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RESEARCH ARTICLE

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Self-assembling tripeptide forming water-bound channels and hydrogels

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D-Ser(tBu)-L-Phe-L-Trp is described as a self-assembling tripeptide that yields nanofibrillar hydrogels at physiological conditions (phosphate buffer at pH 7.4). The peptide is characterized by several spectroscopic methods, such as circular dichroism and fluorescence, oscillatory rheometry, and transmission electron microscopy. Single-crystal X-ray diffraction reveals supramolecular packing into water-bound channels and allows the visualization of the intermolecular interactions holding together peptide stacks.

KEYWORDS
gels, hydrogels, nanofibril, self-assembly

1 | INTRODUCTION

In the last two decades, minimalistic peptides have raised great interest for their capability of self-assembling into nanostructured hydrogels for a variety of applications.[1] The vast majority,[2,3] but not all,[4–7] of peptide gelators employ amphipathic sequences that typically feature Phe residues to promote the spontaneous organization at low concentrations in water.[8] In particular, sequences as short as two to three amino acids are attractive for their low cost and simplicity of production and for the possibility of easily scaling-up their synthesis also in the liquid phase.[9] Numerous dipeptide and tripeptide gelators exploit N-capping groups to increase their hydrophobicity and promote their self-organization in water into hydrogels,[10,11] and examples of gelling dipeptides and tripeptides with free termini are considerably less frequent.[12–14]

Our group has explored the use of heterochiral sequences to modulate the spatial orientation of the sidechains of hydrophobic amino acids and to enable the segregation between hydrophilic and hydrophobic groups on opposite sides of the peptide backbone, toward the formation of amphiphilic gel superstructures in water.[15,16] In general, we found that dipeptide gels are less durable than those formed by tripeptide analogs, for instance, in cell culture conditions, thus finding more limited applicability.[12] Furthermore, tripeptides offer a greater diversity of sequence design, also toward the attainment of bioactivity, because, on average, they display the ideal number of non-hydrogen atoms (i.e., 25), for maximal efficacy of interactions as ligands with their receptors' counterparts.[17] Indeed, taking into account that, on average, an amino acid has 8.3 of such atoms, a tripeptide accounts for 24.9.[17] To increase the chemical diversity of this type of gelators, which typically are based on the Phe-Phe motif,[18] in this manuscript, we report the supramolecular behavior of D-Ser(tBu)-L-Phe-L-Trp, with free termini and a protecting group on the Ser sidechain that was required to gain sufficient hydrophobicity for gelation. Furthermore, the inclusion of Trp might endow the gel with luminescent properties,[19] although this is not a given, considering that several mechanisms could lead to quenching.[20–22] The tripeptide sequence was thus chosen taking into consideration all these factors, and the order and identity of the

aromatic residues were dictated by Reches' and Gazit's observations that Phe-Trp, but neither Trp-Phe nor Trp-Trp, maintained the ability to self-assemble into nanotubes manifested by the original peptide sequence Phe-Phe.[18]

2 | MATERIALS AND METHODS

2.1 | Materials and general methods

Merck (Milan, Italy) provided all the reagents and solvents, which were employed without further purification. 2-Chlorotrytil chloride resin was purchased from GL Biochem (Shanghai, China). All buffers and aqueous solutions were made with pure water that was dispensed from a Millipore MilliQ-system RiOs/Origin (St. Louis, MS, USA) by setting a resistivity >18.2 MΩ·cm at 25°C. ^{1}H- and ^{13}C-NMR spectra were acquired on a Varian Innova spectrometer at 400 and 100 MHz, respectively, and liquid chromatography coupled to mass spectrometry (LC-MS) data were recorded using an Agilent 6120 Infinity.[23] UV–visible (UV–Vis) spectra were measured using a quartz cell (0.1 mm) on an Agilent-Cary 5000 UV–Vis over the range of wavelengths from 350 to 200 nm, with a resolution of 1 nm. Fourier-transformed infrared (FT-IR) spectra were registered on a Varian Cary 660 spectrometer as previously described.[23]

2.2 | D-Ser(tBu)-L-Phe-L-Trp preparation

The tripeptide was prepared by solid-phase peptide synthesis, using Fmoc-protection strategy and 2-chlorotrytil chloride resin, using a standard protocol.[24] The crude was purified by high-performance LC (HPLC) in reverse phase (Agilent 1260 Infinity) on a C-18 column (Phenomenex Kinetex, 5 µm, 100 Å, 250 × 10 mm), employing a gradient of acetonitrile (MeCN)/water with 0.05% trifluoroacetic acid (TFA) as follows: $t = 0$–2 min 30% MeCN; $t = 18$ min 70% MeCN; $t = 20$–22 min 95% MeCN ($t_R = 8.9$ min). The product was freeze-dried into a white powder. ^{1}H- and ^{13}C-NMR spectra and ESI-MS spectra confirmed product identity and purity (Supporting Information S1).

2.3 | Hydrogelation and oscillatory rheology

D-Ser(tBu)-L-Phe-L-Trp (60 mM) was dissolved in sodium phosphate (0.1 M at pH 13.0), with the assistance of ultrasounds (Branson sonicator bath 3800, 10 min, 40°C). Subsequent cooling down to room temperature yielded hydrogels (30 mM) at a pH of 7.3 ± 0.1 by adding an identical volume of slightly acidic sodium phosphate buffer (0.1 M, pH 5.9). Oscillatory rheology was carried out using a Kinexus Ultra Plus rheometer, with parallel-steel plates (20 mm flat) at 5 Pa and 1 Hz. Kinetics were studied for 60 min by forming the gel directly onto the rheometer plate. Frequency ramps were recorded at 5 Pa, and stress ramps employed a frequency of 1 Hz.

2.4 | Circular dichroism

Samples for circular dichroism (CD) were prepared in quartz cells (0.1 mm) using the procedure described above. CD spectra were immediately recorded at 25°C on a Jasco J-815 with 1 nm resolution and a scanning rate of 50 nm/min. Spectra shown are the average of at least five measurements. CD spectra were also acquired on the peptide in solution by dissolving it at 1 mM in milliQ water and using NaOH 0.1 M to adjust the pH to 7.0.

2.5 | Fluorescence studies

Fluorescence studies were performed in solution and in the gel phases using a Tecan 1000 Infinity. In solution, various peptide concentrations were used to prepare samples in milliQ water, and the pH was adjusted to neutrality by adding NaOH 0.1 M. Samples (150 µL) were placed in a Nunclon 96-well plate (polystyrene, black), and excitation spectra were recorded using an emission $\lambda = 350$ nm, whereas emission spectra were obtained with an excitation $\lambda = 280$ nm at 25°C, with a resolution of 2 nm. Thioflavin-T studies were performed as previously described.[23]

2.6 | Crystallization

The tripeptide was dissolved (3 mM) in a solution consisting of 80% milliQ water and 20% methanol in a small glass vial with a screwtop slightly open. Slow evaporation at room temperature yielded needle crystals over a month. Details of single-crystal X-ray diffraction (XRD) can be found in Supporting Information S1.

2.7 | Transmission electron microscopy (TEM)

TEM micrographs were recorded on a JEM 2100 (Jeol, Tokyo, Japan) with a voltage set to 100 kV on freshly prepared samples that were carefully transferred onto carbon-lacey grids, previously charged using a UV-Ozone Procleaner Plus for 360 s and dried in vacuo. Contrast was obtained by adding a drop of potassium phosphotungstate at 2% at pH 7.2. Image analyses were performed using ImageJ2 software freely available from FIJI (https://imagej.net).

3 | RESULTS AND DISCUSSION

3.1 | D-Ser(tBu)-L-Phe-L-Trp preparation and characterization

The tripeptide D-Ser(tBu)-L-Phe-L-Trp (Figure 1A) was prepared in solid phase using a standard protocol with Fmoc as N-protecting group and 2-chlorotrytil chloride resin.[24] Purification by HPLC in reverse phase yielded the pure tripeptide as supported by

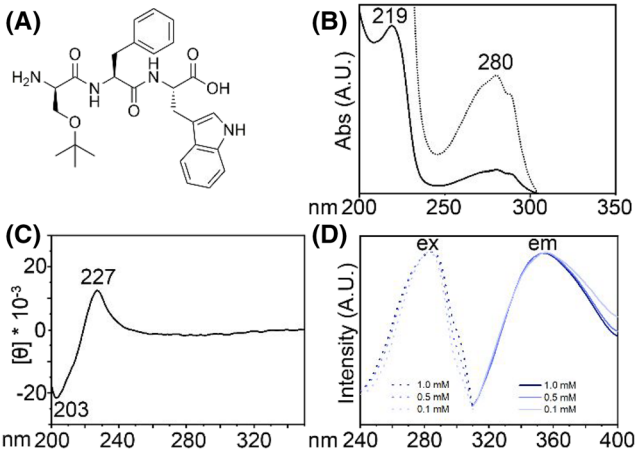

FIGURE 1 (A) Chemical structure of D-Ser(tBu)-L-Phe-L-Trp. (B) Ultraviolet–visible light absorption spectra at 1 mM (black line and 5× enlargement as dotted line). (C) Circular dichroism spectra at 1 mM. (D) Fluorescence excitation (dotted lines) and emission (solid lines) of peptide solutions (0.1, 0.5, and 1.0 mM).

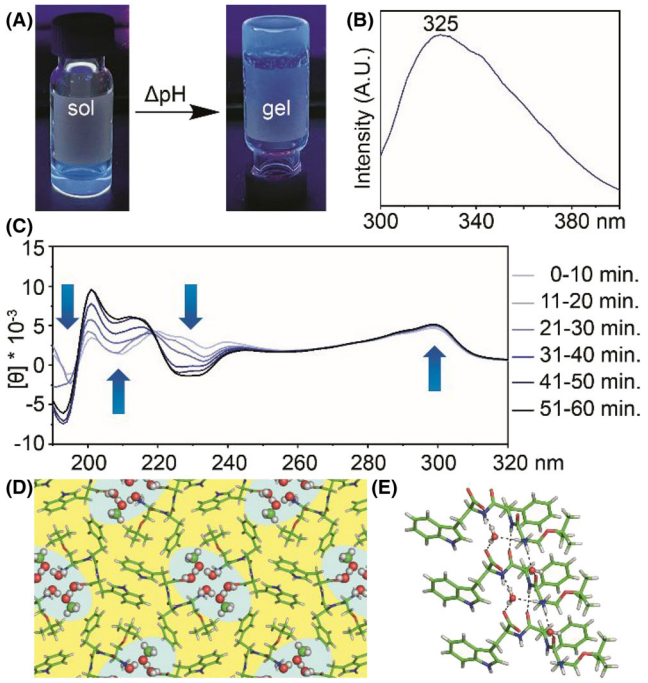

FIGURE 2 (A) Photographs of the peptide solution (left) and hydrogel formed through pH-triggered self-assembly (right) under ultraviolet-light irradiation (λ ≥ 254 nm). (B) Fluorescence emission spectra of the hydrogels (λex_{280}). (C) Circular dichroism spectra evolution during self-assembly. (D) Single-crystal X-ray diffraction packing (CCDC 2254368) along the *a* crystallographic direction identifies hydrophilic (light blue) channels with bound water, surrounded by hydrophobic regions (yellow). Peptide molecules are represented as sticks and solvent (water and methanol) molecules as spheres. (E) Peptide stacks are held together by H-bonds (black dashed lines represent H-bonds with distances <3 Å).

electrospray-ionization MS (ESI-MS) and ^{1}H- and ^{13}C-NMR spectroscopy (see Supporting Information S1). The photophysical properties of D-Ser(tBu)-L-Phe-L-Trp were probed by UV–Vis absorption spectroscopy (Figure 1B), CD (Figure 1C), and fluorescence (Figure 1D). The peptide absorbed UV–Vis light with two maxima at 219 and 280 nm and two shoulders at 272 and 288 nm (Figure 1B), which are classical features of the aromatic residues Phe and Trp, although the latter has a significantly higher extinction coefficient than the former, thus being responsible for the observed absorption. The CD spectrum displayed a positive first (at longer wavelengths) and negative second (at shorter wavelengths) Cotton effects in the 200–240 nm region, with a positive maximum at 227 nm and a negative minimum at 203 nm (Figure 1C). These features are indicative of a positive exciton coupling, which arises when the long axes of the two interacting units constitute a clockwise screw sense.[25] Fluorescence excitation spectra of the tripeptide solutions showed a maximum centered at 283 nm (Figure 1D). Emission spectra (λex_{280}) of more diluted samples (0.1 mM) displayed a maximum at 355 nm, and those of more concentrated samples (0.5–1.0 mM) manifested a 2 nm blueshift to 353 nm.

3.2 | D-Ser(tBu)-L-Phe-L-Trp self-assembly and supramolecular packing

Self-assembly was probed in sodium phosphate buffer, by first dissolving the tripeptide in its anionic form at alkaline pH and then lowering the pH to neutral to trigger hydrogelation of the zwitterions (Figure 2A). Stable hydrogels were obtained with a minimum gelling concentration of 30 mM (see also Figure S5), which was used for further studies (vide infra). Interestingly, under these conditions, fluorescence emission spectra (Figure 2B) displayed a marked blueshift relative to the peptide at lower concentrations (Figure 1D), with a broad and asymmetric maximum at 325 nm. Blueshifts in Trp fluorescence to λ < 330 nm are indicative of the localization of the amino-acid sidechain in buried, hydrophobic environments,[26] as expected as a result of the self-organization.

CD spectra were acquired in sodium phosphate solution during self-assembly (Figure 2C) and revealed the appearance of new features compared with the spectrum in solution shown in Figure 1C. In particular, in the far-UV range, the CD signal evolved with self-assembly over the first hour to a spectrum reminiscent of that reported for gelling D,L-tripeptides, with two positive peaks centered at 201 and 210–216 nm, which had been assigned to a population of β structures.[23] FT-IR measurements in the amide I region revealed the presence of two main signals at 1677 and 1644 cm^{-1} (see Figure S6). We inferred that self-assembly was associated with an alteration of the conformational distribution toward those that could self-associate into gelling fibers. Furthermore, the region above 240 nm, which was featureless in the CD spectrum in solution (Figure 1C), with self-assembly displayed two other positive maxima at 240 and 299 nm, likely a result of the confinement of the aromatic residues within the supramolecular architecture. These peaks were assigned to contributions from the aromatic sidechains of Phe and Trp, respectively.[27] For proteins, this region corresponds to the tertiary structure

fingerprint.[28] In particular, the near-UV absorption of Trp derives from ^1L_a and ^1L_b transitions, the former involving the NH group centered at ~280 nm and highly sensitive to the environment's polarity.[26] Conversely, the latter usually occurs at 290 nm; it involves only hydrophobic components, and it is less sensitive to the polarity of the surroundings.[29] The presence of a notable redshifted positive signal at 299 nm suggested that the Trp sidechain was located in a hydrophobic and rigid environment, as a result of self-assembly. Conversely, the Phe maximum that normally occurs at 250–260 nm displayed a marked blueshift that could indicate its exposure to hydrophilic surroundings.[30]

Interestingly, single-crystal XRD analysis unveiled a supramolecular packing that confirmed these features (Figure 2D and Supporting Information S1). In particular, piles of parallel peptides along the *a* crystallographic direction define hydrophilic water-bound channels (depicted in light blue in Figure 2D), whereby the N- and C-termini of different peptides face each other with the interposition of water molecules and the hydroxyl group of methanol molecules. The peptide stacks are held together through hydrophilic interactions (hydrogen bonds and salt bridges) involving the charged termini, the carbonyl oxygen and the amide nitrogen of the phenylalanine residue, and two water molecules (Figure 2E). These water-bound channels are surrounded by hydrophobic regions (depicted in yellow in Figure 2D), held together by weak hydrophobic interactions between the aromatic moieties, the *tert*-butyl substituent of the serine residue, and the methyl group of the methanol molecule.

3.3 | D-Ser(tBu)-L-Phe-L-Trp nanostructured hydrogel characterization

The viscoelastic behavior of the tripeptide hydrogels was probed by rheology (Figure 3). In particular, gelation occurred within 6 min as observed by naked eye, in agreement with the rheological kinetics study, with the storage (G') and loss (G") moduli reaching 17.0 and 0.7 kPa, respectively, within an hour (Figure 3A). Frequency ramps confirmed the gel stability, with both G' and G" not being affected by changes in the applied frequency interval of 0.1–10 Hz (Figure 3B). Lastly, stress ramps revealed a linear viscoelastic range up to 10 Pa and a transition from gel to sol at ~70 Pa (Figure 3C). Upon heating, the hydrogels started their transition to a solution at 55°C, and further heating to 94°C was required to completely dissolve the tripeptide, and upon cooling down to room temperature, only aggregates were attained (Figure 3D). Therefore, these hydrogels are not thermoreversible, contrarily to others composed of heterochiral tripeptides with free termini.[16,23,31]

TEM analyses of the gels revealed a dense network of fibers (Figure 3E), with an average diameter of 58 ± 19 nm ($n = 100$; Figure 3F), indicative of an amyloid nature, confirmed by a positive Thioflavin T binding assay. This well-known dye binds to hydrophobic grooves that are present on β-stacks,[32] and the consequent impeded rotation between the two aromatic constituents of Thioflavin T results in fluorescence.[33] This phenomenon can be used for the qualitative and quantitative assessment of amyloids, and it enabled us to derive the critical aggregation concentration of the tripeptide,[34] which corresponded to ~2 mM (Figure 3G).

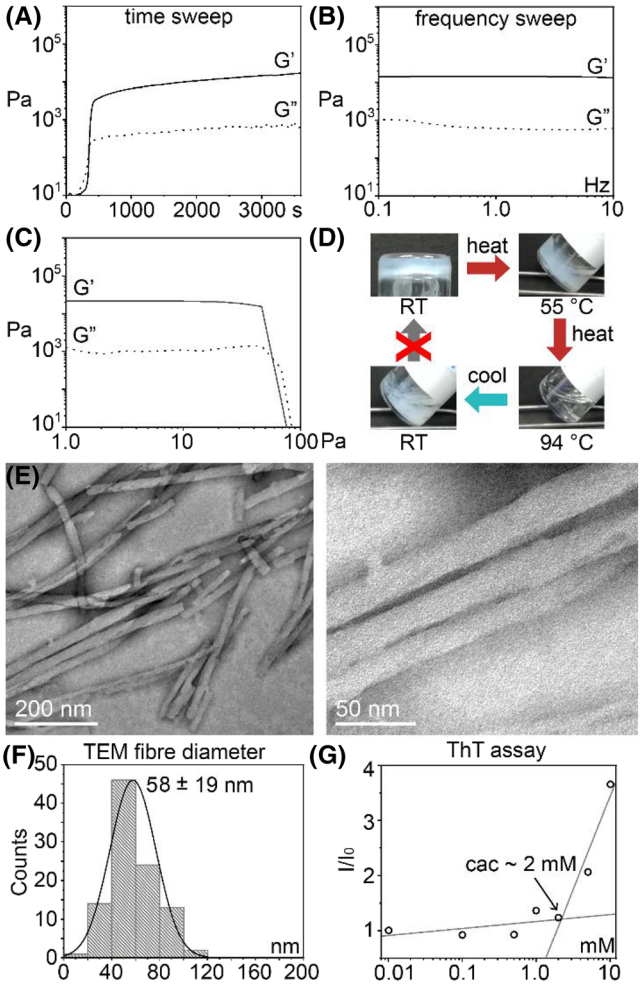

FIGURE 3 (A–C) Oscillatory rheology of the hydrogel: (A) time ramp, (B) frequency ramp, and (C) stress ramp. (D) Photographs of the peptide gel upon heating and cooling down to room temperature (RT). (E) Transmission electron microscopy (TEM) micrographs of the nanostructures present in the gel after drying on the TEM grid. (F) Fiber diameter distribution derived from TEM analyses ($n = 100$ counts). (G) Thioflavin T (ThT) fluorescence assay to calculate the critical aggregating concentration (cac).

4 | CONCLUSIONS

In conclusion, this work reports the characterization of D-Ser(tBu)-L-Phe-L-Trp as a self-assembling tripeptide that yields nanofibrillar hydrogels with amyloid character, responsive to fluorescence measurements. The single-crystal XRD structure showed an amphipathic packing with water-bound channels and features that are consistent with the spectroscopic behavior of the hydrogel, thus suggesting similar packing in the gel phase. Given that the inclusion of the D-residue may endow the gel with increased resistance against protease-

mediated degradation and that heterochiral short peptides displayed good cytocompatibility in vitro,[23] this type of hydrogel may find useful applications as a biomaterial. For instance, inclusion of bioactive motifs could be used to guide cell fate and promote cellular adhesion and growth[35] or to attain antimicrobial hydrogels.[36] Furthermore, coassembly in the presence of drugs could be employed in therapy for topical use, as recently demonstrated for heterochiral, self-assembling tripeptides combined with anti-inflammatory[37] or antitumoral agents.[38] Indeed, peptide-based hydrogel applications in the biomedical field are vast[39–41] and range from wound healing[42] to tumor therapy,[43] drug delivery,[44,45] tissue engineering,[46] hemostatic activity,[47] vaccine formulations,[48] bioimaging,[49] microarray bioassays,[50] and theranostics.[51,52]

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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