

Length-encoded phase transitions in proline–alanine–serine peptides: from nanoaggregates to condensates

Hilda Garay-Perez^{a,b,1}, Marco Savioli^{a,1}, Greta Petrella^{a,1},
 Dhayme de la Caridad Murillo Fernández^{a,b}, Yanet Támbara Hernández^{a,b},
 Matilde Lopez Abad^{a,b}, Annachiara Ferrara^a, Miriam Quattrocioni^a, Daniel Cicero^a,
 Mariano Venanzi^a, Sukhvir Kaur Bhangu^{c,d}, Gianfranco Bocchinfuso^{a,*}, Francesca Cavalieri^{a,*}

^a Department of Chemical Sciences and Technology, University of Rome Tor Vergata, 00133 Rome, Italy

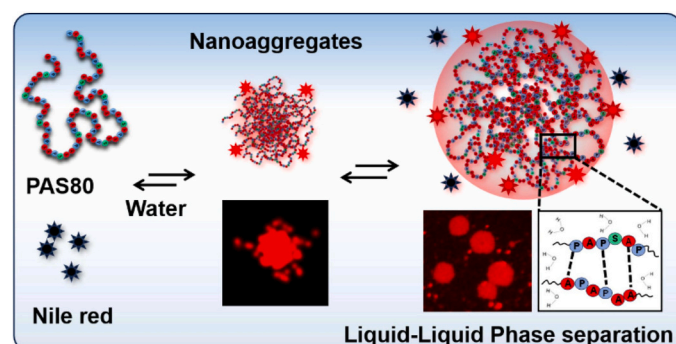
^b Centre for Genetic Engineering and Biotechnology, PO Box 6162, 11600 Havana, Cuba

^c Department of Chemical Engineering, The University of Melbourne, Parkville, Victoria 31010, Australia

^d The Department of Diabetes, School of Translational Medicine, Monash University, 10, Melbourne, Victoria 3004, Australia

GRAPHICAL ABSTRACT

Chain length dictates liquid–liquid phase separation in PAS peptides.



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ABSTRACT

PAS-based polypeptides, composed of uncharged Proline, Alanine, and Serine residues, are intrinsically disordered polymers used to enhance biologics' solubility and pharmacokinetics. Despite their biomedical relevance, their aggregation and phase separation behaviour remain underexplored. Here we combined spectroscopy, microscopy and molecular dynamics simulations to investigate the structure of PAS peptides of varying lengths (20–80 residues). We found that all peptides form nanoscale aggregates (50–350 nm) above their critical concentrations, yet only the longest PAS80 chains undergo liquid–liquid phase separation, yielding micrometre-scale condensates over a broad concentration range (0.15–6 mM), as revealed by the phase diagram.

Monitoring the phase-separation process over time provided direct evidence that condensates undergo fusion with droplet sizes increasing from approximately 1 μm to 5 μm . Fluorescence recovery after photobleaching (FRAP) microscopy and nuclear magnetic resonance (NMR) spectroscopy revealed that PAS80 condensates retain

* Corresponding authors.

E-mail addresses: gianfranco.bocchinfuso@uniroma2.it (G. Bocchinfuso), francesca.cavalieri@uniroma2.it (F. Cavalieri).

¹ HGP, MS and GP contributed equally to the work.

a high degree of molecular mobility and exhibit characteristic liquid-like behaviour. Furthermore, treatment of the condensates with a hydrogen-bond-disrupting agent, an amphiphilic solvent, and heating demonstrated that weak interactions between hydrophobic amino acids and the entropic gain associated with the hydrophobic effect are the primary driving forces underlying the formation of condensates. In contrast, hydrogen bonding was found to play a secondary role, likely contributing to the further stabilization of the assembled structures.

Molecular dynamics simulations of isolated and clustered PAS chains revealed that individual PAS peptides mainly exist as highly flexible but compact random-coil structures, and that the transition from extended to compact conformations is driven by entropic effects. Furthermore, PAS peptides rapidly self-assemble into clusters that maintain random-coil conformations and display substantial structural flexibility and configurational heterogeneity.

These findings elucidate the molecular basis of PAS peptides phase behaviour and establish PAS peptides as promising building blocks for the rational design of bioengineered coacervates with potential applications in drug delivery and compartmentalized biocatalysis.

1. Introduction

PAS-based polypeptides, composed of Pro, Ala, and Ser, are recombinant or synthetic polymers of uncharged L-amino acids that typically lack defined secondary structure, adopting natively disordered conformations with backbones exposed to extensive hydrogen bonding with water molecules [1]. They have been developed as biodegradable, monodisperse, cost-effective hydrophilic alternatives to polyethylene glycol (PEG) to overcome its limitations [2–4] and enhance solubility, stability, and pharmacokinetics of various biologics, such as recombinant interferon- β 1b [5], anti-VEGFR2 Adnectin [6], ferritin-fusion proteins [7], and antibody Fab fragments [8–10]. PAS peptides were also incorporated into mesoporous silica nanoparticles to enable a noninvasive molecular imaging tool of thrombosis [11] and liposomes to prolong their in vivo circulation life [12]. The cytotoxicity and hemocompatibility of (PAS) $_8$ and (PAS) $_3$ peptides were recently studied in vitro [13]. These peptides exhibited low cytotoxicity toward HUVEC and RAW 264.7 cell lines, as well as negligible RBC hemolysis and agglutination, highlighting their potential as building blocks for the fabrication of innovative biomaterials. Unlike PEG, which has been extensively characterized in terms of aggregation and phase separation properties [14–16], knowledge of PAS peptide structural behaviour in aqueous solution remains limited. The random coil conformation of PAS peptides with molecular weights ranging from 16 to 50 kDa has been verified using circular dichroism (CD) spectroscopy [1]. When monitoring the CD signal of different PAS sequences between 20 °C and 90 °C, a gradual shift of the negative random coil band toward longer wavelengths was observed, independent of chain length [1]. However, the molecular basis of this structural rearrangement and the potential tendency of these peptides to undergo aggregation and/or liquid–liquid phase separation (LLPS) have never been investigated.

Biomacromolecules, including polysaccharides, proteins, peptides, and nucleic acids, can undergo liquid–liquid phase separation (LLPS) to form a solvent-rich liquid phase and solute-rich liquid droplets [17,18] when the free energy of mixing becomes unfavourable. In particular, LLPS phenomena are typically observed in proteins and peptides containing large intrinsically disordered regions (IDRs) enriched in a limited number of amino acid types—primarily glycine, serine, glutamine, proline, asparagine, phenylalanine, and tyrosine [19]. The interactions that drive LLPS in protein and peptides can be described by the stickers-and-spacers model where the “stickers” motifs consist of aromatic and charged amino acids (e.g., Tyr, Phe, His and Arg) interacting via transient and weak interactions such as charge-charge, dipole-dipole, cation- π , and π - π interactions while the “spacers” consist of polar residues (e.g., Gly, Ser, Gln) that provide flexibility and a dynamic properties to the peptide chains [17,19]. Numerous studies have been recently reported with the aim to establish minimal, yet predictive rules primarily based on sticker-spacer framework for the design of peptide-based coacervates [20]. Inspired by earlier studies that identified

tyrosine and arginine as key sticker residues driving the LLPS of the FUS protein [21], through π - π interactions and cation- π interactions, a library of short cysteine-terminated peptides was subsequently designed. These studies demonstrated that LLPS is governed primarily by the ratio of arginine (Arg) to aromatic residues. Furthermore, the identity of the aromatic residue—phenylalanine (Phe), tyrosine (Tyr), or tryptophan (Trp) was found to influence phase-separation propensity, with more hydrophobic aromatic residues promoting LLPS more effectively [22]. Very short peptide motifs, such as dipeptides and tripeptides, were found able to undergo liquid–liquid phase separation (LLPS). Diphenylalanine (FF) is a well-studied example due to its strong tendency to self-assemble through aromatic π - π interactions between phenylalanine residues. To control its self-assembly and aggregation behaviour, FF has been modified with non-natural functional groups, which reduce its hydrophobicity and enable tuning of its phase-separation and aggregation properties in different environments [23]. Apart from sequence composition, peptide molecular weight, concentration, ionic strength, pH, temperature and the content of organic solvents and stimuli responsive sticker spacer can affect and trigger the LLPS process and the formation of the coacervates [20,24]. The dynamic, reversible nature of peptide coacervates and their capacity to recruit and encapsulate diverse biomolecules make them highly suitable for applications in biosensing, drug delivery, bioadhesion, and tissue engineering [24,25]. For instance, short His-rich, pH- and redox-responsive beak peptide (HB $_{pep}$) coacervates have been recently developed for encapsulation and successful delivery of all three forms of CRISPR-Cas9 editing machinery, namely, pDNA, mRNA/sgRNA, and the ribonucleoprotein complex [25–27]. Short peptide coacervates, incorporating an enzyme-inspired catalytic triad (Ser-His-Asp) into the peptide sequences were designed to create coacervates with catalytic activity. The presence of redox-responsive disulfide linkages within the peptide, enabled the condensates to reversibly assemble and disassemble in response to intracellular stimuli to enable the delivery of mRNA [22].

Although there is substantial work on the formation of peptide condensates bearing charged and aromatic amino acids [17,20–23], the occurrence of LLPS in peptides devoid of any charged or aromatic side chain amino acids, such as PAS peptides, has been largely overlooked.

Herein, we introduced PAS peptides as an emerging class of LLPS systems that operate in the absence of the canonical sticker residues—such as aromatic or charged amino acids—commonly associated with condensate formation. Using a combination of spectroscopy, microscopy, and molecular dynamics simulations, we investigated PAS peptides of varying lengths (20–80 residues). While all peptides formed nanoscale aggregates above their critical concentrations, accompanied by conformational reorganization of the polypeptide chains, only the longest PAS80 chains exhibited liquid–liquid phase separation, resulting in micrometre-scale condensates. The dynamic and liquid state behaviour of nanoaggregates and condensates is supported by FRAP microscopy and NMR spectroscopy studies. To gain insight into the structure

and intermolecular interactions that govern PAS peptide aggregation, MD simulations were performed for both isolated and clustered PAS chains. The analysis indicates that PAS peptide chains primarily adopt highly flexible yet compact structures, with the transition from extended to compact conformations being entropically driven. In contrast to previously reported peptide condensates, whose phase separation is mediated by a combination of charge–charge, dipole–dipole, cation– π , π – π , and hydrogen-bonding interactions, our results suggest that PAS peptide LLPS is driven primarily by the entropic gain arising from the hydrophobic effect, with hydrogen bonding serving a secondary role in stabilizing the resulting assemblies.

Taken together, these findings highlight the critical role of peptide length in driving phase behaviour and suggest that PAS-based peptides can serve as versatile building blocks for engineering coacervates. By tuning chain length and concentration, it is possible to design condensates with controlled size and properties, opening avenues for biomedical applications such as drug delivery, and compartmentalized biocatalysis.

2. Materials and methods

2.1. Peptide synthesis and characterization

PAS20, PAS40, and PAS80 were synthesized on solid phase using the Fmoc/tBu strategy and were purified by reversed-phase high performance liquid chromatography (RP–HPLC) on an acetonitrile/trifluoroacetic acid gradient, with a final purity higher than 95%. Their molecular weights, reported in Fig. 1a, were confirmed by electrospray mass spectrometry (Micromass, UK). Sodium phosphate monobasic monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$), sodium phosphate dibasic heptahydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) sodium fluoride (NaF), pyrene and Nile Red were purchased from Sigma–Aldrich (St. Louis, MO). Alexa Fluor 647 NHS ester (NHS-AF647) and dialysis tubing (14 kDa) were purchased from Thermo Fisher Scientific. Methanol RS (MeOH) – Spectrosol was purchased from CARLO ERBA Reagents, while 2,2,2-trifluoroethanol (TFE) was purchased from Alfa Aesar (Haverhill, MA). All chemicals were used as received without further purification. High-purity water with a resistivity of 18.2 $\text{M}\Omega\text{-cm}$ was obtained from a three-stage Millipore Milli-Q plus 185 purification system (Millipore Corporation, Billerica, MA).

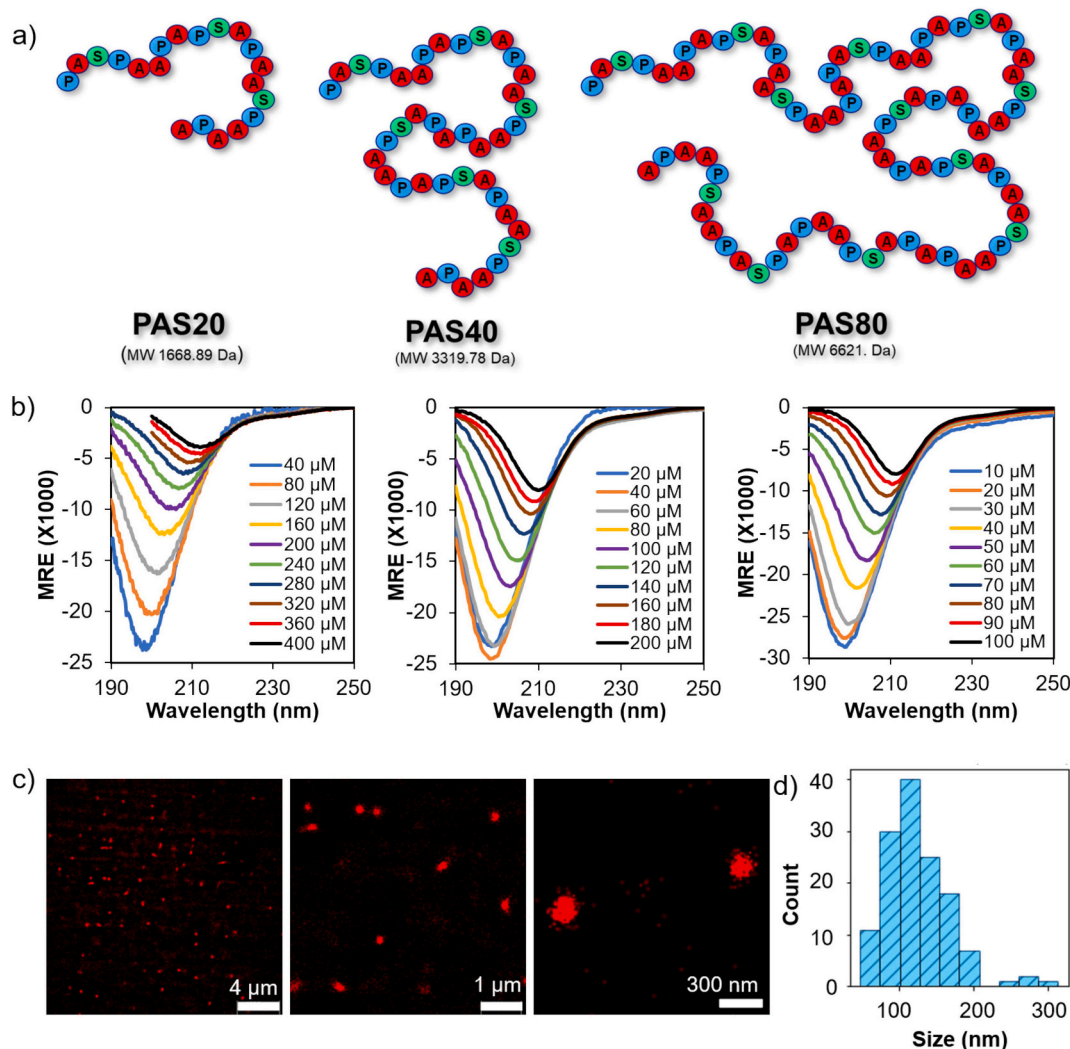


Fig. 1. PAS peptide concentration controls the conformational change and clustering of peptide chains. a) The sequence structure of neutral and hydrophilic PAS peptides. b) PAS peptides conformational changes upon increasing concentration. Far-UV CD spectra of PAS₂₀ (left), PAS₄₀ (middle), and PAS₈₀ (right) as a function of concentration. All the spectra were collected in phosphate buffer 10 mM pH 7.4 and NaF 150 mM at 298 K. c) SMLM-PAINT images (a total of 3000 frames with an exposure time of 100 ms were collected) of PAS80 nanoaggregates at the concentration of 6 mM (NR 10 nM) show nanoscale clusters with hydrophobic properties. d) Frequency histograms of the size of PAS80 nanoaggregates visualized by SMLM-PAINT images.

Synthesis: Peptides were synthesized manually on 2-Chlorotrityl chloride resin by stepwise solid-phase procedure using the Fmoc/tBu strategy. The Fmoc group was eliminated with 20% of piperidine in DMF. The Fmoc-amino acids were coupled using the activation with DIC/OxymaPure. Side-chain deprotection and peptide cleavage from the resin were accomplished by treatment with TFA/H₂O/TIS (95:2.5:2.5 v/v) for 2 h. Finally, the peptide was precipitated with cold ether, dissolved in 40% acetonitrile/H₂O and freeze-dried. Crude peptides were purified by RP-HPLC and the peptide identity was confirmed by ESI-MS.

Chromatography: The purity of peptides were assessed on an AKTA 10 (GE Healthcare, USA) HPLC system. Analytical separation was achieved in a reverse-phase (RP) C18 column (Zorbax, 4,6 × 150 mm, 5 μm). A linear gradient from 5% to 60% of solvent B over 35 min and a flow rate of 0,8 mL/min were used. Solvent A: 0.1% of TFA in water. Solvent B: 0.05% of TFA in acetonitrile. Preparative purification was performed on a AKTA pure 150 (GE Healthcare, USA) HPLC system. Separation was achieved by RP C18 column (ZORBAX 300SB-C18, 30 × 250 mm, 10 μm). A linear gradient from 15% to 45% of solvent B over 50 min and a flow rate of 15 mL/min were used. Chromatograms were recorded at 226 nm using UNICORN 5.31 (GE Healthcare, USA) software package for data processing. Solvent A: 0.1% (v/v) of TFA in water. Solvent B: 0.05% (v/v) of TFA in acetonitrile.

Mass spectrometry: ESI-MS was performed in a hybrid quadrupole-time-of-flight (Q-TOF2) instrument (Manchester, UK). Capillary and cone voltage were set to 3000 and 35 V, respectively. Spectra were manually interpreted and processed by using the MassLynx software (v 4.1) from Micromass (Manchester, UK).

2.2. Pyrene assay

The measurements were performed on a FluoroMax 4 (Horiba Jobin Yvon) spectrofluorometer, using a 1 cm quartz cuvette and a bandwidth of 3 nm for both emission and excitation slits. Pyrene stock solutions were prepared from 4.5 mg of Pyrene powder diluted in 2 mL of MeOH. To obtain the working solution (2 μM), Pyrene was diluted in water.

2.3. Circular dichroism (CD)

CD analyses were performed with a Jasco J-1500 CD Spectropolarimeter instrument (Jasco International Co.) in the wavelength range from 190 to 250 nm, with a built-in temperature interval scan program. Measurements were performed in a 0.1 cm quartz cuvette (Hellma, Mühlheim, Germany) at scan speed of 50 nm min⁻¹, bandwidth of 1 nm, digital integration time (D.I.T.) of 1 s, data pitch of 1 nm, and 8 repeated scans for each spectrum. CD data are shown as the mean residue ellipticity (MRE – deg. cm² dmol⁻¹) obtained after subtracting the baseline.

2.4. Dynamic light scattering (DLS)

The hydrodynamic diameters of PAS aggregates were measured on a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) equipped with He-Ne ion laser (λ = 633 nm). For the DLS measurements, aqueous solutions of PAS peptides a concentration 20 times the critical aggregation concentration were measured in plastic microcuvettes (ZEN0040, Malvern Instruments).

2.5. Fluorescence microscopy

The formation of coacervates was observed using an inverted Zeiss Axio Observer epifluorescence microscope equipped with ZEISS ZEN 3.11 software. To image the formation of coacervates typically, samples (20 μL) at various concentrations ranging from 0.6 mM to 6 mM were supplemented with Nile Red (NR, 1 μM) and deposited onto a 76 mm × 25 mm microscope slide. Observations were performed under the Objective EC PlnN 100×/1.3 oil DIC1 with excitation at 559 nm and

emission at ~570–650 nm using TRITC/Rhodamine filter.

2.6. Laser scanning confocal microscopy

Confocal images of PAS80 condensates were acquired using a Nikon A1R confocal microscope equipped with a 60×/1.4 NA oil-immersion objective. Samples (20 μL) at various concentrations were supplemented with Nile Red (NR, 1 μM), deposited onto a 76 mm × 25 mm microscope slide. Imaging was performed using 555 nm laser excitation. To investigate condensate stability, an aliquot of 20 μL of PAS80 solution (0.6 mM) was mixed with either 20 μL of DMSO (control) or 20 μL of 0.2 M urea, and the resulting samples were examined by microscopy. To establish the phase diagram, 20 μL aliquots of PAS80 at concentrations ranging from 0.15 to 6 mM supplemented with Nile Red (NR, 1 μM), were imaged after treatment at 5, 20, 40, and 65 °C.

For imaging of fluorescently labeled PAS80, labelling of PAS peptide was performed by dissolving 2 mg of PAS in 120 μL of 100 mM bicarbonate buffer (pH 9.2) and subsequently adding 20 μL of AlexaFluor647 succinimidyl ester (AF647 NHS) at the concentration of 1 mg/mL. The reaction was then stirred for 24 h. Removal of excess unreacted Alexa Fluor 647 (AF647) and bicarbonate buffer was done via a NAP10 column to obtain 1.2 mL of aqueous solution of AF647PAS 80 (230 μM).

2.7. SMLM-PAINT imaging

PAINT images were acquired on a Nikon N-STORM-TIRF system equipped with a Nikon 100× 1.4 NA oil immersion objective. Nano-aggregates suspensions (10 μL) of PAS20, PAS40 and PAS80 at the concentration of 0.6 mM were deposited on microscopic glass slide, after 30 min NR (20 μL, 10 nM) was added to the suspension. The focus and total internal reflection fluorescence imaging angle were adjusted to obtain a high signal-to-noise ratio. A 561 nm laser was used for the excitation of NR. All time lapses were recorded onto a 256 pixel × 256-pixel region using an electron-multiplying charge coupled device camera. The PAINT images were analyzed using the NIS Elements Nikon software. A list of localizations by Gaussian fitting fluorescent spots of the blinking dye molecules was processed by the software, which also allowed for drift correction. Blinking events that are detected in consecutive frames are counted as single molecules by the software, a total of 3000 frames with an exposure time of 100 ms were collected. For each sample, 10 images were acquired. The list of localizations was exported as a .txt file and analyzed using an in-house-built clustering analysis script to obtain the nanoaggregate size distribution.

2.8. NMR analysis

NMR experiments were recorded at 25 °C with a Bruker Avance spectrometer operating at 700 MHz for ¹H, equipped with a 5 mm inverse TXI probe and z-axis gradients.

¹H NMR spectra were acquired with a spectral window of 15 ppm (carrier frequency at 4.7 ppm) using 8192 data points and 64 transients, with a presaturation scheme of excitation sculpting to suppress the water signal [28].

The hydrodynamic radius measurements of PAS40 and PAS80 were performed using pulsed-field gradients. NMR diffusion-ordered spectroscopy experiments were performed using the BPPSTE (bipolar pulse pairs stimulated echo) method [29]. The total diffusion phase-encoding gradient pulse was 2 ms, the diffusion delay was 0.15 s for PAS40 and 0.25 s for PAS80, and the minimum gradient strength was set from 0.25 to 50 G/cm². Diffusion coefficients were measured using 0.01, 0.2, 0.5, 1.5 mM PAS40 and 0.05, 0.1, 1 mM PAS80 in PBS with 0.2% dioxane which was used as a radius standard [30]. To determine the diffusion coefficient, the Stejskal-Tanner equation which correlates the signal intensity to the diffusion coefficient (Eq. (1)) was used:

$$\ln I = \ln I_0 - (\gamma \delta G)^2 (\Delta - 2\delta/3) D \quad (1)$$

where I_0 is the signal intensity at a gradient strength of zero, G is the gradient strength, D is the diffusion coefficient, δ ("little delta") is the gradient pulse duration, and Δ is the diffusion time.

2.9. Molecular dynamics simulations

MD simulations were performed using the GROMACS package version 2019.6 [31] and CHARMM36m force field was used [32]. This version of the CHARMM force field has been optimized to improve the sampling of the peptides disordered states, introducing the correction of the CMAP potential and an improved parameterization of the Lennard–Jones (LJ) parameters. MD simulations with 1, 3, 6, 8, and 12 PAS molecules were carried out in a roughly 150 mM NaCl aqueous solution to mimic the experimental condition of ionic strength (Table 1). Time steps of 2 fs were used for equilibration and production runs. We performed MD simulation at 300, 320, 340, and 360 K; the target temperature was reached via a 600 ps NVT annealing step from 10 K. The equilibrium temperature was controlled by velocity rescaling [33] with a coupling constant of 0.1 ps. The pressure was controlled by an isotropic Parrinello–Rahman barostat [34] with a coupling constant of 2 ps and a reference external pressure of 1 atm. Lennard–Jones long-range interactions were treated with a cut-off radius of 1.2 nm. Coulombic interactions were calculated by the Particle Mesh Ewald method [35]. The LINCS algorithm was used to constrain all bond lengths during the MD simulations [36]. Three independent 500 ns-long simulations have been carried out for each system. Each trajectory started from different configurations. The first 100 ns of each trajectory was discarded as equilibration time, the remaining part of the three trajectories were merged and considered for the analysis. MD simulation analyses were carried out using homemade scripts, built-in GROMACS tools, and Vega ZZ [37]. The secondary structure was assessed according to the Dictionary Secondary Structure of Proteins (DSSP) analysis, carried out by using the *dssp* tool in the GROMACS package [38].

3. Results and discussion

3.1. PAS peptides undergo aggregation and liquid-liquid phase separation

PAS peptides with varying lengths (PAS20, PAS40, and PAS80) but similar sequences were synthesized (Fig. 1a). The PAS peptides were selected based on previously reported sequences developed by Skerra and co-workers [1–3] which were designed to adopt intrinsically disordered conformations. The different peptide lengths were chosen to investigate the effect of chain length on the conformational, self-assembly, and phase-transition behaviour of PAS peptides while maintaining the same amino acid composition and repeating primary structure.

The peptides were prepared using step-by step solid-phase synthesis, purified up to more than 95% of purity, and their molecular weights

confirmed by electrospray ionization mass spectrometry (Table S1, Fig. S1). We then examined how peptide length and concentration influence their structural and aggregation properties. At increasing concentrations, all PAS peptides exhibited the formation of less polar domains, as detected by the pyrene fluorescence assay [39] (Fig. S2). Critical aggregation concentrations (CAC) in phosphate-buffered saline (PBS) were determined as 220 μ M for PAS20, 150 μ M for PAS40, and 53 μ M for PAS80. These results suggest that peptide assembly/aggregation is primarily governed by the overall concentration of PAS repeating residues, which remains approximately in the range of 4–6 mM for all sequences, regardless of differences in chain length. Circular dichroism (CD) spectra of PAS peptides (Fig. 1b), recorded at increasing concentrations, confirmed their characteristic disordered structure regardless of chain length. The spectra displayed a pronounced negative band near 200 nm, which is indicative of a random coil conformation [1]. Interestingly, as PAS peptide concentration increases, the negative band in the CD spectra exhibits a redshift accompanied by a decrease in signal intensity (Fig. 1b). This indicates that peptide assembly at higher concentrations is coupled with conformational changes and structural rearrangements of the peptide chains. To compare the propensity of the three PAS peptides to undergo structural rearrangement upon aggregation, molar residue ellipticities at the CD minimum were plotted against residue concentration (Fig. S3a). Overall, the analysis revealed that PAS40 and PAS80 display similar tendencies to rearrange, whereas PAS20 undergoes a more pronounced structural change during aggregation. CD spectra of PAS peptides were also recorded at concentrations below the CAC across a temperature range from 5 °C to 85 °C (Fig. S3b). For all systems, the spectra exhibited a redshift of the minimum accompanied by a decrease in signal intensity. An isodichroic point near 210 nm was evident, suggesting an equilibrium between two distinct conformations (or ensembles of conformations). Furthermore, the CD spectra remained unchanged after several annealing cycles (Fig. S3c), indicating a reversible interconversion between these conformations and implying low energetic barriers between the conformers. Collectively, these findings indicate that PAS peptides of varying chain lengths aggregate above a critical concentration, and this aggregation is accompanied by a structural rearrangement of the polypeptide chains.

Dynamic light scattering (DLS) analysis of PAS peptide nanoaggregates formed above the CACs revealed multiple populations of aggregates with average hydrodynamic diameters ranging from 50 to 500 nm (Fig. S4). These aggregates were in equilibrium with individual PAS20, PAS40, and PAS80 chains having hydrodynamic diameters of approximately 1.7 ± 0.4 nm, 3.3 ± 0.8 nm, and 4.5 ± 0.9 nm, respectively (Fig. S4a–c).

As indicated by the pyrene assay, the aggregation process generated hydrophobic domains within the nanoaggregates. To provide morphological evidence of the nanoaggregates, we sought to map and image these hydrophobic domains by using a single-molecule localization microscopy (SMLM) technique, i.e., points accumulation for imaging in nanoscale topography (PAINT), and the polarity-sensitive fluorescent

Table 1
Composition of the simulated systems.

Simulation index ¹	Peptide	Number of peptides	Number of water molecules	Number of Na ⁺ ions	Number of Cl [−] ions	Edge dimension of the cubic box (nm)
1PAS20-T ²	PAS20	1	8534	24	24	6.42
1PAS40-T ²	PAS40	1	26,313	77	77	9.48
1PAS80-T ²	PAS80	1	177,457	492	492	17.60
3PAS20-T ²	PAS20	3	23,526	66	66	9.00
3PAS40-T ²	PAS40	3	32,096	90	90	10.00
6PAS20-T ²	PAS20	6	23,264	66	66	9.00
6PAS40-T ²	PAS40	6	31,629	90	90	10.00
8PAS20-T ²	PAS20	8	23,123	66	66	9.00
8PAS40-T ²	PAS40	8	31,334	90	90	10.00
12PAS20-T ²	PAS20	12	22,749	66	66	9.00
12PAS40-T ²	PAS40	12	30,648	90	90	10.00

¹ Each simulation has been carried out for 500 ns and triplicated.

² T assumes the value of 300, 320, 340, and 360 as a function of the simulation temperature.

probe Nile Red. This dye remains non-fluorescent in aqueous solutions but becomes highly fluorescent upon partitioning into hydrophobic environments, such as those within nanoaggregates, condensates or microdroplets [40]. The PAS20, PAS40 and PAS80 nanoaggregates were therefore imaged in aqueous solution in the presence of NR (10 nM). The individual probe molecules remain dark in solution until they encounter and bind to the hydrophobic sites on the nanoaggregates. Binding of the probe to hydrophobic regions occurs on a transient time scale, resulting in blinking events that can be recorded by the microscope camera for the localization of single molecules and the reconstruction of super-resolution images of the nanoaggregates (Fig. 1c, Fig. S5a-b). The list of localizations was then processed using a clustering analysis script to estimate the size of the nanoaggregates (Fig. 1d, Fig. S5a-b). The PAINT super-resolution microscopy confirmed the formation of nanoaggregates as depicted in Fig. 2a with size ranging from 50 nm up to 350 nm in agreement with the DLS measurements.

Next, we investigated whether PAS peptides nanoaggregates could

undergo liquid-liquid phase separation and form condensates or microdroplets at high concentrations, specifically in the range of 0.3–6 mM. To assess the formation of PAS peptide condensate, we employed brightfield, fluorescence and confocal microscopy combined with Nile Red (0.1–1 μ M) staining. Our observations revealed that, among the peptides analyzed, only PAS80 formed visible liquid condensates or microdroplets, exhibiting substantial Nile Red partitioning into the denser and hydrophobic droplet phase compared to the surrounding medium (Fig. 2b, c). Condensates (microdroplets) were observed immediately after dissolving PAS80 in Milli-Q water at a concentration of 6 mM, as confirmed by both fluorescence microscopy (Fig. 2b1, Fig. S6a) and brightfield microscopy (Fig. S6b). Analysis of 100 microdroplets revealed a size distribution with an average diameter of $3.2 \pm 0.8 \mu$ m (Fig. S6c). Significantly smaller condensates ($\approx 1 \mu$ m) were observed at 0.6 mM (Fig. 2b2, Fig. S7a-f), indicating enhanced coalescence at higher concentrations. This clearly suggest that PAS80 condensates undergo fusion.

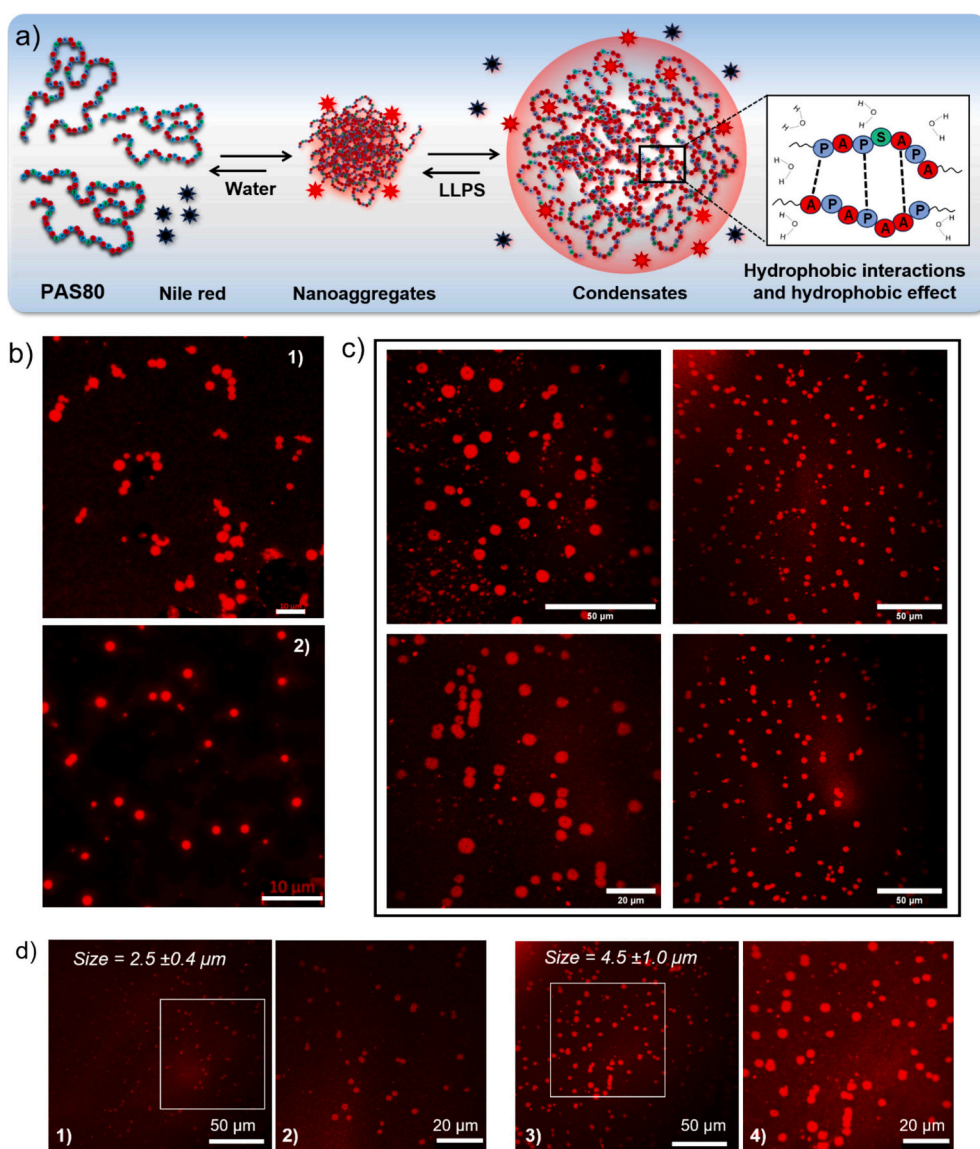


Fig. 2. PAS peptide length governs the propensity for liquid-liquid phase separation. a) Schematic representation of the formation of red fluorescent PAS80 nanoaggregates and condensates upon liquid-liquid phase separation underlying the equilibrium and interactions involved. b) Fluorescence microscopy images of PAS80 condensates (6 mM) stained with NR (1 μ M) obtained in MQ water at 25 $^{\circ}$ C at the concentration 6 mM (b1) and 0.6 mM (b2); c) confocal microscopy images of PAS80 condensates (0.6 mM) stained with NR (1 μ M) showing coalescence after 30–40 min. d) confocal microscopy images of PAS80 condensates (0.3 mM) stained with NR (1 μ M) showing change in size from 2.5 μ m (2d1,2) to 4.5 μ m (2d3,4) over 30–40 min. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

By monitoring the phase-separation process over time at a fixed focal plane using laser-scanning confocal microscopy, we obtained direct evidence of condensate formation and coalescence (Fig. 2c). Condensates formed at a concentration of 0.6 mM and deposited on a glass slide underwent progressive fusion, resulting in larger droplets over time. After 30 min, the droplets reached an average diameter of approximately $5 \pm 1 \mu\text{m}$ (Fig. 2c). A similar trend was observed at a lower concentration (0.3 mM), where the average droplet diameter increased from approximately $2.5 \mu\text{m}$ (Fig. 2d1,2) to $4.5 \mu\text{m}$ over the same period (Fig. 2d3,4). These observations are consistent with condensate growth through droplet coalescence. They further suggest that the coacervates originate from the coalescence of nanoaggregates, as illustrated in Fig. 2a.

To further confirm condensate formation without resorting to NR droplet staining, PAS80 was labeled at the N-terminal using AF647 NHS. A solution of AF647-PAS80 (0.3 mM) in phosphate-buffered saline was deposited on glass slides and imaged by confocal microscopy after washing away excess of soluble peptide. Fig.S7 g-h show the image of the deposited condensates and their size distribution respectively, with an average diameter of approximately $2 \mu\text{m}$.

To demonstrate the liquid-like behaviour of the condensates, fluorescence recovery after photobleaching (FRAP) experiments were performed. Following photobleaching of NR in a selected region of interest, a rapid recovery of fluorescence of NR was observed, indicating the

presence of a dynamic liquid phase within the condensates (Fig. 3a). To further elucidate the molecular interactions driving LLPS, the condensates were post-treated with urea and DMSO. Treatment with urea, a hydrogen-bond-disrupting agent, had only a minor effect on condensate integrity, with the condensates largely maintaining their structure (Fig. 3b). In contrast, exposure to 50% DMSO, an amphiphilic solvent known to disrupt hydrophobic interactions, resulted in rapid dissolution of the condensates (Fig. 3c).

Collectively, these results indicate that weak and transient interactions between hydrophobic amino acids are the primary driving force governing condensate formation. Within the PAS peptide, these weak interactions are mainly contributed by the alanine and proline residues, in contrast, serine residues are expected to enhance peptide solubility and may participate in hydrogen-bonding interactions. Although hydrogen bonds may be present within the condensates and contribute to their internal organization, our results suggest that they are not essential for condensate formation or stability.

Next, we sought to elucidate the effects of peptide concentration and temperature on the phase-separation behaviour of PAS80 by constructing a phase diagram as a function of peptide concentration at four different temperatures (4, 20, 40, and 65°C). The resulting phase diagram (Fig. 3d) shows that condensate formation occurs throughout the concentration range of 0.6–6 mM ($4\text{--}40 \text{ mg mL}^{-1}$) at all temperatures tested. In contrast, at the lowest concentrations examined (0.15 and 0.3

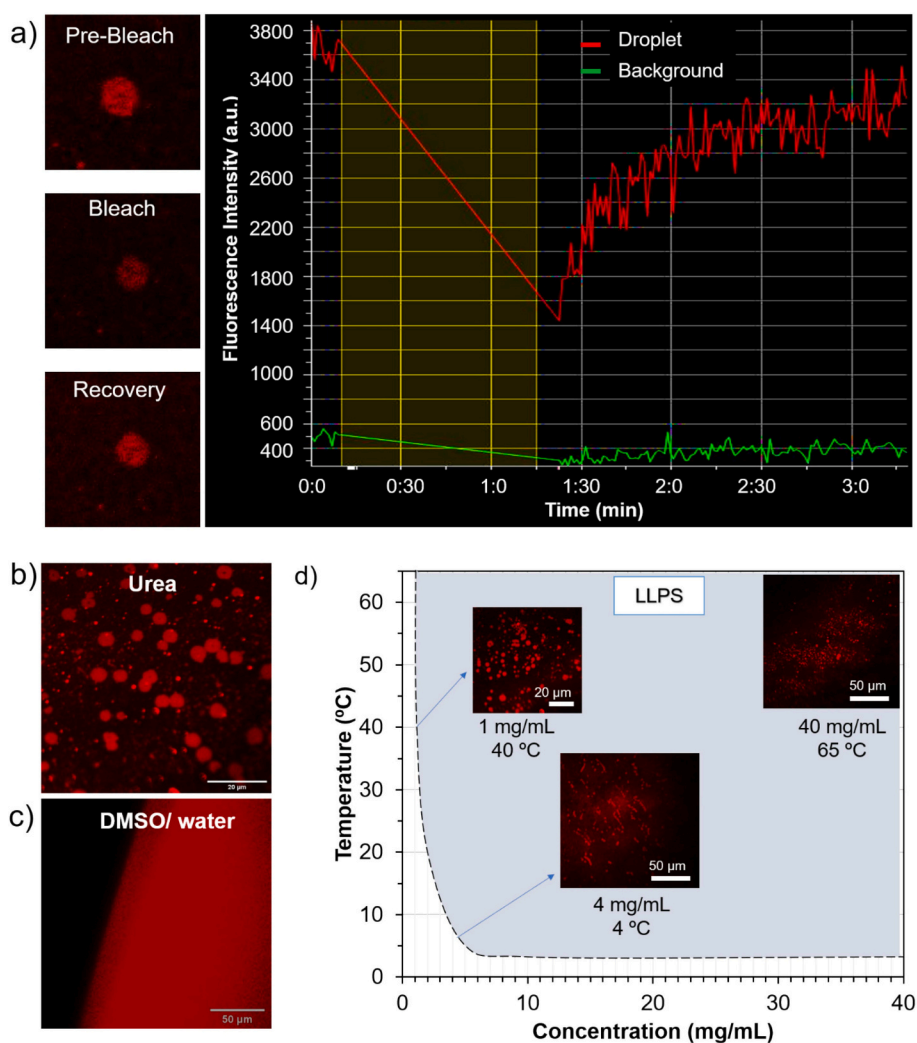


Fig. 3. a) Representative image of FRAP experiments conducted on condensates showing the fast recovery of fluorescence after photobleaching of PAS80 condensates (0.6 mM), b) stability of PAS80 condensates against Urea treatment and c) dissolution of PAS80 condensates after treatment with DMSO, d) Phase diagram of PAS80 as a function of T and concentration with the confocal microscope images of the condensates formed at different T and concentrations.

mM, 1 and 2 mgmL⁻¹), condensates were observed only above 40 °C, and 20 °C respectively. These results demonstrate that the LLPS is promoted by increasing temperature. The observed temperature dependence is characteristic of an entropy-driven phase-separation process and is consistent with a dominant contribution from the solvent-mediated hydrophobic effect. In particular, condensation is expected to be accompanied by the release of ordered water molecules from the peptide hydration shell into the bulk solvent, resulting in a net increase in system entropy. Taken together, these results indicate that although PAS peptides of different lengths tend to form nanoaggregates, only the longest PAS80 chains can form micrometre-sized condensates, likely through the establishment of sufficient multivalent, transient interchain interactions that trigger liquid–liquid phase separation. Our study

indicates that the interactions between hydrophobic amino acids and solvent mediated hydrophobic effect are the major determinants of formation of nanoaggregates and condensates, whereas hydrogen bonding plays a secondary role in stabilizing the assembled structures.

3.2. Probing the dynamic and liquid-like state of nanoaggregates and condensates by NMR

To monitor the dynamic behaviour of PAS40 nanoaggregates and PAS80 condensates, ¹H NMR spectra of both peptides were recorded across increasing concentrations (Fig. 4a, Fig. S8a). No change in signal width or chemical shift was observed, suggesting that the high dynamism of the peptide is preserved at high concentrations in the aggregate

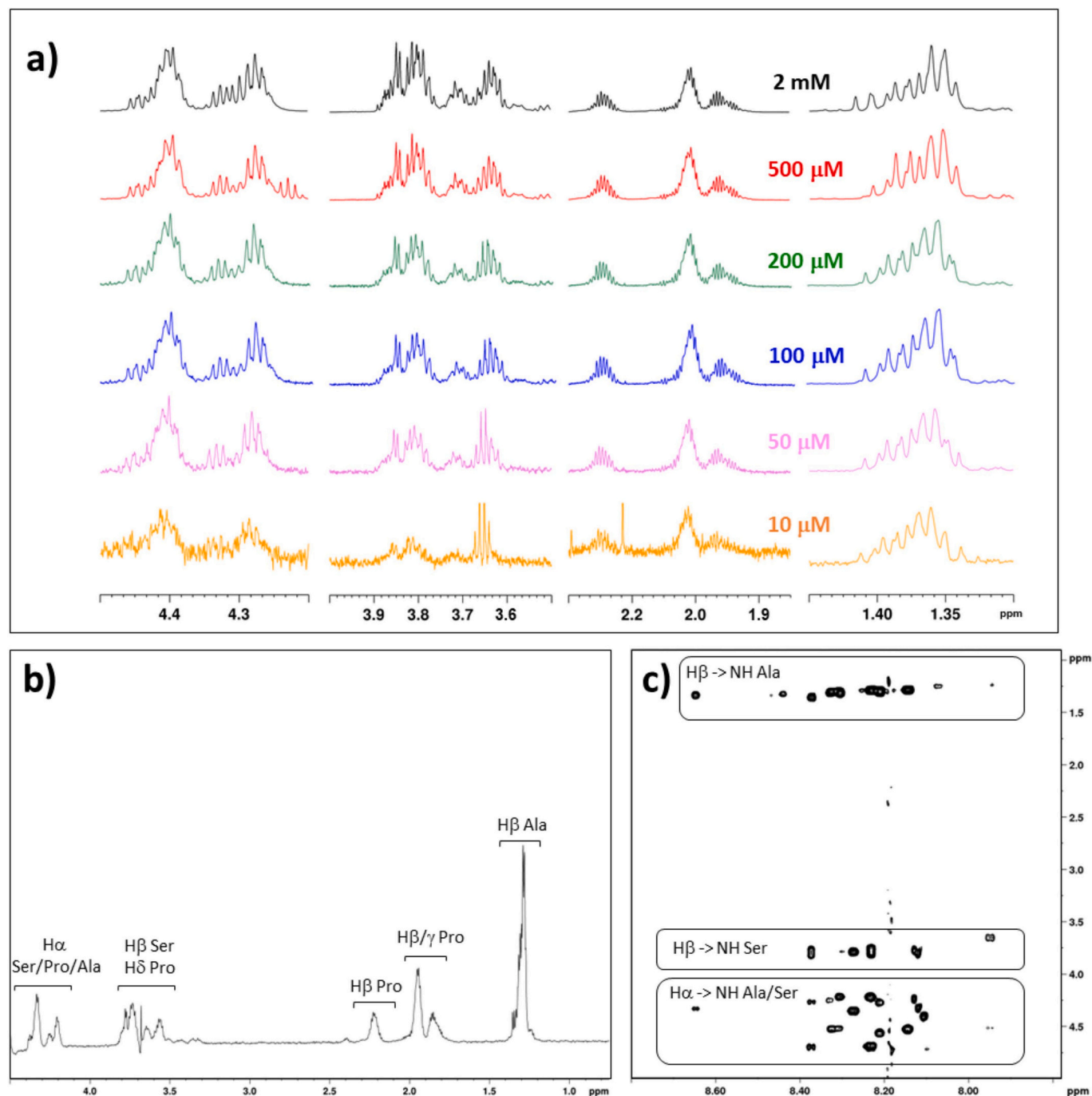


Fig. 4. NMR analysis of PAS40. a) ¹H NMR spectra of PAS40 at six different concentrations ranging from 10 μM to 2 mM. b) ¹H NMR at 700 MHz of PAS40 500 μM in D₂O at 298 K with the assignment. c) Fingerprint region of the ¹H-¹H TOCSY with the corresponding assignment.

and condensate states.

The spectra display the expected signals of the three amino acids: proline, alanine, and serine at the chemical shifts compatible with a random coil structure (Fig. 4b, Fig. S8a). The spectra show sharp signals

consistent with long transversal relaxation time (T_2), indicating that condensates are highly dynamic and liquid-like in solution at all concentrations. The ^1H - ^1H -TOCSY spectra were also acquired (Fig. 4c) to confirm the assignment. The high intensity observed from the cross-

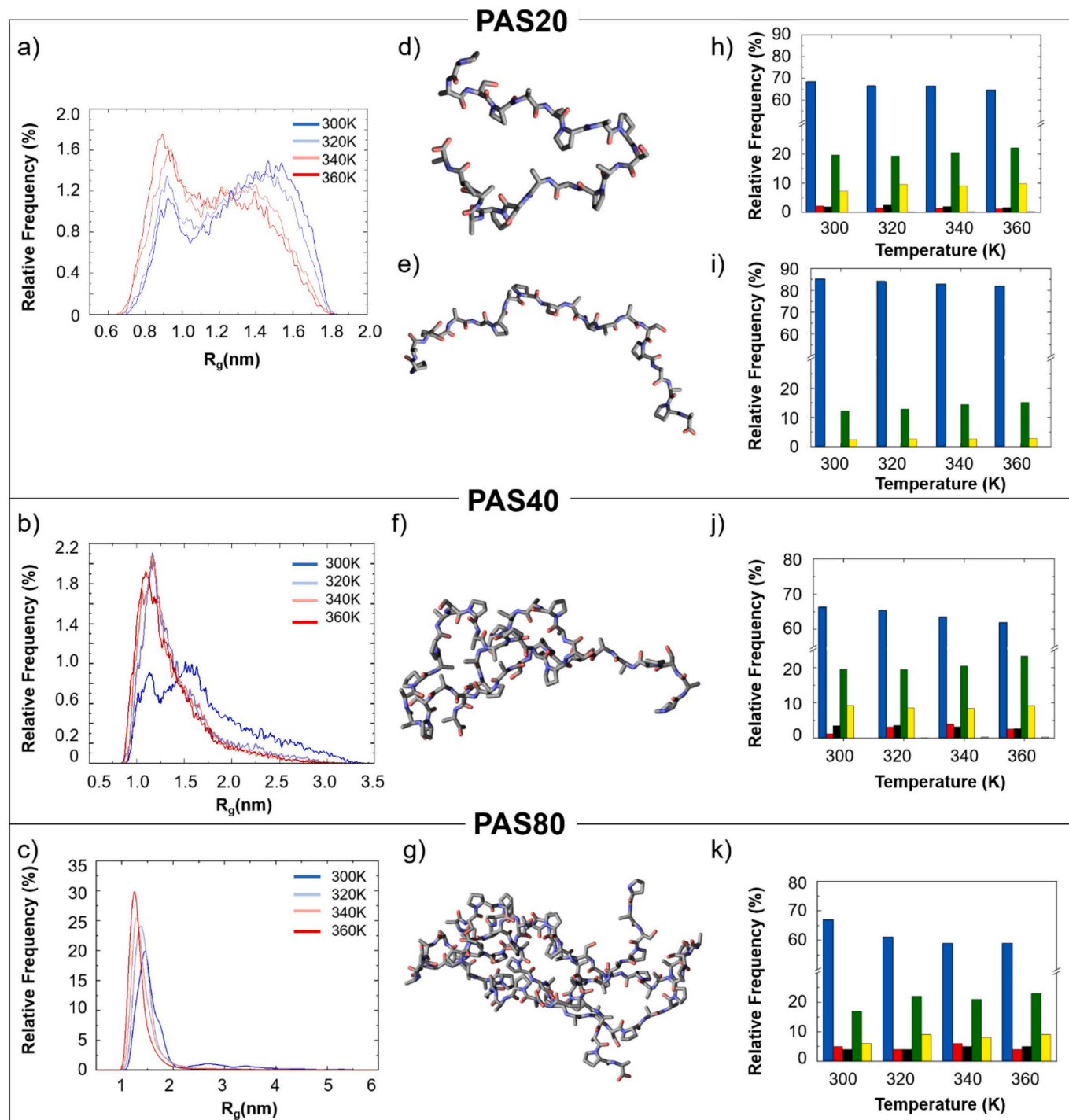


Fig. 5. Molecular dynamics (MD) simulations of isolated PAS. Radius of gyration distribution as obtained from MD simulations of PAS20 (a), PAS40 (b), and PAS80 (c) at different temperatures (simulation codes 1PAS20–300, 1PAS20–320, 1PAS20–340, 1PAS20–360, 1PAS40–300, 1PAS40–320, 1PAS40–340, 1PAS40–360, 1PAS80–300, 1PAS80–320, 1PAS80–340, and 1PAS80–360 in Table 1). Cluster mean structures from simulations of PAS20-compact (d), PAS20-extended (e), PAS40 (f), and PAS80 (g) isolated peptides. The structures are reported as sticks, with the C, N, and O atoms coloured in grey, blue, and red, respectively. For the sake of clarity, H atoms are not shown. The mean structures were obtained by means of the *cluster* tool in GROMACS by using RMSD based clustering criterion with a cut-off of 0.4 nm. Secondary structure analysis of the MD trajectories for PAS20 (h and i), PAS40 (j), and PAS80 (k). Coil, β -sheet, β -bridge, bend, turn, α -helix, and 3–10 helix are coloured in white, red, black, green, yellow, blue and grey, respectively. For PAS20 two different ensembles have been considered, with R_g comprised between 0.80 and 1.10 (h - compact conformations ensemble) and 1.20–1.50 nm (i - extended conformations ensemble), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

peaks between the NHs and the protons of the side chain, despite the long spin-lock time used (80 ms) provided further evidence of the long hydrogen T_2 indicating the dynamic nature of the peptides. The low dispersion of the chemical shift of the different signal groups reflects the lack of a stable secondary and tertiary structure.

The hydrodynamic radii (R_h) of PAS peptides were also measured at different concentrations using NMR diffusion experiments (see experimental part) with dioxane as a reference (Fig. S8b-c). We found that the R_h remained constant ($\approx 25.3 \pm 0.7$ Å for PAS80 and $\approx 16.8 \pm 0.5$ Å for PAS40) across all concentrations (Fig. S8d-e). These R_h values are consistent with those obtained from DLS measurements (Fig. S4). Overall, the NMR studies indicate that no measurable changes in chemical shift, linewidth, or relaxation behaviour occur in the ^1H NMR spectra across the concentration range investigated, including conditions where aggregation and phase separation take place. This indicates that any aggregated species formed above the CAC retain chain dynamics indistinguishable, within NMR sensitivity, from those of the free monomeric chains, i.e., the peptide remains highly mobile and NMR-visible across the transition. This observation argues against the formation of a rigid or solid-like population that would be expected to become NMR-silent or broadened beyond detection upon aggregation, and is consistent with the liquid-like, dynamic nature of the nanoaggregates and condensates proposed in this work, which is also confirmed by FRAP experiments shown in Fig. 3a.

3.3. Molecular dynamics analysis of PAS peptides in the single chain and the condensate states

To give a deeper insight into the structure and conformational transitions of PAS peptides, MD simulations were first carried out for isolated PAS chains in water at four different temperatures, 300, 320, 340, and 360 K. Three 500 ns-long independent simulations were acquired for each peptide at the four temperature values. In Fig. 5a-c the gyration radius (R_g) obtained from the simulations of all PAS peptides at the four temperatures are reported. For PAS20 (Fig. 5a), the presence of two conformers was observed, one characterized by compact structures with R_g roughly centered at 0.8 nm, and the other one characterized by more extended chains with higher R_g at approximately 1.5 nm. While at low temperatures the extended conformations are predominant, it is noted that compact conformations are favored with increasing temperature. These results are consistent with the CD data showing conformational changes upon heating (Fig. S3). Conformational sampling during the simulations of the isolated PAS20 chains (Movie 1 SI) shows that the interchange between these two populations occurs on the nanosecond time scale with low inter-conversion energetic barriers as also suggested by CD spectra. By monitoring PAS40 and PAS80 trajectories, the interchanging between compact and extended conformations can also be detected. We found that the long peptide PAS40 (Fig. 5b) and PAS80 (Fig. 5c) chains reside primarily in the compact conformational state whereas the extended conformations are less populated.

In Fig. 5d-g the more representative structures were obtained from the trajectories of the isolated PAS peptides. For PAS20 the whole ensemble was divided into two subsets, comprising the conformations with R_g ranging from 0.80 to 1.10 nm (Fig. 5d) and from 1.20 to 1.50 nm (Fig. 5e), representing the compact and the extended conformations ensembles, respectively. For PAS40 (Fig. 5f) and PAS80 (Fig. 5g) the whole ensemble obtained by merging the three independent trajectories was considered. The analysis of the secondary structures of the four considered conformations (compact and extended PAS20 (Fig. 5h-i) and compact PAS40 (Fig. 5j) and PAS80 (Fig. 5k)) unveils that the ensembles are primarily featured by random coil structures. However, the ensemble comprising the extended conformations is characterized by the highest level of random coil structures (80%) at all the analyzed temperatures, whereas the compact conformation exhibited approximately 60–70% random coil structures. Furthermore, we found that the compactness is coupled with a detectable content of β conformations

(β -sheet and β -bridge, red and black bars, respectively, in Fig. 5h-k), in good agreement with CD data shown in Fig. 1B. Interestingly, for PAS20 the analysis of the extended (E) and the compact (C) ensembles, at the different temperatures, enabled the study of the thermodynamics of this conformational transition by using the Eq. (1),

$$\Delta G^0 = -RT \ln \frac{f_C}{f_E} \quad (1)$$

where R and T are the gas constant and the temperature and f_C and f_E represent the frequencies during the simulations of conformations with R_g in the range 0.80–1.10 (compact conformations) and 1.20–1.50 nm (extended conformations). The calculated free energy data were plotted as a function of temperature, as shown in Fig. S9a. We found that the compact conformations are disfavoured at low temperatures and dominate at T greater than 320 K. If we assume that ΔH° and ΔS° are constants in the analyzed temperature range, ΔH° and ΔS° can be estimated from the slope measured in Fig. S9a and simply by using the relationship $\Delta G^\circ = \Delta H^\circ - T \Delta S^\circ$. We calculated the ΔH° and ΔS° values equal to 10.1 ± 0.5 kJ/mol and 31 ± 2 J/mol respectively indicating the $E = C$ equilibrium is entropically driven.

To elucidate the origin of the entropic contribution to the conformational change, both the flexibility of the chain and the interaction with the solvent need to be considered (i.e., the hydrophobic effect). Fig. S9b also reports the root mean square fluctuation (RMSF) of the α carbon atom of PAS20 during simulation, for both the compact (Fig. S9b) and extended (Fig. S9c) ensembles. For the compact conformations (Fig. S9b), values greater than 0.4 nm are observed for almost all residues at any temperature, compared with the RMSFs around 0.35 nm in the extended ensembles for all residues except the terminals (Fig. S9c). These data indicate that compact conformations allow greater chain flexibility than extended ones. To evaluate the role of the solvent on the compact conformations, the gyration radii (R_g) as a function of the PAS chain length were analyzed (Fig. S9d). The trend of the R_g versus the number of residues is often described by the simple law $R_g = a(N-1)^b$, where the prefactor (a in the formula) depends on local structure, and the exponent (b) depends on solvent quality. Values of b greater than 0.5 are attributed to good solvent conditions, while values lower than 0.5 indicate poor solvent conditions. The b values observed for the PAS sequences are in the range of 0.22–0.26, depending on the temperature, meaning that PAS peptides experience poor solvent conditions in the compact structures. Overall, MD analysis indicate that PAS peptide chains adopt highly flexible compact structures where the solvent and the hydrophobic effect play a key role in driving the observed entropic gain.

To assess the self-aggregation behaviour of PAS peptides and to characterize the structural and dynamic features of the resulting nanoaggregates, MD simulations were performed by randomly placing different copies of PAS20 and PAS40 (3, 6, 8, or 12) in the simulation box. In all cases, different temperatures (300, 320, 340, and 360 K) were simulated to inspect the effect of the temperature on the aggregate structure and stability. We observed that aggregates formed rapidly, incorporating the available peptides. Fig. 6a and b shows the most representative structures of peptide in the octamer clusters resulting from the MD simulations of PAS20 and PAS40 at 300 K respectively. Visual inspection of trajectories reveals high variability in the aggregate configurations. The dynamics of PAS40 hexamers (Movie 2SI) further demonstrate that, beneath the semi-transparent surface, the clustered peptides remain highly flexible.

In Fig. 6c and d is also reported the secondary structure analysis during the octamer trajectories at different temperatures. The peptides mainly populate random coil conformations, with a detectable fraction of β -strand, β -bridge, bend, and turn conformations. The extent of random coil fraction slightly decreases with temperature, oppositely, the bend structures increase. The comparison of these data with those reported in Fig. 5, referring to the extended and compact conformations of

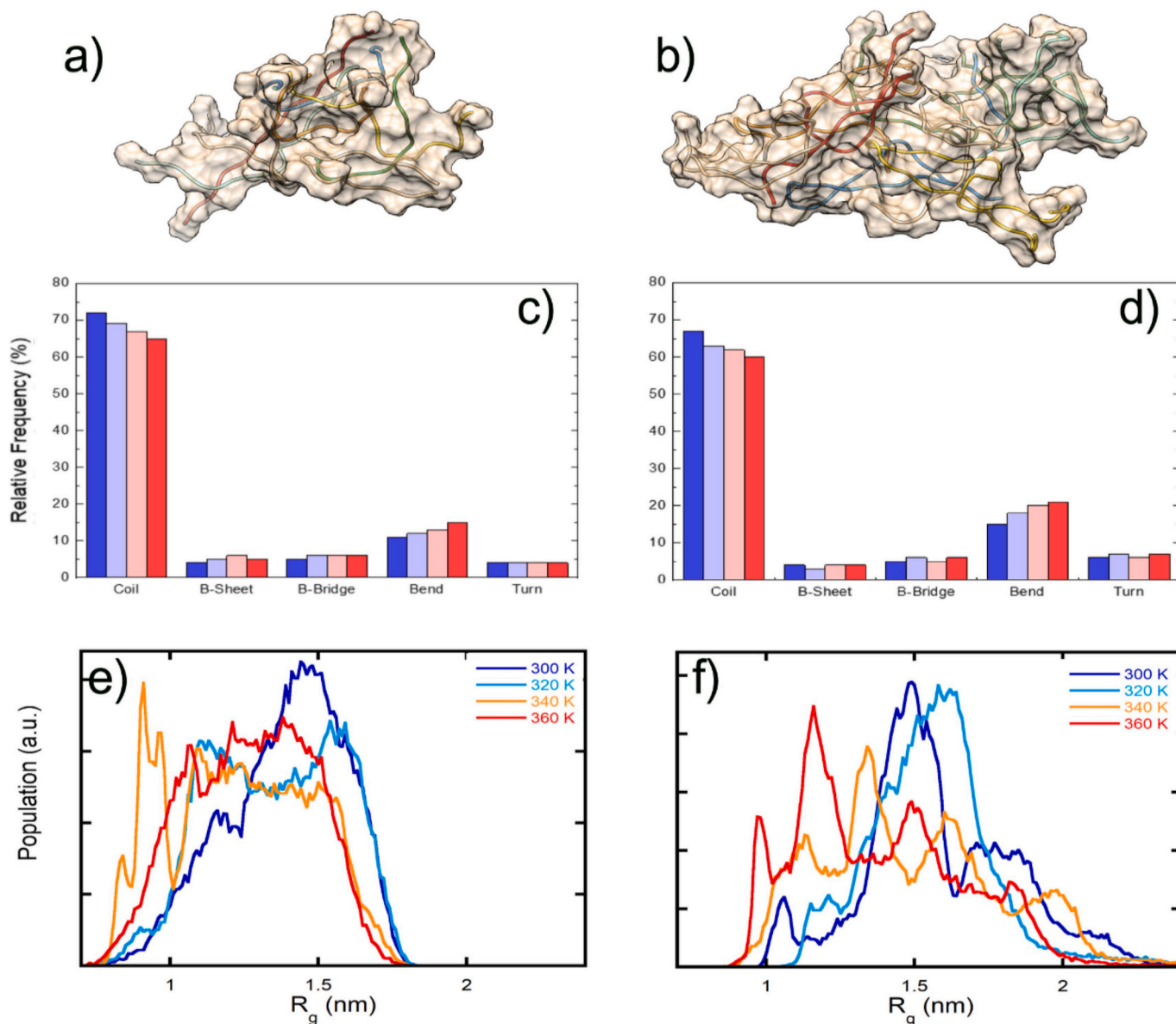


Fig. 6. MD simulations of the aggregates. Data from MD simulations of the PAS20 (a, c, and e) and PAS40 (b, d, and f) octamers (simulations 8PAS20-300, 8PAS20-320, 8PAS20-340, 8PAS20-360, 8PAS40-300, 8PAS40-320, 8PAS40-340, and 8PAS40-360 in Table 1). Most representative structures of PAS20 (a) and PAS40 (b) octamers at 300 K. The aggregates are shown as semi-transparent khaki structures. Under the surface, the backbone of the eight chains is reported in different colors. Secondary structure analysis of the MD trajectories of PAS20 octamer (c) and PAS40 octamer (d) at different temperatures, blue, cyan, orange, and red histograms refer to 300, 320, 340, and 360 K, respectively. Radius of gyration distribution of the aggregates at different temperatures for PAS20 octamer (e) and PAS40 octamer (f). The colour code is the same as in the middle panels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the individual PAS chains, suggests that the compact structures dominate the ensemble at each temperature. Fig. S10 presents the same analysis for all simulated PAS20 and PAS40 aggregates. Despite a greater percentage of random coil structures being observed for PAS20, the two peptides behave similarly. Fig. 6e and f also report the R_g evaluated during the PAS20 and PAS40 octamers simulations respectively. In all the cases, a wide range of R_g values is populated during the simulations, confirming the flexible nature of these nanoaggregates. Furthermore, data clearly show that more compact aggregates are favored at higher temperatures, nicely paralleling what was obtained for the isolated chains. Finally, to elucidate the possible role of hydrogen bonding in stabilizing the PAS20 and PAS40 nanoaggregates, we quantified the inter-chain H-bonds within the aggregates composed of 6 and 12 PAS20 or PAS40 molecules at different temperatures. The results shown in Table S2 indicate that inter-chain H-bonds are present and a

significant fraction of these interactions involves the hydroxyl groups of the serine side chains (from 17 to 27%), depending on the peptide length. Interestingly the number of hydrogen bonds does not decrease with increasing temperature, in contrast the data suggest a slight tendency for the aggregates to become even more extensively hydrogen-bonded at 360 K than at 300 K. These observations are therefore consistent with previous results indicating that hydrophobic interactions and solvent mediated hydrophobic effect are the major determinants of formation of nanoaggregates and condensates, whereas hydrogen bonding likely plays a secondary role in stabilizing the assembled structures.

4. Conclusions

The propensity of PAS peptides to aggregate and undergo LLPS was

examined across varying molecular weights and concentrations using fluorescence spectroscopy and microscopy. Regardless of chain length, PAS peptides undergo aggregation upon exceeding a critical concentration, and this process is coupled to a structural reconfiguration of the polypeptide backbone. While PAS20 and PAS40 self-assemble into dynamic nanoaggregates, PAS80 also exhibits pronounced liquid–liquid phase separation resulting in the formation of liquid-like condensates as evidenced by microscopy observations of droplet coalescence, diffusivity studies, and NMR analyses.

Molecular dynamics simulations revealed that PAS peptides predominantly adopt highly flexible yet compact conformations, with the transition from extended to compact states being entropically driven. In addition, molecular dynamics simulation of the nanoaggregates indicate that the aggregates formed rapidly, and the clustered peptides remain highly flexible.

In conclusion, PAS peptides represent an innovative LLPS system despite lacking the canonical “sticker” motifs, such as aromatic or charged residues, that typically drive condensate formation through charge–charge, dipole–dipole, cation– π , π – π interactions, and hydrogen bonding. In contrast to previously reported peptide condensate systems, where favorable enthalpic contributions from these interactions are often the primary driving force for LLPS, phase separation in PAS peptides is mainly governed by weak and transient interactions between alanine and proline residues, together with the entropy gain associated with the hydrophobic effect. Indeed, our results indicate that hydrogen bonding plays only a secondary role in stabilizing the assembled structures.

Through a comprehensive experimental and computational investigation, we identified three distinct, length-encoded and concentration-dependent processes spanning molecular to mesoscale length scales, that ultimately results in condensate formation. The first process involves the establishment of an equilibrium between extended and compact conformations of isolated peptide chains, with the compact state facilitating subsequent aggregation. This equilibrium is strongly dependent on peptide length, as the compact conformation becomes increasingly populated with increasing molecular weight and it is entropically favored.

In the second stage, the compact/coiled conformers undergo self-assembly through transient interactions among alanine and proline residues, accompanied by the release of hydration water molecules. This aggregation process is also length-dependent, as evidenced by the progressive decrease in the critical aggregation concentration (CAC) with increasing molecular weight.

The final stage consists of the conversion of nanoaggregates into coacervate condensates. Unlike the first two stages, this process was observed, under the investigated conditions, only for PAS80. In contrast, PAS20 and PAS40 formed dynamic nanoaggregates but did not undergo visible macroscopic phase separation. Based on our observations, it is reasonable to hypothesize that condensate formation originates from the coalescence of pre-existing nanoaggregates. The selective occurrence of this process in PAS80 may arise from the higher density of its nanoaggregates together with more effective multivalent contacts established among the longer peptide chains. These features likely promote nanoaggregate fusion and ultimately drive the formation of liquid-like condensates.

Taken together, these findings highlight the critical role of peptide length in driving phase behaviour and suggest that PAS-based peptides can serve as versatile building blocks for engineering coacervates. By tuning chain length and concentration, it is possible to design condensates with controlled size and properties, opening avenues for biomedical applications such as drug delivery, tissue engineering, and compartmentalized biocatalysis. In future work, we will investigate how subtle variations in the primary structure of PAS peptides, introduced through the incorporation of tyrosine and phenylalanine residues, influence their liquid–liquid phase separation properties.

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CRediT authorship contribution statement

Hilda Garay-Perez: Writing – review & editing, Methodology, Investigation. **Marco Savioli:** Methodology, Investigation, Data curation. **Greta Petrella:** Methodology, Investigation, Data curation. **Dhayme de la Caridad Murillo Fernández:** Investigation. **Yanet Támara Hernández:** Investigation. **Matilde Lopez Abad:** Investigation. **Annachiara Ferrara:** Investigation. **Miriam Quattrociochi:** Investigation. **Daniel Cicero:** Methodology, Conceptualization. **Mariano Venanzi:** Supervision, Methodology. **Sukhvir Kaur Bhangu:** Data curation, Investigation, Methodology. **Gianfranco Bocchinfuso:** Supervision, Methodology, Data curation, Conceptualization. **Francesca Cavalieri:** Project administration, Investigation, Funding acquisition, Conceptualization.

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.

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Data availability

Data will be made available on request.

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