation time was 20 mins under constant stirring at 22 °C. The pH was adjusted to pH 7.00 ± 0.05 with HCl (1 N) and stirred for another five minutes. The precipitated Cur was separated by centrifugation (20,379 xg, 15 mins) and the supernatant (PPICur t0) was filtered using a cellulose acetate (CA) filter with a pore size of 0.45 μ m. The freshly prepared sample was either analysed directly or stirred for 15 h at 22 °C. The next day, the precipitated orange powder was separated by centrifugation and the supernatant (PPICur

t24) was filtered (CA 0.45 µm). All samples were prepared in triplicate.

2.2.2. Preparation of pea protein nanoparticles (PPI)

Pea protein nanoparticles (PPI) without Cur loading were prepared as a control. The same protocol described above was followed, except without the saturation step with Cur. After heating the PPIp solution to 85 °C, it was cooled to 22 °C, and the pH was adjusted back to pH 7.00 ± 0.05 using HCl (1 N). The samples were then centrifuged, filtered, and labeled as t0 and t24.

2.2.3. Determination of the solubility and chemical stability of Cur in PPICur

UV–Vis spectroscopy and high-performance liquid chromatography (HPLC) were used to determine the solubility and stability of Cur. 2 mL of PPICur was extracted with 25 mL of EtOH in two extraction cycles (20 + 5 mL). PPI precipitated and was separated from the ethanolic extract by centrifugation. The supernatants from both extraction cycles were combined and filtered using a PTFE filter with a 0.2 µm pore size. The concentration of Cur was measured using UV–Vis spectroscopy at an absorbance maximum of 427 nm with a Lambda18 spectrophotometer from PerkinElmer, Überlingen, Germany, using quartz cuvettes with a 1.000 cm path length. A calibration curve was prepared for Cur in EtOH with the equation $y = 56499x$ ($R^2 = 0.99994$, SI 2.2.3). The mean values and standard deviations of samples prepared in triplicate were calculated.

The stability of Cur for 24 h was monitored using HPLC. The resulting extracts and the precipitated orange powder (resolubilised in EtOH) were analysed with a Waters HPLC system (Eschborn, Germany) equipped with a Waters 2996 photodiode array detector, a Waters 515 HPLC pump, a Waters pump control module II, a Waters 717plus auto-sampler, an Altmann Analytik Nucleosil C18 5 µm HPLC column (Munich, Germany), and a Gynkotek STH 585 column oven. Data were processed with Empower 3 software. Cur and selected degradation products (vanillin, vanillic acid, ferulic acid, and p-hydroxybenzaldehyde) were separated isocratically using two different mobile phase compositions. The mobile phase for eluting Cur was a 60/40 mixture of 60% acetonitrile and methanol (1:1 v/v) and 40% aqueous 0.1% (v/v) trifluoroacetic acid solution. For the degradation products, the mobile phase was the same solutions in a 30/70 ratio, inspired by Sinha et al. (Sinha et al., 2007) (30% of a 1:1 (v/v) mixture of acetonitrile and methanol, and 70% aqueous 0.1% (v/v) trifluoroacetic acid solution). Chromatograms of Cur and vanillin are shown in SI 2.2.3. A 10 µL sample volume was injected and eluted at a flow rate of 0.4 mL/min at 25 °C. All samples were analysed in duplicates. The precipitated orange powder was also analysed via catalytic tube combustion with oxygen supply using an elemental analyser vario MICRO Cube from Elementar Analysensysteme GmbH, Langenselbold, Germany. This analysis was conducted at the central analytical department of the University of Regensburg.

2.2.4. Determination of pea protein isolate (PPIp) concentration and protein concentration in the solution

The mass concentration of PPIp solubilised in water was determined using eq. (S1) (see SI 2.2.4). Protein solubility was determined as a relative measure using the nitrogen solubility index (NSI) (Grossmann & McClements, 2023; Smith et al., 1966). The ratio of soluble to insoluble nitrogen was calculated in % (w/w) using eq. (S2) (see SI 2.2.4). Relevant parameters were obtained from the gravimetric data of lyophilised residues, along with the nitrogen content determined via catalytic tube combustion with oxygen supply. The NSI was converted into a mass concentration using eq. (S3) (see SI 2.2.4, protein conversion factor according to Ebert et al., 2020). The concentration of PPIp and the protein was monitored during preparation and after 24 h. After each step in the protocol, samples were centrifuged, lyophilised, and analysed. All samples were prepared in triplicate and analysed two or three times. The mean values and standard deviations were calculated.

2.2.5. Characterisation of PPI and PPICur

Binding efficiency and loading capacity: The performance of PPI was evaluated using binding efficiency (BE) and loading capacity (LC). BE and LC were determined using eqs. (1) and (2), respectively.

$$BE [\%] = \frac{\beta(Cur)_{sol}}{\beta(Cur)_{input}} \cdot 100 \quad (1)$$

$$LC [\%] = \frac{\beta(Cur)_{sol}}{\beta(PPI)} \cdot 100 \quad (2)$$

with $\beta(Cur)_{sol}$ indicating the mass concentration of Cur solubilised by PPI, $\beta(Cur)_{input}$ being the mass concentration of Cur during saturation, and $\beta(PPI)$ being the mass concentration of PPI.

Preparative size exclusion chromatography (SEC): SEC analysis was performed on a HiLoadTM 16/600 Superdex 200 pg column in TRIS buffer (20 mM Tris pH 7.5, 150 mM NaCl, DTT), at room temperature. A 2 mL sample volume was loaded onto the column at a flow rate of 0.5 mL/min. Subsequently, the flow rate was increased to 1 mL/min, while the elution from the column was monitored with UV–Vis detection at $\lambda = 280$ nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): reducing SDS-PAGE was performed on home-cast discontinuous gels (12%-separating gel, 4%-stacking gel). Protein samples for analysis were prepared by mixing 20 µL of the sample with 10 µL of the protein loading dye solution (composition in SI 2.2.5). The sample was heated to 95 °C for 10 mins, and 10 µL was loaded onto the gel. In addition, a molecular weight standard PageRuler Prestained Protein ladder from ThermoScientific was loaded in a separate lane. The gels were run at a constant voltage (190 V) for 55 mins. Protein fractions were visualised with Coomassie blue.

Mass spectrometry (MS): samples were prepared using trypsin digestion, separated via C18 reversed-phase chromatography, and analysed online via ESI-QTOF. The Uniprot *Pisum sativum* database was used for data evaluation. Additional information can be found in SI 2.2.5.

Nanoparticle Tracking Analysis (NTA): particle sizes were measured with a NanoSight NS300 device from Malvern Panalytical GmbH, Kassel, Germany. Samples were diluted with Millipore water to a concentration of 20–100 particles per frame and manually injected into the measurement flow cell. Particle tracks were recorded for 60 s per measurement. Each sample was measured three times ($N = 3$). The detection threshold was set to 4. Hydrodynamic diameters of the particles are given as mean (average particle diameter) and mode (particle diameter with the highest particle concentration). The polydispersity index (PDI) was calculated from the NTA data with eq. (S4) (see SI 2.2.5) obtained from Clayton et al. (Clayton et al., 2016), taking into account the error propagation.

Zeta potential (ζ): PPI and PPICur were diluted with water to a PPI concentration of 0.5 mg/mL and an ionic strength of 1.16×10^{-3} M. PPIp was diluted to 0.15 mg/mL, and a corresponding amount of NaCl was added to match the ion strength of PPI and PPICur. ζ was measured using a Malvern ZetaSizer Nano ZS (Malvern Instruments GmbH, Kassel, Germany). All samples were prepared in triplicate and analysed three times. The mean values and standard deviations were calculated.

2.2.6. UV–Vis spectroscopy of PPICur

To evaluate the spectroscopic properties of PPICur, UV–Vis spectroscopy was performed with PPI as a blank. The spectra were recorded on a Lambda18 spectrophotometer from PerkinElmer, Überlingen, Germany in disposable micro-UV-cuvettes (0.100 cm path length) from Brand, Wertheim, Germany. To measure the kinetic profile in section 3.5, UV–Vis measurements were taken at defined intervals with the corresponding media as the blank solution. The simulated intestinal fluid (SIF) was prepared according to the protocol of Minekus et al. (Minekus et al., 2014) without enzymes. Samples were filtered (CA 0.45 µm) before each measurement.

2.2.7. Fluorescence spectroscopy of PPICur

Fluorescence measurements were performed using an Infinite M Nano+ from Tecan Trading (Männedorf, Switzerland). The samples were measured in a black Brand® 96 well microplate with a flat bottom in top reading mode with a gain of 100 and bandwidths of 20 (emission) and 5 (excitation). Emission spectra were recorded in the range of 430–800 nm with excitation wavelengths between 390 and 450 nm. Excitation spectra were recorded from 300 to 500 nm with emission wavelengths between 500 and 550 nm. A stock solution of Cur in MeOH was prepared at a concentration of 5 mM. All samples were diluted to the same absolute concentrations. Cur was diluted with Millipore water and PPICur was diluted with PPI.

2.2.8. X-ray diffraction (XRD)

The samples t0 and t24 were lyophilised for 48 h. The obtained powders were ground in a mortar and measured with transmission geometry via a STOE STADI P diffractometer (Stoe & Cie) equipped with a Mythen 1K detector for data collection. CuK α 1-radiation ($\lambda = 1.540598$ Å) was used. Raw data were processed with the WinXPOW software package from Stoe & Cie (Vol. Version 3.0.2.5) (STOE-WinXPOW, 2011).

2.2.9. Circular dichroism (CD) spectroscopy

CD spectra of PPIp, PPI, and PPICur were recorded using a Jasco J-710 spectropolarimeter. Samples were properly diluted with Milli-Q water to a concentration of 0.14–0.15 mg/mL. The CD spectra were recorded in a wavelength range of 185–260 nm using quartz cuvettes with a 0.100 cm path length. The scan speed was 100 nm/min and the bandwidth and step size were 1.0 nm and 0.5 nm, respectively. Ten spectra per sample were measured and averaged, with the baseline spectra of the blank subtracted before data processing. The secondary structure proportions were evaluated using DichroWeb and the CDSSTR algorithm with the reference set 6 (Miles et al., 2022; Sreerama & Woody, 2000) and a statistical amino acid molar mass of 115 Da.

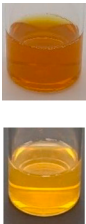
3. Results and discussion

3.1. Solubility and chemical stability of Cur

The solubility of Cur was determined using UV–Vis spectroscopy of ethanolic extracts from PPICur (calibration curve of Cur in EtOH in SI 2.2.3). A Cur loading mass of 80 mg was found to yield the highest Cur concentration in PPICur t0 (see SI 3.1). The mass concentration of Cur in PPICur t0 was 1.39 ± 0.093 mg/mL, and 0.0528 ± 0.0088 mg/mL in PPICur t24. The visual appearance of the two different states of PPICur is

Table 1

Visual appearances of PPICur t0 and t24 against two different backgrounds (top: white, bottom: black), concentration of Cur, binding efficiencies (BE), and loading capacities (LC) of PPICur t0 and t24. LC was calculated for the total amount of PPIp (LC_{tot}) and the proportionate amount of PPI (LC_{frac}).

	PPICur t0	PPICur t24
Pictures		
$\beta(\text{Cur})$ [mg/mL]	1.39 ± 0.0930	0.0528 ± 0.00878
BE [%]	17.41 ± 1.28	0.695 ± 0.109
LC _{tot} [%]	14.27 ± 0.0276	0.615 ± 0.00387
LC _{frac} [%]	37.2 ± 0.0720	1.76 ± 0.0111

shown in Table 1. HPLC analysis of the ethanolic extracts was performed to evaluate the chemical degradation of Cur. Traces of vanillin were detected in both the ethanolic extracts of PPICur and the pure Cur powder during calibration (chromatograms in SI 2.2.3 and 3.1). Therefore, no additional chemical degradation was observed, and Cur was considered chemically stable. The orange precipitate could be completely redispersed in EtOH and was also analysed via HPLC. Only Cur and traces of vanillin were detected (see SI 3.1). A full recovery of the initial amount of Cur ruled out the presence of other degradation products not detected by HPLC.

3.2. Characterisation of PPICur: SEC, SDS-PAGE, protein concentration, particle size, ζ , BE, and LC

Treating the protein with pH shifting and heating definitely causes some degree of aggregation (Jiang et al., 2018). The size distribution in commercial pea protein isolate powder, induced by the preparation process and loading with Cur, was examined using SEC and SDS-PAGE (see Fig. 1 and SI 3.2). PPIp was also measured for comparison. For PPIp, a low-intensity peak at the edge of the dead volume (around 42 mL) and a broad, higher-intensity peak between 90 and 125 mL were detected in the SEC. SDS-PAGE showed well-defined bands across the entire range of the molecular weight standard. In samples PPICur t0 and t24, a high-intensity peak around the dead volume (1), two smaller peaks at 65–70 mL and 85 mL (2,3), and a defined peak at 110 mL (4) were observed. Both chromatograms looked very similar; only the fractions producing the first two peaks were coloured (based on the optical appearance of the collected fractions). These peaks were collected and summarised into fractions I (coloured) and II (non-coloured). After 24 h, peak 1 in PPICur t24 decreased, while peak 2 increased, whereas peaks 3 and 4 remained unchanged. SDS-PAGE of PPICur at t0 and t24 appeared identical, showing an intense band above the standard and two bands around 25 kDa. Peak 1 and the first fraction of PPIp were too large to assign a specific molecular weight because they eluted with the dead volume of the SEC and did not migrate into the separation gel in the SDS-PAGE. Peak 2 was also unresolved by SDS-PAGE and eluted near the dead volume, indicating both were larger than 600 kDa (the SEC column's separation limit). Comparing the chromatograms of untreated and treated samples, the fraction larger than 600 kDa (at the edge of the dead volume) was already present in PPIp and became more prominent during treatment. Since only peaks 1 and 2 were coloured, it can be concluded that only the large protein fractions bind Cur, while the smaller ones do not contribute to Cur solubilisation. MS analysis of fractions I and II from PPI and PPICur showed both fractions contained protein mixtures, with no major compositional differences identified. It can be presumed that all peaks represent aggregates; no specific affinity of Cur for particular proteins could be deduced, but aggregate size was the key factor (see data in SI 3.2).

When analysing SEC and SDS-PAGE data, several factors must be considered: heating causes aggregation and gelling of proteins (Creusot et al., 2011; O'Kane et al., 2005; Sun & Arntfield, 2010). The heat denaturation temperature (T_d) and gelation concentration (β_g) are two important parameters describing these phenomena. Since most of the commercial PPIp is already denatured, T_d refers more to the breaking and unfolding of aggregates formed during extraction (Tang, 2021a; Tang, 2021b) rather than the unfolding of native structures. The gelling temperature (T_g) describes the temperature required to form heat-induced gels (Sun & Arntfield, 2011). Regarding the protein concentration, aggregates form below β_g , and a gel network develops above this concentration (Creusot et al., 2011; O'Kane et al., 2005). In the presented case, the PPIp concentration was 1% (w/v) (10 mg/mL), the ionic strength was 2.3×10^{-2} M, and the treatment temperature was 85 °C. Based on considerations in the literature (Creusot et al., 2011; O'Kane et al., 2005; Shand et al., 2007; Sun & Arntfield, 2010; Sun & Arntfield, 2011) regarding T_d , β_g , and T_g , it was concluded that soluble heat-

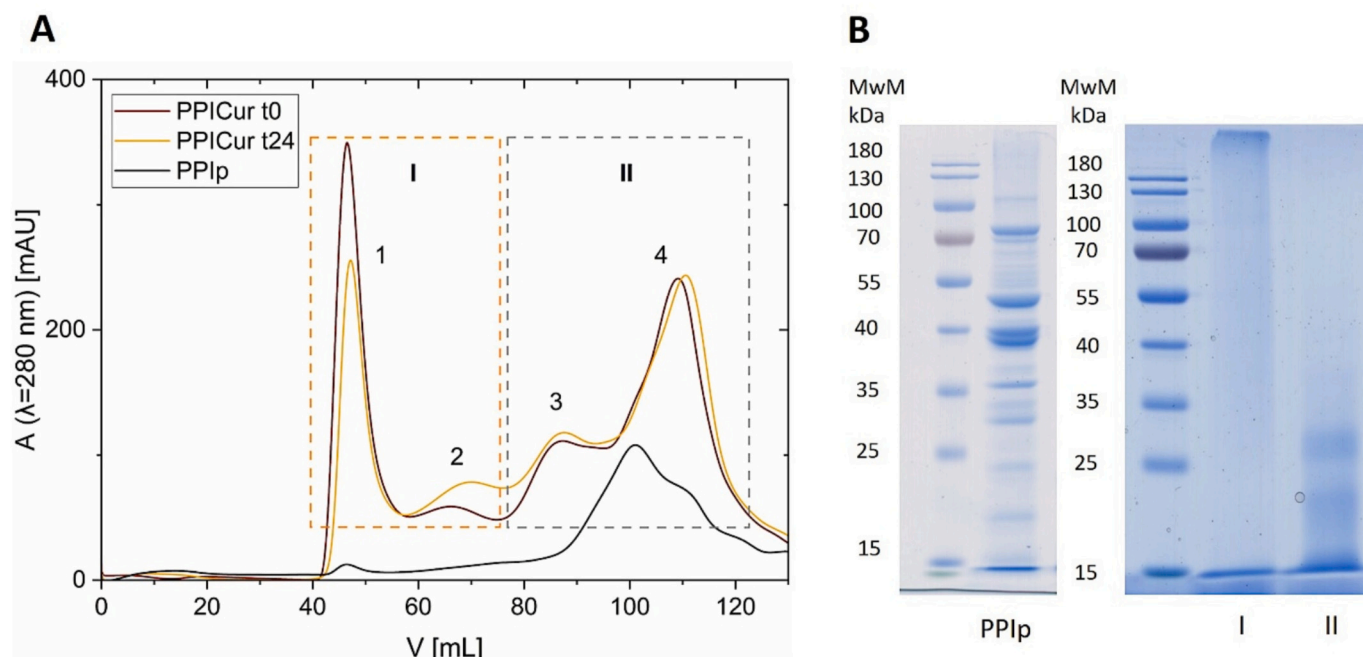


Fig. 1. Chromatograms of PPIp, PPICur t0, and PPICur t24 obtained from SEC (A). The peaks of PPICur t0 and t24 are summarised in fractions I (peaks 1 and 2, coloured) and II (peaks 3 and 4, not coloured). Electrophoretic patterns of PPIp and fractions I and II (B). PPIp was run before the SEC, and fractions I and II were obtained from the SEC and run afterwards.

denatured protein aggregates, rather than a gel, were formed. Messian et al. extracted globulins from pea flour and denatured them by heating to 92 °C. They also observed large soluble aggregates that did not migrate into the separation gel and remained in the stacking gel. Considering the data from SEC and SDS-PAGE, the formation of compact, soluble heat-induced aggregates was suggested (Messian et al., 2013). Peng et al. also observed an increase in intensity in the first peak in SEC when comparing unheated and heated pea protein isolates, attributing it to the formation of soluble, heat-induced aggregates (Peng et al., 2016).

To characterise the nanoparticles, NTA and ζ measurements were performed to determine the particle size and surface charge of PPIp, PPI, and PPICur at t0 and t24 (see Fig. 2A, B, and C). The solubility of PPIp was monitored throughout the entire preparation process (D). The solubility of PPIp after stirring for 30 mins in water was approximately 3 mg/mL, with 2 mg/mL being protein, and a mean particle size of 147 nm. When the pH was adjusted to 12, the solubility of PPIp increased to 9.1 mg/mL. The size distribution was broadened with diverging values for mean and mode particle sizes and an increased PDI. By adding NaOH, the pH was shifted far from the isoelectric point of the storage proteins, which constitute the majority in PPIp. The deprotonation of ionisable side chains induces repulsive electrostatic interactions. The globular proteins or aggregated structures are now unfolded or disrupted, becoming flexible, hydrated, and with hydrophobic binding sites made accessible (Jiang et al., 2018; Tang, 2021a).

Due to a high charge, the aggregates might not only disrupt but also elongate, as in the case of a polyelectrolyte in water. This explains the discrepancy between the mean (226 nm) and mode (137 nm) particle sizes, as well as the larger polydispersity index (PDI). Heating to 85 °C resulted in a slight increase in solubility to 9.7 mg/mL and decreased the particle size to 128 nm (mean) with a comparably low PDI. Wei Wang examined how various stimuli influence protein structure and aggregation. Tang et al. studied the thermal aggregation behaviour of soy protein, and Chen et al. adapted its influence on the performance of soy protein on Cur binding. According to their summaries and findings, heating the protein causes further denaturation and unfolding, resulting in increased surface hydrophobicity as the hydrophobic binding sites

become more exposed. Additionally, chemical cross-links are likely (Chen, Li, & Tang, 2015a; Tang et al., 2007; Wang, 2005). Following this explanation, we explain the reduction in particle size with the hydrophobic effect: large, loose structures dehydrate and shrink to form compact aggregates, minimising thermodynamically unfavourable interactions between hydrophobic surfaces and surrounding water (Chandler, 2005). The primary influence of electrostatic repulsion prevents aggregation driven by hydrophobic interactions (Grossmann & McClements, 2023). Solubility and particle size remained stable after cooling and returning to pH 7, resulting in partial neutralisation of the protein surface charge. The solubility of the protein and PPIp behaved consistently. Overall, about 99% of PPIp could be solubilised using the preparation protocol. Within 24 h of stirring, roughly 1% of the protein precipitated, as determined by elemental analysis. In the case of saturation with Cur, after cooling to room temperature, approximately 7% of the protein precipitated immediately. Within 24 h, 10% of PPIp, including 3% protein, precipitated along with Cur. This is the only case where protein solubility and PPIp solubility diverged. The final protein concentration was 6.94 mg/mL, and total PPIp concentration was 8.20 mg/mL. The increased precipitation of protein and PPIp with Cur could be attributed to reduced colloidal stability of protein particles when exposed to high Cur levels (Quan et al., 2019; Tang, 2020). The ζ magnitude increased with pH shifting and heating, from −43 mV (PPIp) to −47 mV (PPI). Loading with Cur did not significantly change it. Following the argumentation of Pan et al. (Pan et al., 2014), the increase in ζ magnitude is attributed to breaking the supramolecular structure of denatured protein aggregates and the subsequent formation of the nanoparticles. Bound Cur did not affect the surface charge of the nanoparticles. Within 24 h, all ζ -values decreased. ζ (PPIp) significantly dropped to −36 mV, and ζ (PPI t24) and ζ (PPICur t24) decreased to −44 mV and −41 mV, respectively. The trend was consistent across all samples, indicating that Cur precipitation could not explain the decrease in ζ magnitude. Whether changes in surface properties within 24 h could lead to Cur precipitation remains uncertain based on these results. Since particle size did not change significantly during this period, aggregation processes are unlikely to be responsible. BE and LC values are listed in Table 1. Monitoring the carrier concentration, LC was calculated for the

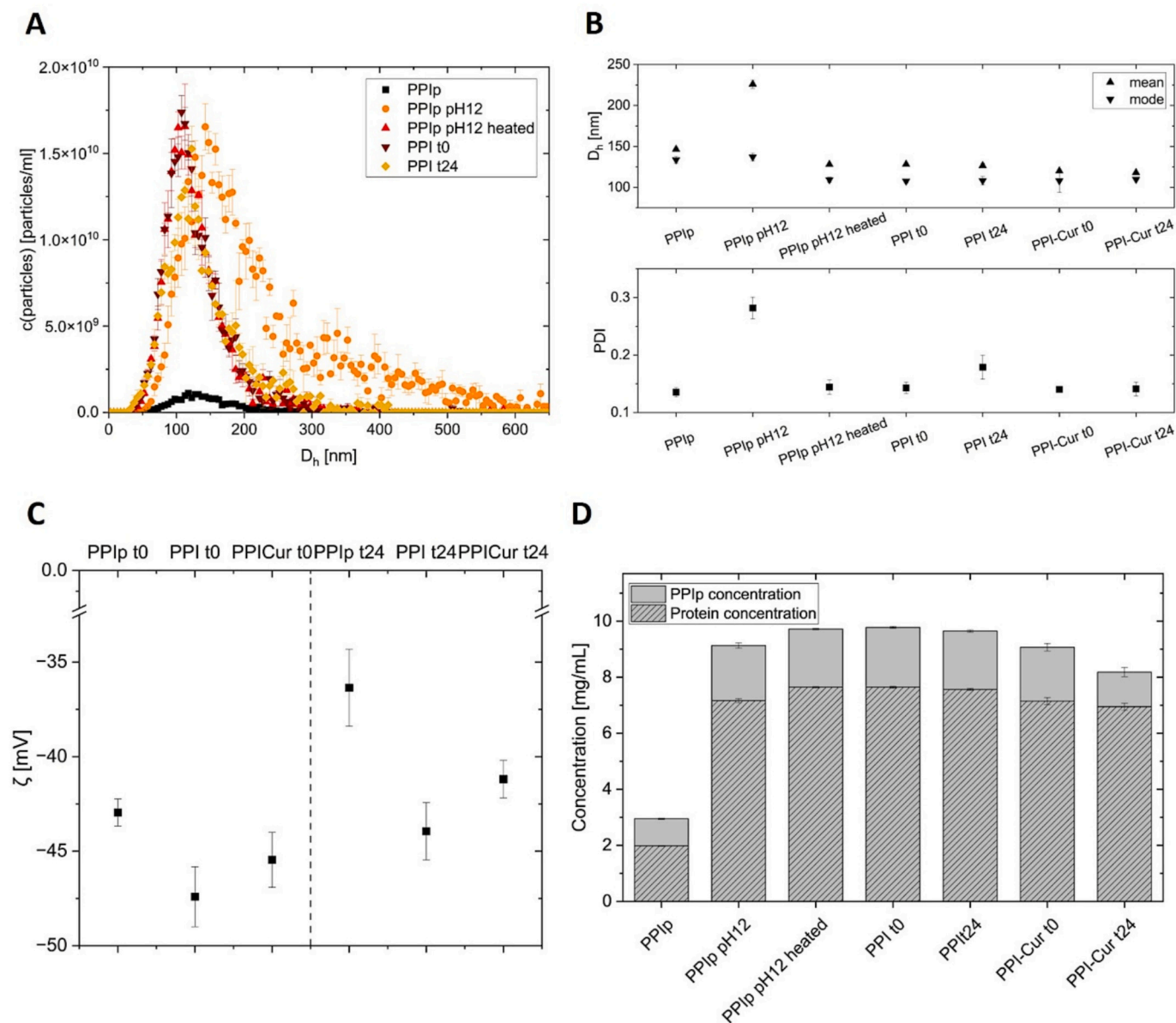


Fig. 2. Particle sizes (A, B), ζ (C), and protein and PPIp concentration (D) of the single steps in the preparation and PPICur t0 and t24.

whole PPIp (LCtot) and proportionately for the PPI (LCfrac), using the areas under the SEC curve (see SI 3.2). All LC values are comparable with literature reports, though the latter lack comments on stability (Guo, Bayram, et al., 2021; Ren et al., 2024; Vijayan et al., 2021).

This fundamental study demonstrated that Cur is bound to large soluble nanoaggregates formed through pH shifting and heating, without showing a preference for specific proteins. Cur is bound in a supersaturated state, and 96% precipitates again within 24 h under constant stirring, producing a stable solution of PPICur t24. The loading and precipitation of Cur did not alter the surface properties of PPI. Overall, 10% of the protein and 17% of PPIp precipitated alongside Cur.

3.3. Binding mechanism of Cur: XRD, UV-Vis & fluorescence spectroscopy, CD

3.3.1. XRD

The XRD patterns of PPICur t0 and t24, PPIp, and Cur are presented in Fig. 3A. Cur exhibited a characteristic XRD pattern with sharp reflexes, indicating its crystallinity. The diffractogram of PPIp showed no

distinct reflexes but broad humps indicating its amorphous state. The XRD patterns of PPICur t0 and t24 largely overlap and show both types of patterns. The reflexes at 31.7, 45.46, 56.45, 66.29, 75.29, and 84.08° were attributed to NaCl resulting from pH shifting (Guo, Bayram, et al., 2021; Rabiei et al., 2020). The absence of Cur-related reflexes in the diffractograms of PPICur samples was attributed to the amorphous binding of Cur to the protein in the literature (Guo, Bayram, et al., 2021; Patel et al., 2010; Ren et al., 2024; Sinha et al., 2007; Teng et al., 2012; Zhan et al., 2020). Because there is a significant concentration difference between the plant protein and the amount of bound Cur, caution should be exercised when interpreting amorphous structures as the sole indicator of Cur binding. In this case, it is more relevant to compare the supersaturated state (t0) and the stable state (t24), noting no differences between PPICur t0 and t24. The lack of crystalline Cur at t0 indicates that the supersaturated state is not due to coexistence of unbound crystalline Cur and Cur bound to PPI. The precipitated Cur was in an amorphous state (see Fig. 3B), indicating to rapid precipitation. These data suggest that the structural order of Cur has fully transitioned from crystalline to amorphous. A temporary increase in Cur solubility caused

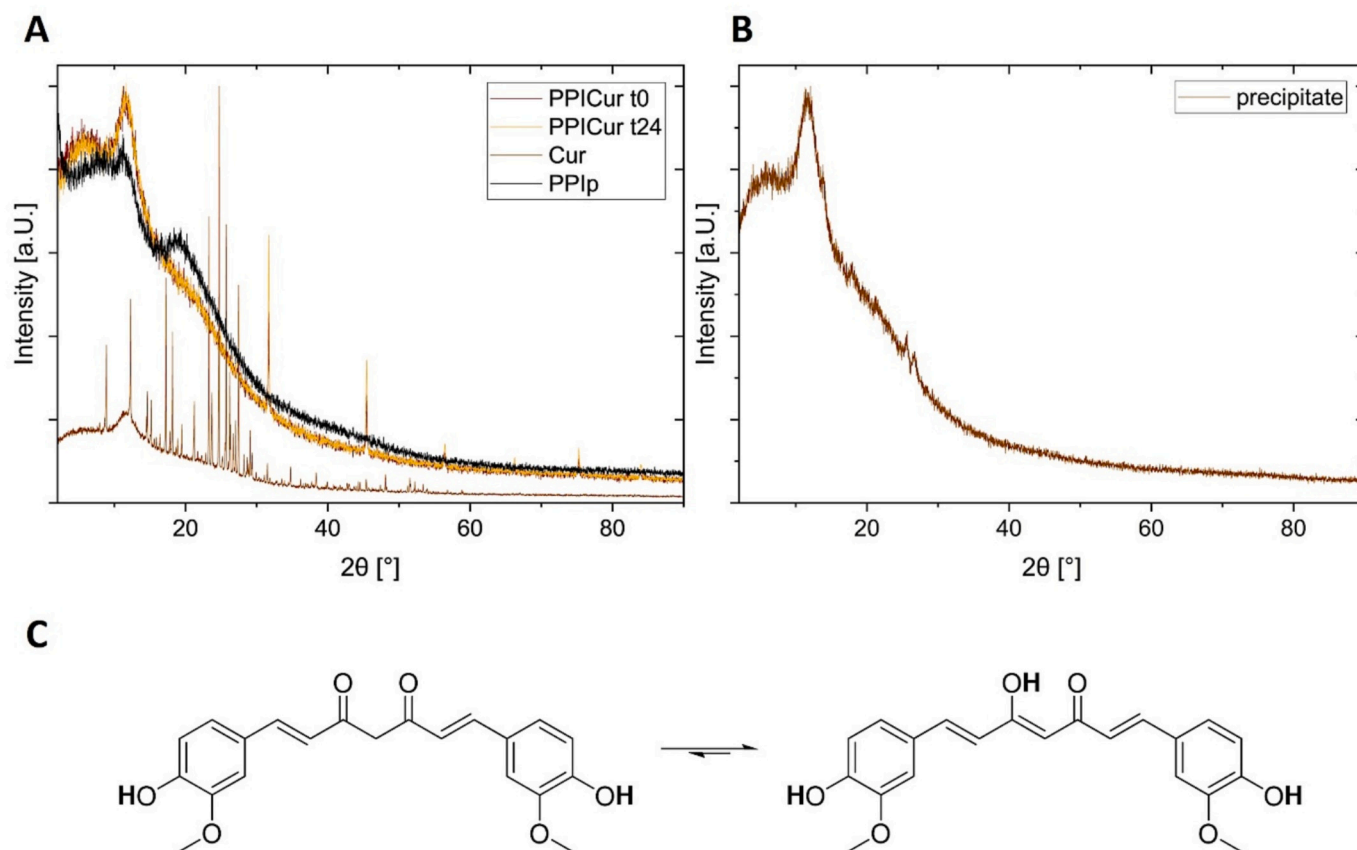


Fig. 3. XRD patterns of PPICur t0 and t24, PPIp, and Cur (A) and the precipitate (B). It should be noted that the XRD data for PPICur t0 and t24 (A) largely overlap. Keto-enol-tautomerism of Cur with pH-dependent protons marked in bold (C).

by unstable amorphous Cur formation can be ruled out, as pH shifting of Cur in water without PPIp resulted in only a minor amount of soluble Cur due to degradation and insolubility (see SI 3.3.1).

3.3.2. UV-Vis and fluorescence spectroscopy

The absorption properties of Cur in PPICur were evaluated using UV-Vis spectroscopy with PPI as the dilution medium and blank. The spectrum of PPICur t0 exhibited a distinct profile with an absorption maximum (λ_{max}) at 428 nm and a shoulder at 449 nm. The spectrum of PPICur t24 exhibited a less pronounced profile with λ_{max} at 410 nm (see Fig. 4A). The spectrum of PPICur t24 had a much lower absorption maximum due to the precipitation of 96% of Cur (see Fig. 4B). HPLC analysis confirmed the presence and stability of Cur in both samples, thus excluding changes in the absorption due to chemical degradation. All fluorescence measurements were performed at the same Cur concentration, with the proportion of organic solvent in the pure Cur sample below 1%. The fluorescence emission and excitation spectra of PPICur t0 and t24 exhibited maxima at 502 nm and 430 nm, respectively, regardless of the excitation or emission wavelengths (see Fig. 5A and B). However, the fluorescence intensity of the emission spectra of PPICur t0 was by a factor of 2.2 higher than that of PPICur t24. The intensity of the excitation spectra was higher by a factor of 1.8. Cur is a weakly fluorescent molecule in water (Barik et al., 2003) and exhibited a maximum emission around 550 nm and maximum excitation at 430 nm. In the excitation spectrum, an emerging shoulder is observed around 350 nm at a lower emission wavelength (see Fig. 5C). The UV-Vis spectrum showed a λ_{max} at 430 nm and a shoulder at 350–365 nm at a Cur concentration of 5 μM (see Fig. 4D). In the range of 225 to 325 nm, instrumental noise from an aged deuterium lamp is visible.

To understand the different spectral behaviours observed in UV-Vis, we examined Cur's molecular structure and its behaviour in solution

more closely. Cur's molecular structure (see Fig. 3C) is sensitive to the surrounding medium. Its absorption properties vary based on the degree of protonation, which depends on the pH and the balance of keto-enol tautomerism. Assuming Cur is fully protonated, the cis-keto-enol tautomer was reported to be predominant in all purely organic solvents analysed, due to intramolecular hydrogen bonds (Chignell et al., 1994; Khopde et al., 2000; Nardo et al., 2008; Patra & Barakat, 2011). Cur shows a negative solvatochromic effect in nonpolar solvents and a positive solvatochromic effect in polar solvents, resulting in a maximum absorption range of 408–440 nm. The fluorescence emission spectra follow a similar trend, with blue-shifted emission maxima in nonpolar solvents and red-shifted maxima in polar ones (Indira Priyadarsini, 2009; Nardo et al., 2008; Patra & Barakat, 2011). However, as the water content increases in an aqueous mixture of EtOH, the equilibrium shifts from the keto-enol form toward the diketo form (Kazakova et al., 2022; Manolova et al., 2014). A rising shoulder was detected at 355 nm, indicating an increasing amount of the diketo form (Manolova et al., 2014). The fluorescence emission is blue-shifted with increasing amounts of the diketo form (Bhatia et al., 2016).

In addition to the position of λ_{max} , the various shapes of UV-Vis spectra are also discussed in the literature. While a broad absorption band is usually observed with more polar solvents, fine structuring and shoulders in the absorption band frequently occur with more nonpolar solvents (Chignell et al., 1994; Nardo et al., 2008; Patra & Barakat, 2011). Chignell et al., Khopde et al., and Nardo et al. observed some structuring, similar to that from PPICur t0, in the UV-Vis spectra of Cur solubilised in organic solvents of different polarities and attributed it to different isomers of the keto-enol form being present. The fluorescence excitation and emission spectra provided no evidence of coexisting isomers, which would mean that all excited isomers have similar fluorescence properties, or all isomers produce the same excited state

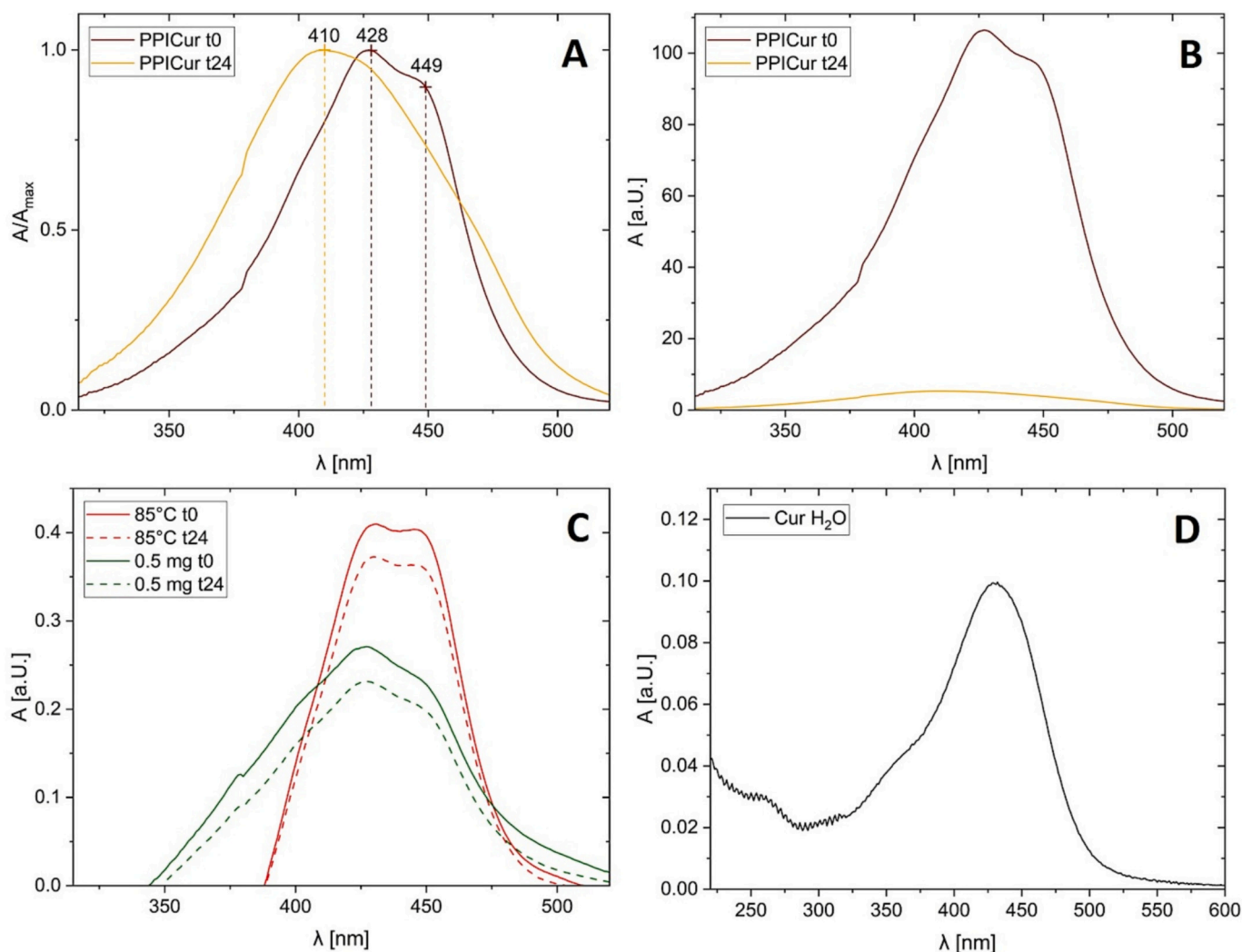


Fig. 4. UV-Vis spectra of PPICur t0 and t24 with normalised absorption (A) and concentration correction (B), prepared by heating (no pH shifting) (red) and without saturation of Cur (green) (C). Cur in water at a concentration of 5 μ M (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Chignell et al., 1994; Khopde et al., 2000; Nardo et al., 2008). This was also observed in the fluorescence measurements of PPICur t0 and t24 presented here.

Moving from organic solvents to aqueous systems, Cur has been studied in the context of proteins and surfactants in the literature. Adhikary et al. examined Cur in anionic, cationic, and neutral micelles. They observed an intense absorption peak around 423–432 nm for all surfactants and a shoulder around 442–445 nm for Triton X and dodecyl trimethyl ammonium bromide (DTAB). They assigned the shoulder to vibronic transitions of Cur and compared it with the absorption spectra of Cur in chloroform and toluene. They concluded that environments similar to those in nonpolar, aprotic solvents exist for Cur in Triton X and DTAB micelles. The shoulder was absent for the third surfactant SDS. The loss of structure in SDS micelles was due to a strong interaction between Cur and water in the Stern layer of the micelle. The maximum fluorescence emissions were at 498 nm and 501 nm for DTAB and Triton X, respectively, and 548 nm for SDS. The positions of λ_{max} were attributed to varying degrees of interaction with water and, thus, a location of Cur inside the micelle (in the case of Triton X and DTAB) or closer to the outside (in the case of SDS) (Adhikary et al., 2010; Khopde et al., 2000). No results were found that indicate the presence of multiple tautomers in micelles. Dutta et al. observed varying proportions of both Cur tautomers in the presence of different anionic surfactants below and above

the critical micellar concentration (Dutta et al., 2013). The interaction between Cur and proteins was studied in more detail for serum albumins and casein than for plant proteins, mainly due to the lack of characterisation and the commercial production of plant proteins as impure powders. The main finding, regardless of the protein type, was binding to hydrophobic sites within the proteins, as discussed in the introduction. The UV-Vis spectra exhibited λ_{max} between 425 and 440 nm, mostly with structuring, and the fluorescence emission spectra showed a blueshift to 500–510 nm compared to the maximum at 550 nm of Cur in water. In the papers revised for this publication that address Cur binding to proteins, the behaviour of keto-enol tautomerism and its balance upon binding to (plant) proteins was never discussed (Barik et al., 2003; Chen, Li, & Tang, 2015b; Guo, Su et al., 2021; Hudson et al., 2019; Liu et al., 2024; Ren et al., 2024; Sahu et al., 2009; Tang, 2020; Tapal & Tiku, 2012; Teng et al., 2012; Wang et al., 2024; Yang et al., 2013; Zhan et al., 2020).

Interestingly, when PPIp was not saturated with 80 mg of Cur, but only 0.5 mg of Cur was added (which roughly corresponds to the solubility of Cur in PPICur t24), λ_{max} at 428 nm and the shoulder at 449 nm, as well as a pronounced shoulder at 410 nm, were visible. Additionally, the shape of the absorption spectrum remained unchanged for t0 and t24. When the PPIp dispersion was heated to 85 °C and Cur was added without pH shifting, the absorption band remained unchanged for t0 and

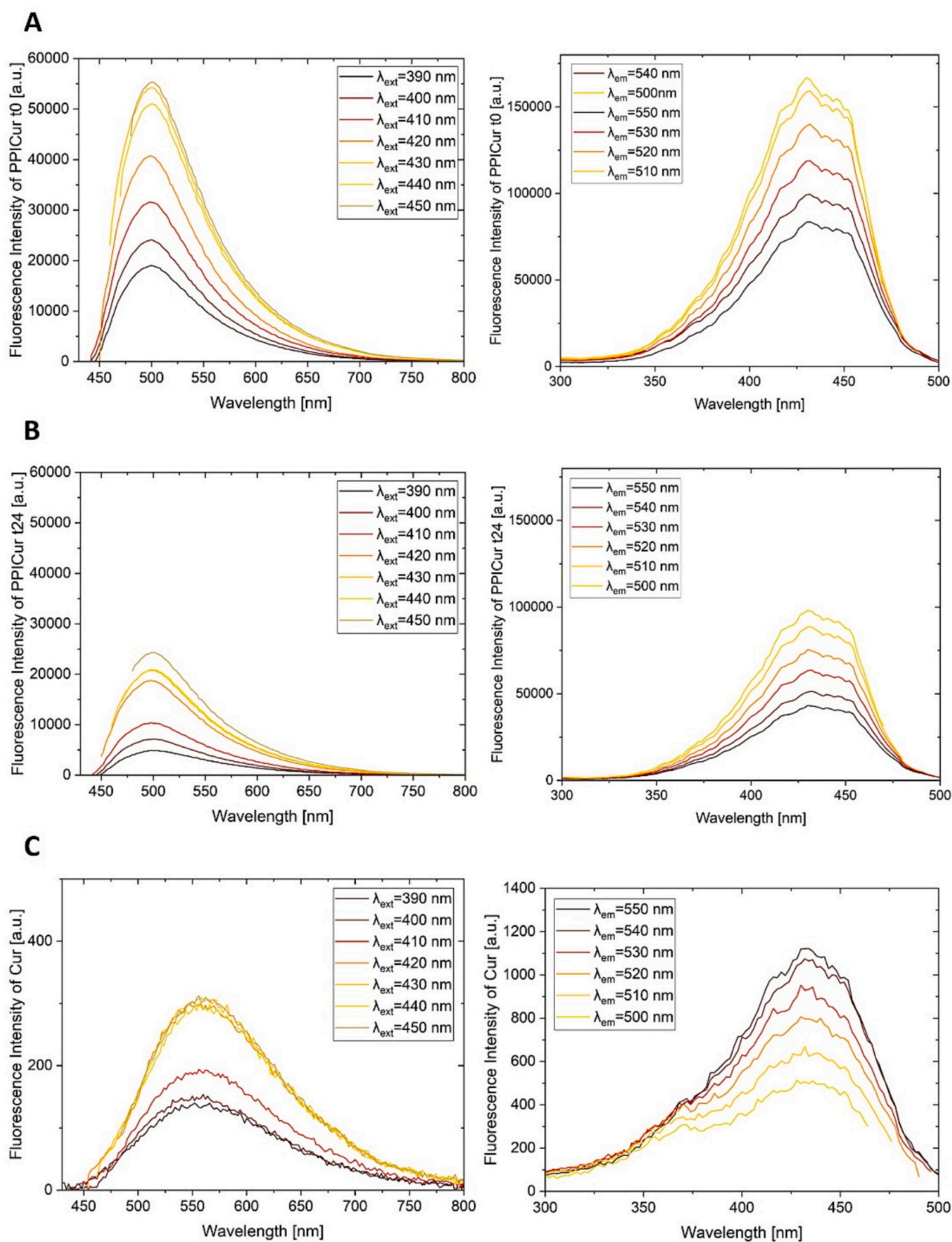


Fig. 5. Fluorescence excitation and emission spectra of PPICur t0 (A), t24 (B), and Cur in water (C). The emission spectra were measured with excitation wavelengths between 390 and 450 nm. The excitation spectra were measured with emission wavelengths between 500 and 550 nm.

t24, displaying only λ_{max} at 428 nm and the shoulder at 449 nm (see Fig. 4C). In both cases, only a small amount of Cur still precipitated over 24 h.

The key factors from the above considerations are summarised as follows: Cur is insoluble in water and can be solubilised with proteins through hydrophobic interactions and hydrogen bonding at hydrophobic binding sites within the proteins. A supersaturation of Cur is generated via a pH shifting method. Within 24 h, 96% of the initially solubilised Cur precipitates again. The absorption spectra of PPICur t0 and t24 differ significantly in intensity, shape and the position of λ_{max} . The fluorescence excitation and emission spectra show no difference in the position of λ_{max} for PPICur t0 and t24. Cur can undergo keto-enol tautomerism and exhibits a solvatochromic effect, which depends on the solvent's polarity.

Considering all experiments and information from the literature presented above, the possible scenarios for Cur binding to PPI are as follows: The interaction between Cur and the protein isolate, when prepared through a pH shifting method, occurs at highly alkaline pH. At this pH, the aggregate structure is disrupted, and Cur becomes deprotonated. Consequently, no inner or outer layers of the nanoparticle exist, and Cur binds randomly to all accessible sites. When the pH is shifted back to pH 7, compact aggregates form, and Cur is located in different positions within and around the nanoaggregates. This includes internal binding, such as encapsulation with a protein environment surrounding the Cur molecule, and external binding, resulting in increased contact with the surrounding bulk water. This has been suggested in the literature but not yet experimentally confirmed (Mehryar et al., 2021; Sadeghi et al., 2014; Tang, 2020). The UV-Vis spectra of PPICur t0 and t24 indicated that the PPI surface is supersaturated with Cur molecules at t0. The maximum absorbance at 428 nm, with a shoulder at 449 nm, indicates a rather polar environment, suggesting hydrogen bonding and contact with water. Referring to the binding situation in micelles, the external binding of Cur is indicated. Then, the sample is stirred for 24 h, during which Cur precipitates due to tight surface packing, energy input, and stacking of Cur molecules, analogous to nucleation theory. λ_{max} of PPICur t24 shifts to 410 nm, with shoulders at 428 and 449 nm, because the internally bound Cur molecules are in a nonpolar environment and become more prominent after the precipitation of the externally bound Cur molecules. Since not all of them precipitate, their contributions at 428 and 449 nm are still visible. As the absorption at t0 and t24 is in different orders of magnitude, it is assumed that the absorbance at 410 nm is also present at t0 but is masked by the larger absorption at 428 nm. These two modified experiments support this idea: when only a small amount of Cur (0.05 mg/mL) is used to load PPI, fewer molecules are bound externally, and the contributions of all binding environments are visible at t0. Only a small amount of Cur precipitates over time, as the surface of PPI is not supersaturated, reducing the aggregation of external Cur molecules. If PPICur is prepared not via a pH shifting method but solely by heating PPIp to 85 °C, no disruption of the protein should occur, and internal binding should be absent. This is evident in the UV-Vis spectra at t0 and t24, showing peaks only at 428 and 449 nm, but not at 410 nm (see Fig. 4C). One fact that challenges this hypothesis is the position of peak maxima in the fluorescence excitation and emission spectra. If Cur is present in different environments and does not undergo keto-enol tautomerism or isomerisation, its corresponding fluorescence peaks should also shift, but they do not. This circumstance, however, does not necessarily weaken the proposed binding scenario for two reasons: first, water quenches Cur's fluorescence (Barik et al., 2003). The quantum yield of externally bound Cur could therefore be significantly reduced, preventing the external binding state of Cur from being visible in the fluorescence measurement. Second, steady-state fluorescence measurements provide a statistical average of various binding states and thus are not suitable for resolving different, simultaneously occurring binding states within a sample that originates from a heterogeneous protein environment. Although the Cur concentration is constant in the fluorescence measurements of PPICur t0 and t24, the intensity in the

emission spectra of PPICur t0 was by a factor of 2.2 higher than that of PPICur t24. This may suggest the rearrangement of Cur bound to the carrier and static quenching of Cur's fluorescence through the stacking of Cur molecules or its interaction with aromatic amino acid residues in the carrier. Additionally, an inner-filter effect could contribute to the lower fluorescence intensity observed for PPICur t24. These considerations mean that the present measurements agree with the consensus in the literature that Cur is bound to the protein isolate carrier, but they neither strengthen nor weaken the thesis of internal and external binding.

Another scenario involves keto-enol tautomerism. The red-shifted shoulder of the peak at 428 nm could be caused by either a vibrational transition stabilised by H-bonding or the presence of different enol isomers. As the pH is rapidly adjusted back to pH 7, the reprotonated enolate molecules do not have enough time to adapt to the environment, leading to the formation of the enol tautomer with different isomers. Molecules bound to the surface gradually isomerise to the diketo form because this tautomer is favoured at higher water content. Since this tautomer tends to aggregate and the surface is supersaturated, externally bound Cur precipitates over time. This process could be accelerated by the energy input from stirring. Within 24 h, the equilibrium shifts toward the diketo form, which agrees with a λ_{max} at 410 nm. Due to the different microenvironments with varying polarities in the protein, both diketo isomers and enol isomers could be stabilised, making a clear assignment impossible. The fluorescence spectra cannot resolve these different isomers, as discussed in the literature (Chignell et al., 1994; Khopde et al., 2000), and only show a blueshift in the emission spectrum compared to Cur in water, suggesting the binding of Cur to the aggregate. Kazakova et al. revealed a concentration-dependent adsorption of Cur onto fumed silica nanoparticles – at low Cur concentrations, the enol form adsorbed as a monolayer on the nanoparticle surface and at high Cur concentrations, aggregates of the keto form adsorbed, forming a bilayer. This was confirmed through UV-Vis spectroscopy and quantum chemical calculations (Kazakova et al., 2022). These results support the idea that Cur undergoes isomerisation depending on the concentration. Since the diketo form lacks an extended electron system, it should not absorb in the same spectral range as the enol form, which does not support this scenario. The shift to 410 nm would only be possible if the electron system were somehow extended. The two modified experiments also do not support this consideration.

3.3.3. CD spectroscopy

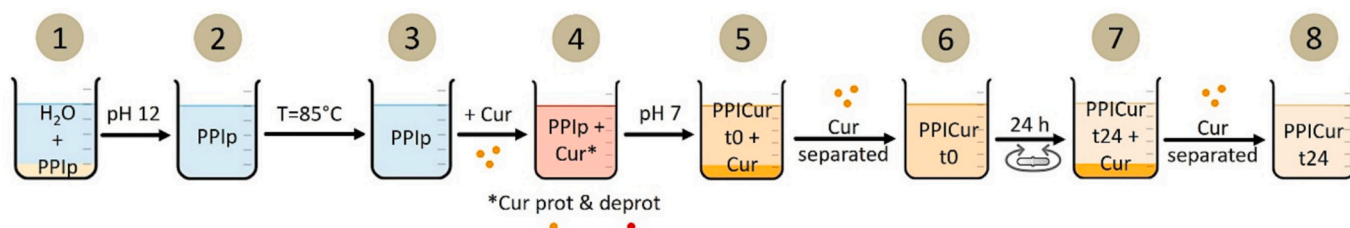
To analyse protein folding during preparation and loading of PPI with Cur, as well as to evaluate whether dynamic protein folding might explain the supersaturated system, CD measurements were performed. The secondary structure components derived from these can be found in SI 3.3.3. No significant differences in the amounts of secondary structure elements of PPIp and PPI t0 and t24 were observed before and after pH shifting and heating (dispersed in water for 30 mins). When loaded with Cur, a slight increase in the proportion of α -helix (approximately from 5% to 7%), a decrease in β -sheet and β -turn (approximately from 27% to 21% and from 17% to 14%, respectively), and a relatively more substantial increase in the proportion of random coil (approximately from 50% to 58%) were observed compared to the pure protein aggregates. The distribution suggests that upon Cur binding to the protein, β -structures became more disordered, with only minor parts refolding into α -helices. Ren et al. applied a slightly modified pH shifting method to a commercial pea protein isolate and studied the binding of Cur via CD. They observed an increase in α -helix content and a decrease in β -sheet content. The change in secondary structure was attributed to hydrogen bond formation and hydrophobic interactions between the pea protein and Cur (Ren et al., 2024). Zhi et al. studied the influence of pH shifting, ultrasound, and heating on the techno-functional properties of a commercial pea protein isolate and observed significant changes in the protein's secondary structure after the treatments (Zhi et al., 2022).

In the present case, there was no significant difference between PPICur t0 and t24. These results led to the conclusion that the precipitation of Cur cannot be attributed to a change in protein structure. Cur separates from the protein carrier over time due to aggregation or isomerisation of supersaturated Cur on the outer layer of the nanoparticles. Comparing the samples PPIp, PPI t0, and PPI t24, it can be concluded that the preparation method affects the aggregate size (see Fig. 2) but does not change the internal protein folding. The degree of denaturation remains high, as indicated by a low content of α -helix (Pezeshk et al., 2021). Therefore, the terms ‘unfolding’ and ‘refolding’ may not be suitable when describing the influence of the pH shifting method. It might be better described as the disruption of existing aggregates by inducing electrostatic repulsion and rearranging aggregates when decreasing ionic interactions. This also means that the system is not dynamic. This fact would make the term “nanoaggregate” more accurate than “molten globule” (Jiang et al., 2018; Qiao et al., 2023; Zhi et al., 2022) for plant protein powders undergoing pH shifting and exhibiting this behaviour. Concluding the CD analysis, different trends are reported in the literature. CD data and explanations derived from them are highly dependent on the pea protein product studied and its denaturation state. Additionally, the uncertainty in the molar masses used, resulting from commercial products, affects the fitting. Therefore, the data within one study are comparable, but absolute numbers differ depending on the product studied.

3.4. All put together: mechanism elucidation

Summarising the experimental results and current knowledge in the literature, a schematic mechanism including all steps of the preparation and interactions between PPI(p) and Cur is illustrated in Fig. 6. PPIp is dispersed in water at a concentration of 1% (w/v) (10 mg/mL), with a protein concentration of 2 mg/mL. Due to the industrial extraction process, PPIp exists as aggregates composed of a mixture of denatured proteins and organic impurities (1). By adjusting the pH to 12, the ionisable groups become charged, causing electrostatic repulsion and disassembly of the aggregates (2). Heating to 85 °C increases surface hydrophobicity by exposing previously hidden hydrophobic groups to the surrounding medium (3). The pH adjustment and heating significantly improve PPIp solubility. Additionally, heating results in a more compact structure of the aggregates. When Cur is added in a quantity exceeding its solubility in alkaline water, it exists in equilibrium between deprotonated and protonated forms. Through electrostatic and hydrophobic interactions, Cur interacts with the detangled aggregates and is stabilised (4). When the pH is readjusted to pH 7, Cur is reprotonated and unbound Cur precipitates. The ionisable groups of the protein are protonated, reducing electrostatic repulsion within the aggregates. Cur binds to both the interior and surface of PPI, with the surface being highly supersaturated. The protein aggregate conformation slightly changes upon Cur loading, as indicated by CD analysis (5). The precipitated Cur is separated from PPICur t0 (6). 96% of the

Macroscopic processing steps:



Nanososcopic images of the individual processing steps:

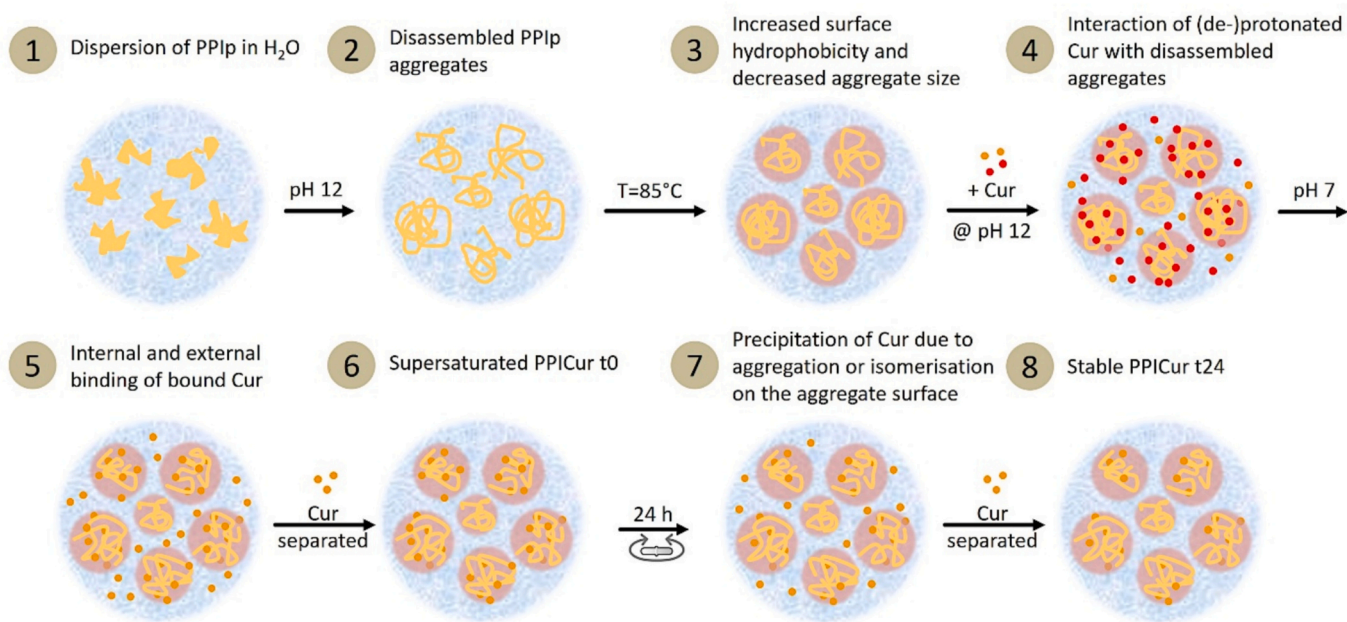


Fig. 6. Representation of PPICur preparation (upper part, macroscopic processing steps) and schematic nanoscopic images of the individual processing steps (lower part). Yellow lines refer to (disassembled) protein aggregates, the pale red background represents increased surface hydrophobicity of protein aggregates, and red and orange dots refer to deprotonated and protonated Cur molecules, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

externally bound Cur precipitates from the carrier due to aggregation or isomerisation within 24 h of stirring (7). The precipitated Cur is again separated from the PPICur t24 (8).

The relaxation of supersaturation could not be attributed to a breakdown of the carrier structure, changes in its internal structure, degradation of the compounds, or crystalline species of Cur present. PPIp underwent significant modifications during preparation. The precipitation of Cur from the nanoaggregate is attributed solely to the polyphenol itself. This may result from the aggregation of Cur, located at the supersaturated surface. It may also stem from the isomerisation of Cur, depending on the time and environment surrounding the Cur-loaded carriers. Additionally, minimal changes in the distribution of protein aggregates, as determined by SEC, could serve as a seed for spontaneous precipitation. This mechanism generally applies to legume proteins, as they consist of the same storage proteins (Jiang et al., 2018). We propose that this schematic mechanism could also be relevant for industrial protein isolates from other plant classes, provided that processing parameters are tailored based on the isoelectric points of the

significant protein fractions, heat denaturation temperature, and critical gelling concentration. Other commercial plant protein isolates are also partially denatured, as extraction typically induces some degree of denaturation (Tanger et al., 2020). To restore the functionality of these raw materials, their properties must be made accessible by disrupting aggregates through external stresses such as pH changes, temperature shifts, and shear stress. If the aggregate structure is not disrupted during preparation, purely external binding is expected, known as complexation (Tang, 2020). If the aggregate structure is disrupted, the binding mechanism of Cur or polyphenols should typically follow the same rules proposed above. Since the polyphenol is incorporated into a disrupted, then reassembled structure, it is illogical to expect purely internal or external binding; it will always be a mixture of both. To the best of our knowledge, the supersaturation and, thus, only kinetic stability of plant protein-Cur aggregates or complexes have not yet been investigated.

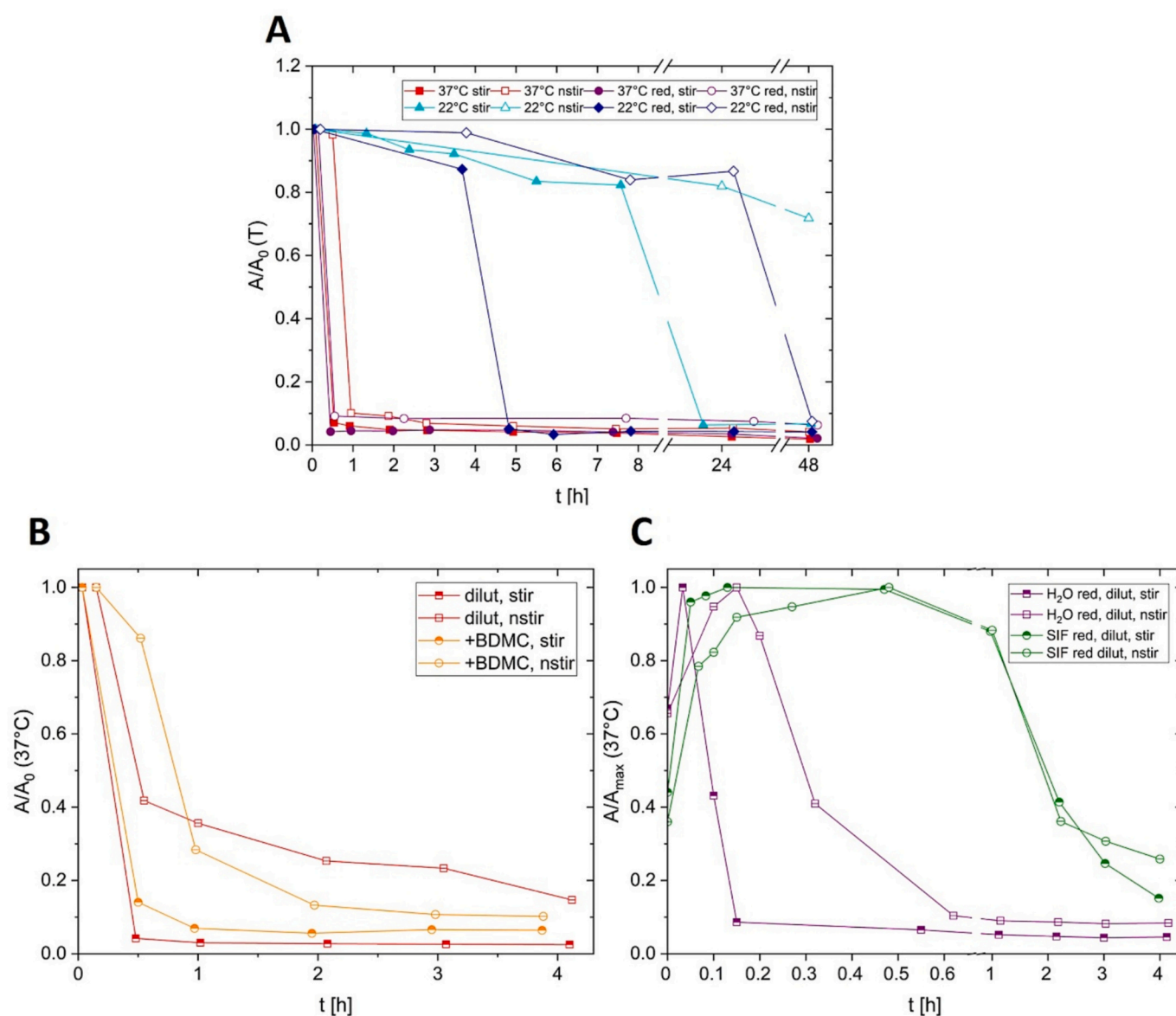


Fig. 7. Kinetic profiles and release kinetics of PPICur t0 with different influencing factors under stirred (stir) and unstirred (nstir) conditions. A: Freshly prepared and redispersed (red) PPICur t0 at 22 and 37 °C conditions. B: Freshly prepared PPICur t0 at 37 °C diluted (dilat, 1:10) and with the addition of bisdemethoxycurcumin (BDMC). C: Release kinetics at 37 °C of redispersed PPICur t0 in H₂O and simulated intestinal fluid (SIF). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Kinetic profiles and stabilisation approaches for PPICur t0

The stability of nutraceutical formulations is a key factor that determines their suitability for use. PPICur t0 is only kinetically stable, and β (Cur) changed within 24 h. The precipitation kinetics depend on temperature and stirring (see Fig. 7A). Under constant stirring at 22 °C, about 80% of β t0(Cur) remained in solution for 8 h. Within 24 h, 96% of β t0(Cur) precipitated (filled turquoise triangles). If the same sample was not stirred, 80% of β t0(Cur) remained in solution after 24 h and 70% after 48 h (blank turquoise triangles). When the temperature increased to 37 °C, the same trend was observed but over a shorter period. In the stirred sample, 96% of β t0(Cur) precipitated within 30 mins, and in the non-stirred sample, the same amount precipitated within 1 h (filled and blank red squares, respectively). HPLC analysis confirmed the stability of Cur at 37 °C (see SI 3.5). The pH value changed by between 1 and 3% over time (data not shown). Regarding its use as a food dye, supplement, or drug, PPICur will be supplied in powder form. Therefore, the kinetic stability was assessed with freeze-dried and redispersed powders. PPICur t0 could be redispersed by 80–90% (see SI 3.5). In all freeze-dried and redispersed samples (purple and dark blue symbols), Cur precipitated following the same trend but at a faster rate compared to freshly prepared samples. This may be due to the drying method and could be improved through a faster process, such as spray-drying, and optimisation of parameters (Desobry et al., 1997; Dong et al., 2022; Fong et al., 2015).

Based on the results discussed in the previous sections, the precipitation of Cur was linked to either the emerging contact of densely packed Cur molecules on the surface of the protein carrier, which then led to aggregation and precipitation, or the isomerisation of Cur, followed by aggregation and precipitation. Both processes could be accelerated by stirring and raising the temperature.

To maintain supersaturation in biorelevant systems, two approaches were assessed. The contact between supersaturated nanoparticles could be minimised by diluting the sample. This was tested on freshly prepared samples (B, red symbols) and redispersed samples (C, purple symbols), both kept at 37 °C. The dilution factor was 1:10. No delay in precipitation was observed for either the stirred or the redispersed samples. Only for the freshly prepared, non-stirred diluted sample was precipitation slowed; after 1 h, about 40% of the initially solubilised Cur remained in solution, compared to roughly 10% in the other samples. This approach could also work for redispersed, non-stirred samples with optimised drying and improved redispersibility.

Another approach to slow down precipitation involved adding an impurity. Following the approach of Heffernan et al. (Heffernan et al., 2018), the addition of BDMC at 300 μ M was tested on freshly prepared samples without dilution. A deceleration of precipitation was observed, with 30% of Cur remaining in solution after one hour. Nucleation is probably slowed down in the same way as described by Heffernan et al. BDMC can also act as a stacking breaker and slow down the Cur accumulation on the supersaturated surface. When Cur is used as a supplement, it is taken orally, passes through the gastrointestinal tract, and is absorbed in the small intestine. Assuming that PPICur powder is protected and reaches the small intestine, the release profile in simulated intestinal fluid (SIF) was evaluated (see Fig. 7C, green symbols). Unlike other kinetic profiles, absorption in this case was measured from the very beginning rather than from when all PPICur was fully solubilised. While both dissolution and precipitation occurred rapidly in pure water, these processes were significantly slowed in the SIF medium. After one hour, 90% of Cur remained in solution, and after 4 h, 30% of Cur was still solubilised in the non-stirred sample. SIF contains bile salts as bio-surfactants, which form micelles that can either incorporate the released Cur or prolong nucleation. Both mechanisms extended the precipitation time. The slower dissolution could be due to competition between Cur loaded onto the protein carrier and incorporation into bile salt micelles (Chen, Mosquera-Giraldo et al., 2015; Dima et al., 2021). Considering all three effects – dilution, a mixture of curcuminoids similar to those in

turmeric powder, and the presence of naturally occurring stabilisers in the target medium – we expect a significant slowdown of precipitation in *in vitro* experiments. Whether this enhances Cur's bioaccessibility and bioavailability cannot be concluded from these data alone. This requires cell uptake and *in vivo* testing (Hu et al., 2023).

Shifting from stability analysis to a pharmaceutical perspective, the precipitation profiles can also serve as a method to evaluate delivery performance through release profiles. A rise in solubility above the drug's equilibrium solubility, followed by rapid precipitation, is known as the spring effect. Extending the metastable solubility of a drug by adding precipitation inhibitors is termed the parachute effect. This behaviour is associated with supersaturated drug delivery systems (SDDSs) (Brouwers et al., 2009; Joshi & Sangamwar, 2018), and based on the results, PPICur t0 can probably be classified as such. Another release phenomenon is burst release, which involves the immediate release of a drug from the carrier upon placement in the target medium (Huang & Brazel, 2001). Various reasons for this have been discussed in the literature. Mehryar et al. prepared sunflower protein isolate nanoparticles loaded with Cur and observed burst release in PBS in the presence of TWEEN. They attributed the burst release to a burst of the protein matrix and the binding of a large amount of Cur to the exterior of the nanoparticle (Mehryar et al., 2021). They referenced Sadeghi et al., who proposed a biphasic release pattern of Cur from albumin nanoparticles, resulting from both external (faster release) and internal (sustained release) binding of Cur (Sadeghi et al., 2014). Teng et al. observed similar behaviour with Cur-loaded soy protein nanoparticles and attributed it to swelling and breakage of the protein matrix (Teng et al., 2012). Jithan et al. and Rahman et al. observed burst release from Cur-loaded albumin nanoparticles and solid lipid nanoparticles, respectively (Jithan et al., 2011; Rahman et al., 2023). Conversely, a slower release has also been reported for Cur-loaded carriers. Ye et al. prepared kafirin and zein composite nanoparticles and monitored Cur release during simulated digestion. They observed low release during the saliva and gastric phases, with an increase in the intestinal phase after 1 h (Ye et al., 2024). Mohammadian et al. discussed the release properties of Cur from different carrier systems and their varying behaviours in simulated gastrointestinal fluids (Mohammadian et al., 2024).

Both discussed mechanisms align with the kinetic profiles observed in Fig. 7, depending on lower or higher temperatures and energy input through stirring. The release of Cur from its carriers is typically assessed after solvent removal and dispersion of the powder in the target medium. The kinetic stability of Cur binding before removing the solvent is rarely studied. Therefore, its release behaviour has not been linked to its kinetic binding to the protein carrier, although it is known that pH shifting and solvent methods induce supersaturation (Joshi & Sangamwar, 2018).

4. Conclusion

A comprehensive concept is introduced to explain how Cur binds to nanoparticles formed from plant proteins, specifically PPIp, using a pH-driven and heating method. Along with existing literature, it provides detailed insights into the preparation steps and the stability of PPICur t0. SEC and SDS-PAGE data confirmed the formation of large, soluble protein aggregates. The binding of Cur was not dependent on specific proteins but rather on aggregate size. PPICur t0 is only kinetically stable, with 96% of the initially bound Cur precipitating within 24 h. The supersaturation and subsequent precipitation of Cur were examined using UV–Vis and fluorescence spectroscopy and linked to the aggregation or isomerisation of Cur molecules on the surface of the protein carrier. Analysis of the precipitation behaviour showed accelerated precipitation when transitioning from room temperature to body temperature. The precipitation was slowed by diluting the sample, adding bisdemethoxycurcumin, and releasing it in SIF medium. The next step is to stabilize the Cur concentration at PPICur t0. By integrating existing

literature with additional experiments from this study, significant progress was made in understanding plant protein carrier systems prepared via a pH-driven method. By framing the findings from a pharmaceutical perspective, the study also contributes to interdisciplinary research on stabilising SDDS. Emphasising the formulation's kinetic and stable states enables various applications of the same product in the food and pharmaceutical industries. A thorough understanding of molecular interactions enables both sectors to optimize formulations in a targeted manner, whether for food colorants, food delivery systems, nutraceuticals, or pharmaceuticals.

CRedit authorship contribution statement

Lea Rohr: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nadine Stefan:** Investigation. **Astrid Bruckmann:** Investigation, Data curation. **Didier Touraud:** Supervision. **Axel Dürkop:** Writing – review & editing, Methodology, Conceptualization. **Remco Sprangers:** Writing – review & editing, Methodology, Conceptualization. **Werner Kunz:** Writing – review & editing, Supervision.

Compliance with ethical standards

This article does not contain any studies with human or animal subjects performed by any of the authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We acknowledge the help of Martin Schmid, Institute of Inorganic Chemistry, University of Regensburg, D-93040 Regensburg, Germany, for the XRD measurements and Barbara Baumann, Faculty of Chemistry and Pharmacy, University of Regensburg, D-93040 Regensburg, Germany, for performing the elemental analyses. Further, we want to acknowledge the possibility of performing measurements on the NTA device at the chair of Joachim Göpferich, Institute of Pharmaceutical Technology, University of Regensburg, D-93040 Regensburg, Germany. We also thank Sabrina Zölch, Institute of Pharmacy, University of Regensburg, D-93040 Regensburg, Germany, for providing chemicals and instructions for preparing SIF.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2026.149156>.

Data availability

Data will be made available on request.

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