INTRODUCTION

Biomolecules have an intrinsic tendency to self-assemble into various morphologies. In the recent years, DNA, lipids, peptides, and proteins have been utilized in the construction of biomolecule-based nanostructures which can be put to various applications such as biosensors, templates for growth of other functional materials, enzymatic catalysis, gene expression, and drug delivery platform. The self-assembly process is governed by an intricate interplay of various noncovalent forces such as hydrogen bonding, hydrophobic interactions, aromatic stacking, and electrostatic interactions. The self-assembly of biomolecules is dependent on various factors such as self-assembling building blocks, solvent, concentration of the peptide, time of incubation, pH of the solution, and so forth. There are several studies in the literature where transition in self-assembly has been observed by modulating self-assembling building blocks, concentration, solvent, pH, chirality, temperature, and by the introduction of chemical groups such as thiol.

A thorough understanding of the mechanism of the self-assembly process and the morphological transitions induced by several factors is fundamentally essential to be able to manipulate the various factors to fine tune the self-assembly process and properties for custom-made applications.

Self-assembling hollow spheres derived from a variety of molecules such as polymers, fatty acids, lipids, polypeptides, and proteins have been reported to be prospective drug delivery vehicles. Most of these spherical drug delivery vehicles entrap the drug molecule and release them in the presence of certain stimuli such as pH or the presence of physiological cations such as potassium.

Of the different classes of biomolecules capable of self-assembly, peptide- and peptidomimetic-based scaffolds are immensely important because of their ease and high yields of synthesis, well-defined structures, biocompatibility, diversity, tunability of properties, and molecular recognition abilities. Diphenylalanine peptide forms the core recognition motif for the molecular self-assembly in Alzheimer’s disease identified through a methodical reductionist approach envisioned to find the minimum recognition motif for self-assembly. It has been studied in great detail because of its structural simplicity, ease of modification, biocompatibility, extremely strong capability of assembling under various conditions, and versatile applications. The aromatic interaction was thought to be one of the important factors driving the diphenylalanine self-assembly. However, the ditryptophan peptide does not show similar aggregation properties in spite of having an aromatic side chain that could have aromatic interactions. Verma and co-workers demonstrated that a tetrapeptide containing the ditryptophan moiety self-assembled to form nanovesicles. They also demonstrated that the ditryptophan moiety when attached to a tridentate ligand also gave rise to spherical vesicular structures.

Here, we report the self-assembly mechanism of a tetrapeptide Boc-Trp-Leu-Trp-Leu-OMe (P, Figure 1) and its application as a drug delivery platform.
concentration-dependent morphological transition from unilayered discrete nanospheres to clustered microspheres. Intrinsic tryptophan fluorescence acts as a reporter entity and helps in studying the self-assembly process of the peptide. Dye/drug binding studies significantly help in understanding the details of the self-assembled morphology. We further demonstrate that the peptide microspheres interact, intercalate, and restrain the hydrophobic dye carboxyfluorescein and drug molecule curcumin on its surface, which are released in the presence of physiologically relevant cations and acidic pH, in addition to encapsulating the drug in the conventional way. This dual mode of drug binding, we believe to the best of our knowledge, is a rather rare approach of drug delivery. As the drug release can be stimulated by lowering the pH, this model might find potential applications in cancer drug delivery.

### RESULTS AND DISCUSSION

Morphology of the peptide assembly was studied using various electron microscopic techniques. Figure 2 shows the morphology of P at 10 mM concentrations in various solvents such as dimethyl sulfoxide (DMSO), toluene, MeOH, EtOH, and so forth. It is clearly evident from the field-emission scanning electron microscopy (FESEM) images that P adopts different morphologies in various solvents. There have been several reports in the literature of microvesicles/microspheres being used as potential drug delivery vehicles. Thus, in our study, we focused only on the spherical morphology which was obtained in two solvents, namely, EtOH and MeOH. As MeOH is carcinogenic and in the practical scenario may not be practical for applications, we decided to continue our studies further with EtOH. Figure S1a,b shows the particle size distribution of the microspheres at 10 mM MeOH and EtOH. At 10 mM concentration of P, microspheres with diameters ranging from 2 to 3 μm were formed predominantly in EtOH. We further found from FESEM that P self-assembled into nanospheres of 200–300 nm diameter at as low a concentration as 0.02 mM (Figure 3a). The size of the microspheres was proportional to the concentration of the peptide solution. We wanted to find out whether this self-assembly process was kinetically or thermodynamically governed. Figure 3a,b shows the morphologies of P after incubation for 2 min and 10 days at 0.02 mM. There was no visible difference in the morphology, indicating that the self-assembled morphology of the reported peptide may be a thermodynamically controlled product. The self-assembled microspheres were disrupted upon the addition of KCl and formic acid, which is clear from FESEM images (Figure 3c,d).

For further insight into the self-assembled morphology, field-emission transmission electron microscopy (FETEM) studies were performed at the 0.02 mM peptide concentration. From FETEM studies, it was revealed that P formed nanospheres of diameter of about 200–400 nm at a concentration of 0.02 mM and microspheres of diameter of about 1–1.1 μm at a concentration of 10 mM, which is in corroboration with the FESEM data (Figures 4a, S2a). The electron diffraction pattern in Figure S2b shows that the microspheres are partly crystalline. However, so far, our attempts to crystallize the peptide have not succeeded. The particle size distribution of the microspheres as seen by FETEM at 10 mM is shown in Figure S2c. The average particle size was found to be 1 μM.

The topology of the self-assembled structures was studied using atomic force microscopy (AFM) (Figure 4b) at the 0.02

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**Figure 1.** (a) Schematic presentation of Peptide Boc-Trp-Leu-Trp-Leu-OMe (P). (b) Electrostatic potential map of gas-phase optimized geometry (method: Hartree–Fock basis set: 6-31+g*) of P. Blue and red indicate positive and negative charges, respectively.

**Figure 2.** FESEM image of P in (a) DMSO, (b) toluene, (c) MeOH, and (d) ETOH at the 10 mM peptide concentration. P adopts different morphologies in various solvents.
mM peptide concentration. Nanospheres of 200 nm diameter were seen, which also supported FESEM and FETEM data. Figures 4c,d shows the 3D plot and the surface profile analysis of the particles to see the width and flatness of the particles at that concentration.

To study the dependence of the size of the self-assembled nanospheres on the concentration of the peptide solution, dynamic light scattering (DLS) experiments were performed. At peptide concentrations of 0.01, 0.02, 0.03, and 0.04 mM, the diameter of the nanospheres varied from 248, 638, 971, and 1410 nm (Figure 5). Thus from DLS studies, it was clearly seen that with the increase in the concentration, the size of the microsphere increased, as was previously seen in the FESEM studies. The peptide formed smaller nanospheres at lower
concentrations, which started fusing with each other to form larger microspheres at higher concentrations.

Fourier transform infrared (FTIR) was performed to look into the conformation of tetrapeptide P in the solid state (Figure S3). The most informative frequency ranges for peptides are (a) $3500 - 3200 \text{ cm}^{-1}$, which corresponds to the $\text{N} - \text{H}$ stretching vibrations of the peptide and N-protecting urethane groups and (b) $1800 - 1600 \text{ cm}^{-1}$, which corresponds to the stretching vibrations of the peptide, urethane, and ester groups.\textsuperscript{89 - 92} Two intense bands were obtained at 3416 and 3312 $\text{cm}^{-1}$ indicating non-hydrogen-bonded and intermolecularly hydrogen-bonded NHs, respectively.\textsuperscript{90,92} The intensity of the NH band at 3312 $\text{cm}^{-1}$ is more than that at 3416 $\text{cm}^{-1}$, indicating hydrogen-bonded structures for the peptide in the solid state. The characteristic IR absorption bands at about 1641 $\text{cm}^{-1}$ (amide I) and 1531 $\text{cm}^{-1}$ (amide II) of the tetrapeptide are typical of the $\beta$-sheet.\textsuperscript{89 - 91} Moreover, the existence of the band at 1689 $\text{cm}^{-1}$ indicated the presence of antiparallel $\beta$-sheet structures.\textsuperscript{99}

Circular dichroism (CD) was performed at different concentrations of the peptide (Figure S4). Though CD is a sensitive technique for the prediction of the secondary structure of polypeptides,\textsuperscript{93} the limitations on the use of CD for conformational analysis of small linear peptides with aromatic residues have been reported.\textsuperscript{94,95} Moreover, the secondary structure formation has been shown to be dependent on the length of the polymers. As P is very small, the presence of random coil-like CD peaks was not surprising.\textsuperscript{96} Upon increasing the concentration of P beyond 0.15 mM, a noteworthy exciton-coupled band appeared at 215 nm (negative) and 229 nm (positive), which indicated interactions between aromatic chromophores of tryptophan\textsuperscript{97} (Figure S4). These bands were not seen at lower concentrations which suggested that the self-assembly that was obtained at a lower concentration of 0.02 mM did not involve stacking of the indole rings of tryptophan. The observation of the exciton-coupled band at high concentrations of the peptide suggested that some change in self-assembly occurred at high peptide concentrations involved aromatic stacking of tryptophan side chains.

To probe the self-assembly of the peptide further, we decided to monitor the intrinsic fluorescence of tryptophan residues present in the peptide. As the intrinsic fluorescence of tryptophan gets quenched upon aromatic stacking interactions of the indole rings during self-assembly, this study is immensely important in monitoring the self-assembly driven by aromatic stacking of tryptophan. Upon increasing the concentration of the peptide, the fluorescence intensity at 345 nm steadily increased till 0.156 mM, beyond which the fluorescence intensity got quenched upon increasing the concentration (Figure 6). This suggested that aromatic stacking of indole rings occurred beyond 0.156 mM and was absent at lower

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**Figure 5.** DLS study of P at (a) 0.01, (b) 0.02, (c) 0.03, and (d) 0.04 mM peptide concentrations showing increasing diameters of 248, 638, 971, and 1410 nm, respectively.

**Figure 6.** (a) Concentration-dependent fluorescence spectra of P and (b) plot of fluorescence emission intensity vs concentration of P.
concentrations. These data corroborated the CD data and suggested that the self-assembly into nanospheres at a low concentration of 0.02 mM was not assisted by tryptophan stacking. With increase in the concentration of the peptide, not only did the size of the peptide nanospheres increase as seen by DLS, but a significant change in the self-assembly pattern also occurred, which involved aromatic stacking of tryptophan.

**Studies on Binding/Encapsulation of Dye Carboxyfluorescein and Hydrophobic Drug Curcumin to the Peptide Microspheres.** Here, we examined the peptide nanospheres as an in vitro model for delivery of the hydrophobic fluorescent drug curcumin as well as the dye carboxyfluorescein. The interaction of the dye and the drug molecules with the peptide was studied using the intrinsic fluorescence emission of the dye/drug molecules. Figure 7a,b shows the fluorescence emission spectrum of carboxyfluorescein and curcumin, respectively, upon being incubated with the peptide solution (0.02 mM). Initially, after the addition of the dye/drug to the peptide solution, the fluorescence emission of the dye/drug was quenched, which indicated the interaction of the dye/drug with the peptide. The fluorescence kept steadily decreasing over time till about 6 h for carboxyfluorescein and 36 h for curcumin and stayed stable till 48 h (not shown in the figure). Upon the addition of KCl, the fluorescence emission of carboxyfluorescein/curcumin increased again. This might be a consequence of the release of the dye molecules due to disruption of peptide nanospheres upon the addition of KCl. Disruption of nanospheres in the presence of KCl has already been demonstrated earlier with FESEM (Figure 3c). The recovery of fluorescence in the case of carboxyfluorescein was almost 100%, as KCl seemed to have very little effect on it (Figure S5a). However, in the case of curcumin, the recovery of the fluorescence intensity was not 100%, as in the presence of KCl, the fluorescence of curcumin

Figure 7. Time-dependent fluorescence spectra of (a) carboxyfluorescein and (b) curcumin upon being added to 0.02 mM solution of P. The effect of the addition of KCl and HCOOH to the carboxyfluorescein/curcumin–peptide solution has also been studied.

Figure 8. Fluorescence microscopic image of P at 0.02 mM upon the addition of carboxyfluorescein. (a) Bright-field image, (b) fluorescence image, (c) superposition of (a,b), and (d) fluorescence image upon the addition of KCl.
was modified, as shown in Figure S5b. Upon the addition of HCOOH to the peptide solution containing curcumin, the fluorescence emission of curcumin increased, confirming the release of the dye upon disruption of nanospheres, as was previously seen with the FESEM study (Figure 3d).

The peptide−carboxyfluorescein interaction was also studied using fluorescence microscopy. Figure 8a shows the bright-field image of the peptide nanospheres at the 0.02 mM peptide concentration. Figure 8b is the fluorescence image of the dye; it shows localization at certain zones. Figure 8c is the superimposition of the two earlier images which demonstrates the co-localization of dye carboxyfluorescein and the peptide nanospheres. However, this picture does not explain whether the peptide is surface-bound or entrapped within the nanospheres. Figure 8d shows the loss of localization/scattering of carboxyfluorescein upon the addition of KCl which is an effect of the disruption of the vesicles upon the addition of KCl, which is already proved by FESEM (Figure 3c). This disruption of peptide self-assembly leads to the loss of interactions between the peptide and carboxyfluorescein, which explains the increase in the fluorescence emission of the dye, as seen by fluorescence spectroscopy (Figure 7a).

Figure 9. (a) FESEM image of peptide nanospheres formed in the presence of curcumin. (b) Time-dependent fluorescence spectra of curcumin upon the addition of freeze-dried P (final conc. 0.02 mM) to curcumin solution. The effect of the addition of KCl and HCOOH to the curcumin−peptide solution has also been studied.

Figure 10. Time-dependent fluorescence spectra of curcumin upon being added to (a) 0.02 mM and (b) 10 mM solution of P. Time-dependent fluorescence spectra of P (tryptophan) upon the addition of curcumin to (c) 0.02 mM and (d) 10 mM solution of P.
Next, we formed the nanospheres in the presence of curcumin (Figure 9a) and monitored the fluorescence emission of curcumin at different time points (Figure 9b). In this scenario, a lot of dye was encapsulated inside the nanospheres. Interestingly, this time, the fluorescence intensity of curcumin did not diminish appreciably, unlike in the earlier case where curcumin solution was added to the peptide solution, that is, to the already formed nanospheres (Figure 7b). This result suggested that curcumin was not entrapped within the nanospheres, but was rather surface-bound in the earlier case. As interactions of curcumin with the surface of the nanospheres led to appreciable quenching of fluorescence, it may be argued that the surface of the nanospheres contained some aromatic groups that interacted with aromatic moieties of curcumin (Figure S6). This suggested that tryptophan side chains stick out on the outer surface of the nanospheres formed at the 0.02 mM peptide concentration. When curcumin was entrapped inside the nanospheres, there was a slight fluorescence quenching, indicating that the inside of the nanospheres did not have aromatic groups to interact with curcumin. The slight decrease in the fluorescence intensity may have occurred because of the untrapped curcumin molecules in solution that interacted with the tryptophan residues on the surface. This is a clear hint that the interior of nanospheres did not contain any indole moieties of tryptophan but contained leucine side chains instead. Thus, the drug encapsulation studies proved a good way to indirectly understand the self-assembled morphology of P. Upon the addition of KCl and formic acid to the curcumin-entrapped nanospheres, the fluorescence emission of curcumin changed significantly. This was due to the rupture of the nanospheres releasing the encapsulated and bound curcumin. This changed fluorescence of curcumin was similar to that observed earlier upon the release of surface-bound curcumin. Figure S7 shows the FESEM image of freeze-dried curcumin-loaded nanospheres upon resuspension in EtOH, and Figure S8a,b shows the FESEM image of rupture of the curcumin-loaded nanospheres in the presence of KCl and HCOOH that validates the change in fluorescence emission of curcumin, as seen in Figure 9b. Furthermore, to prove that curcumin was indeed encapsulated in the microspheres, we analyzed the contents using mass spectrometry after disruption of the peptide microspheres in the presence of KCl. The presence of curcumin mass in Figure 9d, of the KCl-ruptured curcumin-loaded peptide microspheres with KCl, clearly proved that curcumin was entrapped in the microspheres (Figure S9).

To understand the microsphere morphologies at the 10 mM peptide concentration, curcumin was added to the peptide microspheres formed at 10 mM, and its fluorescence emission was monitored and compared with the data obtained upon the addition of curcumin to peptide solution at 0.02 mM (Figure 10a). Figure 10b shows the quenching of curcumin fluorescence with time, indicating the interaction of the peptide with dye, suggesting that even at the 10 mM concentration of the peptide, the microspheres had tryptophan sticking out of the surface just like in the case of P nanospheres formed at 0.02 mM. To ensure that the binding of curcumin to the surface of nanospheres was indeed through aromatic interactions between the aromatic moiety of the drug and the indole ring of tryptophan, fluorescence emission of Trp upon the addition of curcumin to preformed nano-assemblies at both 0.02 mM (Figure 10c) and 10 mM (Figure 10d) was monitored. In both the cases, quenching of tryptophan fluorescence was observed, proving that curcumin was indeed surface-bound to the nano-/microspheres by aromatic interactions between the tryptophan side chains and the aromatic moieties present in the drug (Figure S6).

Mechanism of Self-Assembly. Tetrapeptide Boc-Trp-Leu-Trp-Leu-OMe spontaneously self-assembled into spherical nano-/microspheres upon dissolution in EtOH, as seen from FESEM. On the basis of the experiments described above and the energy-optimized structure of the peptide (Figure 1b), it was possible to understand the mechanism of self-assembly of the tetrapeptide. Figure 11 is a schematic which attempts to explain the mechanism of concentration-dependent self-assembly of the tetrapeptide. From the energy-minimized structure of the peptide, the orientation of the tryptophan ring was seen to be roughly about perpendicular to the plane containing the peptide bond. From FTIR, the peptide was found to adopt anti-parallel β sheet conformation. When peptide strands hydrogen-bonded to form anti-parallel β sheets, the tryptophan residues pointed toward one side of the β sheet and the leucine side chains pointed toward the opposite side.
The tryptophan aromatic rings were poised in such a fashion that they did not stack with each other upon forming the anti-parallel β sheet. Thus, even upon forming the anti-parallel β sheet, the intrinsic fluorescence of tryptophan in the peptides increased proportionately with the concentration. This peptide sheet folded to form the nanospheres which were seen in FESEM at as low as 0.02 mM concentration (Figure 11c). These nanospheres were formed in such a way that the Trp side chains pointed outwards while the hydrophobic leucine side chains pointed inwards. The formation of nanospheres at the 0.02 mM concentration did not need the aromatic stacking of tryptophan and was driven by the hydrophobic effect. Because of the low concentration, the nanospheres remained discrete and did not cluster with each other (Figure 3a). Upon increasing the concentration of the peptide, self-assembly via the above mentioned mode continued and the size of the nanospheres increased by fusion of the smaller spheres forming larger spheres (microspheres), as seen by DLS studies (Figure 5). Beyond a certain concentration (0.156 mM), the microspheres came close together and formed clusters of microspheres (Figure 11d). This clustering was stabilized by aromatic interactions of tryptophan side chains pointing out of the microspheres. This led to quenching of tryptophan intrinsic fluorescence (Figure 6a) and appearance of exciton-coupled CD peaks (Figure S4) upon increasing the concentration of the peptide beyond 0.156 mM. The proximity of the microspheres at higher concentrations is seen in the FESEM images at 10 mM (Figure 2d). Figure 11E–G indicates surface binding and encapsulation of curcumin at different peptide concentrations.

Quantification of Drug Loading. Our peptide microspheres can both intercalate with the drug on the surface as well as encapsulate it. For the intercalation mode of curcumin binding, drug loading efficiencies after 24 h at 0.02 and 10 mM of the peptide concentration for 20 μM of curcumin were 87.5 and 84.2%, respectively (Figure S10b). It was not possible to quantify the drug encapsulation for our drug delivery system. This was because when the drug was encapsulated inside the microspheres, some of the drug was also left unencapsulated in the medium. This drug immediately intercalated with the tryptophan on the surface of the microspheres. The species obtained in this case were peptide microspheres with both drug encapsulation and binding. Very little amount of drug was left free in the medium, as proved by mass spectrometry. The fluorescence signal obtained in this case was due to the fluorescence of the encapsulated drug—fluorescence quenching due to the surface-bound drug and hence could not be used to quantify the amount of the encapsulated drug alone (Figure S10c).

SUMMARY AND CONCLUSIONS

In conclusion, we have been able to explain the mechanism of self-assembly of a tetrapeptide containing tryptophan. This is a befitting example showing how self-assembly is governed by an interplay of several forces. At the low concentration range, intramolecular hydrogen bonding leads to the formation of anti-parallel β sheets that are driven to assemble into unilaered nanospheres by the hydrophobic effect, with the leucine side chains pointed towards the core of the nanosphere and tryptophan side chains pointing outwards. Upon increasing the concentration of the peptide, the size of the peptide spheres increases, eventually forming unilaered microspheres. Beyond a certain concentration, clustering of microspheres occur which is stabilized by aromatic stacking interactions between the tryptophan side chains sticking out of the microspheres. We have also demonstrated the potential of the nanospheres to be used as a drug delivery vehicle. When curcumin is added to P nanospheres, it is intercalated on the surface of the nanospheres instead of being encapsulated in the spheres. Curcumin interacts with peptide nanospheres via aromatic interactions between the indole ring of the tryptophan sticking out of the nanosphere surface and its aromatic moiety. On the other hand, upon forming the peptide nanospheres in the presence of curcumin, encapsulation occurs. In both cases, the drug is released in the presence of physiologically relevant ions such as potassium and in acidic conditions upon disruption of the peptide self-assembly. With dual drug encapsulation and intercalation capabilities, this prototype is definitely a prospective drug delivery vehicle.

EXPERIMENTAL SECTION

Materials and Methods. All amino acids, di-tert-butyl dicarbonate, dioxane, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl) were purchased from Spectrochem India. Sodium chloride, potassium chloride, sodium hydroxide, 1-hydroxybenzotriazole, triethyl amine, and thionyl chloride, DMSO, toluene, dichloromethane (DCM), and high-performance liquid chromatography (HPLC) grade acetone, and methanol were purchased from Merck. Absolute ethanol was purchased from Tedia (USA). Carboxyfluorescein and curcumin were obtained from Sigma and TCI, respectively.

Synthesis of the Peptide (General Procedure). A racemization-free, fragment condensation technique-based solution-phase method was employed for the synthesis of the tetrapeptide (Boc-Trp-Leu-Trp-Leu-OMe). Tertiary butyloxycarbonyl and the methyl ester group were used for the protection of amino and carboxyl groups. The Boc group and the ester group were deprotected using formic acid and saponification reactions, respectively. Coupling was mediated via EDC·HCl and HOBt. The progress of the reaction was monitored using thin layer chromatography (TLC). The final peptide was obtained with high purity by column chromatography using a silica gel (100–200) mesh as the stationary phase and a mixture of ethyl acetate and hexane in the ratio of 3:2 as the mobile phase. The peptide was further purified using HPLC for further removal of any impurities. The synthesized peptide was fully characterized by the 1H NMR study, mass spectral analysis, and FTIR.

Synthesis of Boc-Protected L-Tryptophan (Boc-W-OH). L-Tryptophan (20 mmol, 1 equiv) was dissolved in 5 N NaOH, stirred, and cooled to 0 °C. To the stirring solution, Boc anhydride (24 mmol, 1.2 equiv) dissolved in 1,4-dioxane was added. The pH of the reaction was maintained at >12. The reaction mixture was allowed to stir overnight. Dioxane was evaporated completely over rotavapor. Water was added to the reaction mixture, washed with ethyl acetate (3 × 30 mL), and acidified with 6 N HCl (pH = 2). The aqueous layer was extracted with ethyl acetate (3 × 30 mL), the organic layers were pooled and washed with 25% brine solution. The organic layer was dried over Na2SO4 and evaporated to get the Boc-protected L-tryptophan. (Boc-W-OH). The yield of the reaction was 5.89 g (19.4 mmol, 97%). HRMS (ESI-TOF) m/z: (m + H)+ calc for C16H20N2O4, 305.1496 Da; found, 305.1451 Da (Figure S11). 1H NMR (DMSO-d6, 600 MHz, δ ppm) (Figure S12): 1.33 (s, 9H, BOC-H), 2.96–3.02 (m, 1H, Cβ-H), 3.12–
3.17 (m, 1H, Cβ-H), 4.12–4.19 (m, 1H, Cα-H), 6.94–6.97 (d, 1H, J = 12 Hz, Cβ-H wto indole ring), 6.98–7.02 (t, 1H, J = 9 Hz, Cα-H wto indole ring), 7.05–7.10 (t, 1H, J = 6 Hz, Cβ-H wto indole ring), 7.14–7.20 (broad singlet, 1H, Cα-H wto indole ring), 7.33–7.37 (d, 1H, J = 12 Hz, Cα-H wto indole ring), 7.52–7.55 (d, 1H, J = 6 Hz, amide NH), 10.83 (s, 1H, NH indole).

**Synthesis of Methyl Ester of l-Leucine (l-OMe·HCl).** l-Leucine (50 mmol, 1 equiv) and dry methanol (50 mL) were taken in a round-bottom flask, covered with a calcium chloride guard tube, and cooled in an ice bath. Into the stirring reaction mixture, SOCl2 (75 mmol, 1.5 equiv) was added dropwise. The reaction mixture was stirred for another 30 min under ice-cooled conditions. After 30 min, the reaction mixture was refluxed for 4 h. After 4 h, the reaction mixture was cooled down and then evaporated to get the methyl ester of l-leucine. The yield of the reaction was 7.67 g (42.5 mmol, 85%).

**Synthesis of Dipeptide Boc-Trp-Leu-OMe.** l-OMe·HCl (15 mmol) was dissolved in a minimum amount of dry DCM (30 mL) to which 5.9 mL (2.8 equiv) of triethyl amine was added. The solution was cooled to 0 °C and stirred for 15 min. This was added to a precooled solution of Boc-protected tryptophan Boc-W-OMe (15 mmol) in dry DCM (30 mL) and preactivated with EDC-HCl (1.2 equiv) and HOBT (1.2 equiv). After 12 h, the reaction mixture was washed with distilled water and brine for three times. The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated in vacuum to yield an off-white solid. The dipeptide was purified with column chromatography using silica gel as the stationary phase and a mixture of ethyl acetate and hexane in the ratio of 3:2 as the eluent. The yield of the reaction was 2.0 g (2.8 mmol, 58.1%), mp 154 °C. The peptide purity was confirmed by analytical HPLC trace (Figure S15).

**Synthesis of Peptide Boc-Trp-Leu-OMe.** Formic acid (10 mL) was added to the dipeptide Boc-Trp-Leu-OMe (4.8 mmol) and kept for 3 h. Thereafter, formic acid was completely removed under pressure. The residue so obtained was taken in water, and the pH of the solution was adjusted to 11 using sodium bicarbonate and then extracted with ethyl acetate (3 x 30 mL). Organic layers were pooled, washed with brine, dried over sodium sulfate, and concentrated to a viscous liquid which responds to the ninhydrine test. The Boc-deprotected dipeptide (NH2-Trp-Leu-OMe) was dissolved in DCM and triethyl amine was added to it at 0 °C, and the reaction mixture was stirred for 15 min. This was added to the precooled Boc-Trp-Leu-OMe in dry DCM, preactivated with EDC-HCl and HOBT. After 72 h, the reaction was washed with distilled water and brine (3 x 30 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated in vacuum to yield an off-white solid. The tetrapeptide was purified using silica gel as the stationary phase and a mixture of ethyl acetate and hexane in the ratio of 3:2 as the eluent. Final purification was done by reverse-phase HPLC using ACN/H2O solvent mixtures. Tetrapeptide was obtained as a white powder. The yield of the reaction was 2.0 g (2.8 mmol, 58.1%), mp 154 °C. The peptide purity was confirmed by analytical HPLC trace (Figure S15) and 1H NMR spectra (Figure S16). 1H NMR (DMSO-d6, 600 MHz, δ ppm): 0.78–0.83 (m, 12H, leucine β-H), 1.07–1.09 (t, 2H, leucine γ-H), 1.26 (s, 9H, Boc-H merged with leucine β-H), 1.3–1.5 (m, 4H, leucine β-H merged with Boc-H), 2.8–3.14 (m, 4H, tryptophan β-H), 3.6 (s, 3H, OMe), 4.1–4.6 (s, 4H, chiral), 6.81, 7.88, 8.07, 8.26 (d, 4H, J = 6 Hz, amide N–H), 6.91–6.97 (t, 2H, J = 12 Hz, Cβ-H wto indole ring), 7.02–7.05 (t, 2H, J = 6 Hz, Cα-H wto indole ring), 7.08–7.11 (d, 2H, J = 6 Hz, Cβ-H wto indole ring), 6.98–7.30 (d, 2H, J = 12 Hz, Cβ-H wto indole ring), 7.52–7.58 (d, 2H, J = 6 Hz, Cα-H wto indole ring), 10.79–10.80 (s, 2H, indole ring N–H). FTIR (cm–1): 3415.59, 3312.23, 1689.24, 1642.73, 1531.41 (Figure S17). HRMS (ESI-TOF) m/z: (m + H)+ calcld for C32H38N6O8, 668.2917 Da; found, 668.2751 Da (Figure S8).

**Nuclear Magnetic Resonance Spectroscopy.** All nuclear magnetic resonance studies were carried out on a Bruker Ascend Aeon 600 MHz spectrometer at 298 K. The compound concentrations were in the range 1–10 mmol in DMSO-d6.

**Mass Spectrometry.** ESI (positive mode)-HRMS masses were recorded using Agilent 6520 Accurate Mass Q-TOF LC/MS.

**FTIR Spectroscopy.** IR spectra were recorded in a KBr pellet in a PerkinElmer spectrometer in the region of 400–4000 cm–1.

**Field Emission Scanning Electron Microscopy.** The morphology of the reported materials was studied by an FESEM Sigma Zeiss Gemini microscope.

(a) For the FESEM study in different solvents, the lyophilized peptide was incubated for 5 min in different solvents (acetonitrile, DMSO, toluene, methanol, and ethanol) at a peptide concentration of 10 mM. The peptide solutions were drop-casted on a silicon wafer and dried under vacuum at room temperature overnight (7 days for DMSO solution).

(b) For the morphology study at lower concentrations, 0.02 mM ethanolic peptide solution was prepared and after 2 min, 5 μL of this solution was taken out and drop-casted on a wafer. Another part of the solution was allowed to age for 10 days and 5 μL of the same was drop-casted on a wafer. KCl (20 μL, 0.01 mM) and 50 μL of HCOOH were added to two batches of 10 days-aged peptide solution (5 μL each), incubated for 48 h, and drop-casted on a silicon wafer.

(c) Lyophilized peptide (1.46 mg) was added to 100 mL of 20 μM curcumin solution to the final peptide
concentration of 0.02 mM and incubated for 2 days. Five microliters of this solution was drop-casted on a silicon wafer. Water was added to the peptide solution, and it was freeze-dried in three batches. One batch was resuspended in EtOH and to the other two batches, 5 μL of 0.01 mM of KCl and 50 μL of HCOOH were added and incubated for 48 h, and 5 μL of each batch was casted on a wafer.

Field-Emission Transmission Electron Microscopy. FETEM studies were performed on JEOL JEM (model 2100F) at an operating voltage of 200 kV by casting 3 μL of 0.02 mM ethanolic solution of the peptide on carbon-coated copper grids (300 mesh). EtOH was removed by slow evaporation, and the grid was allowed to dry under vacuum at room temperature for 3 days. Uranyl acetate solution (3 μL) was added to the grid and dried under vacuum overnight. Images were taken in both the transmission mode and the diffraction mode.

Atomic Force Microscopy. All experiments were performed on Agilent (S500 series). For the AFM study, 5 μL of ethanolic solution of the peptide (10 mM, 0.02 mM) was placed on a microscope glass coverslip and dried by slow evaporation under vacuum for 3 days.

Dynamic Light Scattering. The particle size of the peptide spheres was determined by DLS with corresponding peptide solution in ethanol at different concentrations (0.01, 0.02, 0.03, and 0.04 mM). The experiment was carried out in Zetasizer Nano ZS90 from Malvern using a 632.8 nm He–Ne laser at 298 K.

CD Spectroscopy. The CD spectra of all the samples were recorded by using a 200 μL quartz cuvette of 1 mm path length with a Jasco J-1500 spectropolarimeter at room temperature. Spectra were collected at a scan rate of 100 nm-min⁻¹ and 2 nm bandwidth from 195 to 260 nm with five scans for averaging. Before running the sample, ethanol was run to correct the baseline.

Fluorescence Spectroscopy. All fluorescence measurements were performed on a HORIBA JOBIN YVON Fluoromax-4 spectrofluorometer using a cuvette of path 1 cm.

Intrinsic tryptophan fluorescence of the peptide was monitored to study the peptide self-assembly process. Emission spectra were recorded for peptide solutions at different concentrations in between 10 and 0.04 mM at an excitation wavelength of 280 nm and a slit width of 3.

The peptide microsphere dye/drug interaction was studied by monitoring fluorescence emission of dye carboxyfluorescein and drug curcumin.

Peptide–carboxyfluorescein interaction: 5 mL of peptide solution was mixed with 5 mL of ethanolic carboxyfluorescein solution to attain the final peptide and carboxyfluorescein concentrations of 0.02 mM and 90 μM respectively. Emission spectra of carboxyfluorescein were recorded at different time intervals with an excitation wavelength of 493 nm and a bandwidth of 5 nm. Finally, after 24 h, 5 μL of 0.01 mM KCl was added to 5 mL of the above mentioned solution and incubated for 24 h, and fluorescence was measured. To the other 5 mL of the abovementioned solution, 50 μL of HCOOH was added and incubated for 24 h, and fluorescence was measured.

Curcumin peptide interaction: The experiment was done in two different ways.

1) Lyophilized peptide (1.46 mg) was added to 100 mL of 20 μM ethanolic solution of curcumin to make a final peptide concentration of 0.02 mM. Emission spectra with an excitation of 430 nm and a bandwidth of 5 were recorded at different time intervals up to 36 h. KCl and HCOOH were added to different portions of the abovementioned solution as described previously, and fluorescence was recorded.

2) To ethanolic peptide solution, curcumin solution was added to make a final peptide concentration of 0.02 and 10 mM and a final curcumin concentration of 20 μM. Fluorescence emission was monitored at different time intervals. After 36 h, KCl and HCOOH were added to different portions of the abovementioned solution as described previously, and fluorescence was recorded.

Proof of Curcumin Encapsulation. Peptide (10 mM) was added to 20 μM curcumin solution. The microspheres containing curcumin were coagulated using water and centrifuged down. The supernatant’s mass was checked. After several cycles of washes followed by centrifugation, the precipitate was resuspended in ethanol and water and lyophilized. Lyophilized microspheres loaded with curcumin were again resuspended in EtOH, and its mass was checked. Finally, KCl was added to rupture the microspheres to release curcumin, and mass was checked.

Quantification of Curcumin Loading. We have quantified the amount of the drug intercalated (surface bound) using fluorescence spectroscopy at both the 0.02 and 10 mM peptide concentration. For intercalation (surface binding), curcumin solution was added to peptide solution, and fluorescence was measured after 24 h of incubation. The quenching of curcumin fluorescence was due to surface binding of the drug to the peptide microspheres. Hence, the fluorescence signal was obtained from the unbound drug.

Figure S10 in the supplementary shows the standard curve of curcumin fluorescence and the fluorescence emission experiment done to quantify the drug loading efficiency. Drug loading efficiency = (amount of encapsulated drug/amount of injected drug) × 100.

Fluorescence Microscopy. Ethanolic solution of the peptide (0.02 mM) was incubated with 50 μM of carboxyfluorescein for 24 h. Then, one drop (10 μL) of this solution was drop-casted on a cover slide and dried at room temperature under vacuum. The fluorescence microscopic image was taken with a Nikon eclipse Ts2R fluorescence microscope. For salt-triggered disruption studies with the spheres, KCl solution (1 mM 5 μL) was added to the dye solution of the peptide and incubated for 12 h, and the image was taken subsequently.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01871.

Particle size distribution figures for FESEM in MeOH and EtOH at 10 mM; FETEM and particle size analysis of P microspheres at 10 mM concentration of P; electron micrograph image of P at 0.02 mM; fluorescence spectra showing the effect of KCl on the fluorescence emission of carboxyfluorescein and curcumin; chemical structure of curcumin and carboxyfluorescein; FESEM of freeze-dried and EtOH re-suspended curcumin-loaded nano-
spheres; FESEM of ruptured nanospheres in the presence of KCl and HCOOH; ESI-MS of KCl-ruptured curcumin-loaded P microspheres; quantification of drug loading efficiency; ESI-MS and 1H NMR spectra of Boc-W-OMe; ESI-MS and 1H NMR spectra of Boc-WL-OMe; ESI-MS, 1H NMR spectra, analytical HPLC trace, FTIR spectra, and CD spectra of Boc-WLWL-OMe (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: sunanda.c@iitg.ernet.in, chatterjee.sunanda@gmail.com. Phone: +918811080890 (S.C.).

ORCID

Sunanda Chatterjee: 0000-0001-5068-7208

Author Contributions

S.C. conceived the idea and designed the project; S.C. and G.P. designed the experiments. G.P., K.R., and U.A. performed the experiments; S.C. analyzed the data and wrote the paper.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MeOH, methanol; EtOH, ethanol; DMSO, dimethyl sulphoxide; KCl, potassium chloride; HCOOH, formic acid; ESI, electrospray ionization; HRMS, high resolution mass spectrometry

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