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Supramolecular self assembly of nanodrill-like structures for intracellular delivery

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Abstract

Despite recent advances in the supramolecular assembly of cell-penetrating peptide (CPP) nanostructures, the tuning of size, shape, morphology and packaging of drugs in these materials still remain unexplored. Herein, through sequential ligation of peptide building blocks, we create cell-penetrating self-assembling peptide nanomaterials (CSPNs) with the capability to translocate inside cells. We devised a triblock array of Tat_{48–59} [HIV-1 derived transactivator of transcription_{48–59}] based CPPs, conjugated to up to four Phenylalanine (Phe) residues through an amphiphilic linker, (RADA)₂. We observed that the sequential addition of Phe leads to the transition of CSPN secondary structures from a random coil, to a distorted α -helix, a β -sheet, or a pure α -helix. This transition occurs due to formation of a heptad by virtue of even number of Phe. Atomic force microscopy revealed that CSPNs form distinct shapes reminiscent of a “drill-bit”. CSPNs containing two, three or four Phe, self-assemble into “nanodrill-like structures” with a

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Author Contributions

G.S directed the project and conceived ideas to conduct all studies. G.S and N.A designed strategies to engineer CPPs for intracellular delivery by a triblock array. N.A was involved in conducting all experiments listed. N.A performed peptide synthesis, their characterization and drug encapsulation and release studies. J.S.P., and S.C.E. performed AFM analysis. B.M and DZ performed MD simulation studies. K.M. performed CD spectral analysis. S.P and P.K performed autophagy induction experiment and cell uptake study respectively. C.S provided ideas to SP for doing in-vivo biodistribution. G.S and N.A. analyzed data and wrote the paper. All authors refined the manuscript.

coarse-twisted, non-twisted or fine-twisted morphology, respectively. These nanodrills had a high capacity to encapsulate hydrophobic guest molecules. In particular, the coarse-twisted nanodrills demonstrate higher internalization and are able to deliver rapamycin, a hydrophobic small molecule that induced autophagy and are capable of *in vivo* delivery. Molecular dynamics studies provide microscopic insights into the structure of the nanodrills that can contribute to its morphology and ability to interact with cellular membrane. CSPNs represent a new modular drug delivery platform that can be programmed into exquisite structures through sequence-specific fine tuning of amino acids.

Graphical abstract

Keywords

Cell penetrating peptides; Nanodrills; Intracellular delivery; Supramolecular assembly

1. Introduction

CPPs are versatile platforms for the intracellular delivery of small molecular drugs, liposomes, antibodies, imaging agents, proteins and nucleic acids [1–5]. The Tat_{48–59} peptide (GRKKRRQRRRPQ) is a well-known CPP derived from HIV-1[6] that has the ability to enter cells owing to its strong cationic charge and ability to interact with membrane receptors. Tat has shown a high degree of efficacy in the delivery of the aforementioned cargoes, as demonstrated in preclinical and clinical studies [7–11]. In most cases, the peptide has been chemically conjugated to the cargo for the purpose of intracellular delivery [12–17]. New methods have been employed to develop supramolecular nanostructures with Tat peptides as self-assembling amphiphiles. This is usually achieved by ligating a lipid tail onto Tat, which leads to its self-assembly into nanosized fibers [7], sheets [18], ribbons [9], and micelles[8]. In contrast, the use of further peptide building blocks to develop Tat peptide-based structures is a relatively unexplored strategy [19,20]. The electrostatic repulsion of cationic residues in Tat, which impedes its self-assembly, can be overcome through conjugation with amphiphilic peptides, leading to supramolecular assemblies of different shapes. CSPNs were assembled through conjugation of TAT with peptide building blocks containing ionic and hydrophobic amino acids. The fine tuning of the amphiphilic character through sequential addition of amino acids lends versatile properties such as flexibility, self-assembly, biodegradability and biocompatibility for effective intracellular delivery [21– 24].

Tat peptides were conjugated to an amphiphilic linker, (RADA)₂, followed by sequential addition of phenylalanine (Phe) residues. We chose (RADA)₂ as the amphiphilic sequence since it contains alternating hydrophilic and hydrophobic amino acids with self-complementarity and imparts the property of self assembly [25]. This sequence is a part of RADA-16, a well-explored peptide in the field of drug delivery[26–28] and tissue engineering[29–31] that self-assembles into β -sheeted nanofibers. The hydrophobic cores of the CSPNs were designed through introduction of different numbers of Phe (0–4). Phe was chosen because, unlike other aromatic amino acids (tryptophan, tyrosine and histidine), it contains an aromatic ring without functional groups or heteroatoms. This governs its ability to self-assemble through aromatic π - π stacking with a distinct hydrophobic core. Furthermore, 9-Fluorenylmethyloxycarbonyl chloride (Fmoc) capping at the N-terminal was incorporated to strengthen the aromatic π - π stacking [32]. We were able to generate five different CSPNs, including Fmoc-(RADA)₂-Tat (RT), Fmoc-F-(RADA)₂-Tat (1F-RT), Fmoc-FF-(RADA)₂-Tat (2F-RT), FFF--(RADA)₂-Tat (3F-RT), and Fmoc-FFFF-(RADA)₂-Tat (4F-RT) (Figure.1.a–c, Figure.S1). Their syntheses and unique self assembly to form shape-defined nanodrills (here afterwards we are addressing nanodrill-like structures as “nanodrills”) with capability to deliver drugs delivery is demonstrated in the present study. A sequence-specific structurally controlled design of peptide amphiphiles can lead to their assembly into exquisite morphology with capability to deliver hydrophobic drugs to their subcellular targets.

2. Materials and Methods

2.1. Materials

All Fmoc protected amino acids and Tentagel–S–NH₂ Resin were purchased from Peptide International Inc. Fmoc Rink linker was purchased from Novabiochem. 8-Anilidonaphthalene-1-sulfonic acid ammonium salt (ANS) and rapamycin were obtained from Cayman Chemical Inc. Coupling reagents:- Hydrobenzoxo triazole (HOBt), Diisopropylethylamine (DIEA), O-Benzotriazole-N,N',N''-tetramethyluronium-hexafluorophosphate (HBTU), Cleavage reagents:- [Trifluoroacetic acid (TFA), Triisopropylsilane (TIS)], Ninhydrin, Piperidine, Dichloromethane (DCM), Diethyl ether and N, N-Dimethylformamide (DMF) was obtained from Sigma Aldrich. Cyanine 7-N-hydroxy succinimide ester was obtained from Lumiprobe Corporation.

2.2. Synthesis of peptide

Different CSPNs peptides were synthesized with Standard Fmoc-Solid Phase Peptide Synthesis (SPPS) Strategy. Initially Tentagel –S–NH₂ resin was linked to Fmoc Rink linker using HBTU/ HOBt activation. Then the peptide chain was assembled starting from C-terminal using standard Fmoc methods. Each step was monitored using Ninhydrin test. After completion of the sequence, the peptide fragment was cleaved manually using trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water (95:2.5:2.5). Finally the peptide was precipitated using ice-cold diethyl ether followed by 10–12 washings with the same. After ensuring the purity the peptide was solubilised in minimal water followed by lyophilisation. HPLC purification of the synthetic peptides was performed with a Shimadzu LC-AD HPLC system equipped with a variable wavelength absorbance detector using a reverse phase C-18

column. A binary gradient of acetonitrile: water (1:19, 0.1% TFA) and acetonitrile: water (4:1, 0.08% TFA) at 1 ml/min was used. The eluents from the column were monitored by UV absorbance at 215 and 254 nm. Fractions were collected and lyophilized after their purity and identity was further confirmed by LC-MS performed using an RP-C18 column. Peptide identity was verified using mass spectral analysis.

2.3. Structural characterization

The structural characterization of the peptides was done by Fourier transform-Infrared spectroscopy (FT-IR). For primary structural characterization, the lyophilised peptide samples were loaded on to the ATR- crystal and subjected to light within the infrared spectrum. For secondary structural characterization, the 1 μ L of peptide solution in water was used as sample. Sixteen scans were collected for each scan and averaged to reduce the signal to noise ratio. The spectral range of analysis was 4000 cm^{-1} –400 cm^{-1} .

2.4. Preparation of peptide nanodrills loaded with guest molecules

Different RT-Peptide solutions were prepared by dissolving (0.023–0.026% wt/v) in Ultrapure water or Phosphate Buffered Saline (pH = 7.4) to achieve 0.8 mM final concentration of nanodrill monomers. The nanostructure was formed spontaneously with mere vortexing or gentle agitation. For the preparation of the guest molecule loaded nanodrills, molecules including ANS and rapamycin were dissolved in aqueous buffer and mixed at a 1:10 ratio with the peptides. The resultant solution was sonicated for three minutes in a bath sonicator. After sonication; the solutions were kept in room temperature for 30 min ensuring the re-assembly of peptides with the guest molecule loading. The unbound guest molecules were removed by centrifugation using Amicon® Ultra-4 centrifugal filter units at 2000 rpm (4°C). The effect of mock endosomal pH (pH=4.5) and mock extracellular pH (pH=7.4) on nanodrills was also assessed by subjecting the self assembled nanodrills to this different pH conditions [Phosphate buffered saline (PBS); At pH=7.4 and at pH=4.5 (pH adjusted by 0.001M HCl)]

2.5. Determination of entrapment efficiency

A known amount of the different RT peptides loaded with guest molecules was dissolved in H₂O/Acetonitrile/TFA (7:2.8:0.2) to obtain a clear solution. The dissolved peptide was precipitated with diethyl ether. Further absorbance measurements of the aqueous layer were carried out at the corresponding wavelength. The entrapped amount was calculated with the respective ratio between the actual entrapment ratio (AER) and the theoretical entrapment ratio (TER), expressed in terms of amount of molecule per weight of peptides. The entrapment efficiency can be determined by the following equation: Entrapment efficiency (%) = AER/TER $\times$ 100 [where AER = Measured amount of guest molecule /Measured peptide amount, TER = Initial amount of guest molecule /Initial peptide amount].

2.6. Particle size and zeta potential measurements

The particle size and zeta potential of different nanodrill formulations were measured via Dynamic light scattering on a Zeta Sizer ZSP instrument (Malvern Instruments Inc., Westborough, MA). The peptide nanodrills (0.4mM) were prepared under different pH

conditions and with or without dye or drug; added to a 1 ml quartz cuvette; equilibrated for 2 min and then subjected to size measurements. Zeta potential measurements were conducted with the same methodology using DTS1070 Folded Capillary Zeta Cells (Malvern Instruments Inc.).

2.7. CD spectral analysis

Circular dichroism was performed using a JASCO J-815 spectropolarimeter. Wavelength scans (16 consecutive) were recorded under nitrogen at 0.1 nm intervals from 260 to 190 nm, using a 0.1 cm path length quartz cuvette. The measurements were expressed as ellipticity (Θ) per residue.

2.8. Fluorescence spectroscopy

Fluorescence studies were conducted with an Infinite Pro 200 fluorescence microplate reader (TECAN US Inc., Morrisville, NC). Experiments were performed at ambient temperature in a low autofluorescence 96-well plate. In this study, ANS was used to investigate the amphiphilicity inside peptide nanodrills. The emission spectra of ANS (0.2 μM) in aqueous solutions of CSPNs at different concentrations (2 μM - 16 μM) were collected. The solutions were excited at 356 nm and emissions were recorded from 380–600 nm (λ_{max} for ANS = 366 nm).

2.9. AFM analysis

AFM images were acquired in tapping mode with Bruker Fastscan-D silicon nitride probes on a Dimension Fastscan System (Bruker Nano Surfaces, Santa Barbara, CA). Scans were taken of 2 μm regions at 3.92 Hz and 1024 $\times$ 1024 resolution in a single scan direction with feature tracking enabled (except 4F-RT for which 4 μm region were scanned at 2048 $\times$ 2048 resolution with feature tracking disabled). Regions of interest were then cropped from the original images and analysed using Nanoscope Analysis 1.8 and 1.9 software (Bruker Nano Surfaces). Samples were prepared by incubating 25 μl of 400 μM (2F-RT and 3F-RT) or 800 μM (RT, 1F-RT and 4F-RT) peptide solution on freshly cleaved V1 grade mica discs (Ted Pella, Redding, CA) for 30 min followed by 2 $\times$ gentle washes with UltraPure Distilled Water (Life Technologies, Carlsbad, CA). All samples were imaged under a column of water with a new probe and, to achieve higher resolution, samples were thermally equilibrated for 30 min prior to imaging. Each supplementary time lapse video was assembled in Nanoscope analysis 1.8 using 11 sequential images of a continuously scanned region of interest that were acquired with the aforementioned parameters and played back at 1 frame per second.

2.10. In-vitro release kinetics

The release profiles of rapamycin from different CSPN preparations were monitored in order to study the drug release behavior and stability of the peptide nanodrills under physiological conditions. A known quantity of freshly prepared, rapamycin encapsulated peptide nanodrills were taken in a dialysis membrane (MWCO 2 kDa) and placed in a beaker containing phosphate buffered saline (PBS) pH 7.4. The drug loaded peptide nanodrills were shook at 50 rpm and 37 $^{\circ}$ C from which 1 ml of the reaction mixture was withdrawn at different time intervals. The release profile was monitored for up to 24 hours. The amount of

released molecules was estimated using with an Infinite Pro 200 fluorescence microplate reader by UV absorbance at 316 nm. The λ_{max} for rapamycin, and the free (released) rapamycin concentration was determined from a standard curve.

2.11. Cell Uptake Studies

HeLa cells were plated in 8-well chambered glass slide (Fisher Scientific) at 10,000 cells/well in DMEM media (containing 10% fetal bovine serum (Corning) and 5% Penicillin/Streptomycin (Corning)). Cells were grown overnight at 37 °C. They were then treated with 20 uL of free or peptide-conjugated Cy7 and incubated at 37 °C for 3 or 24 hrs. At each time point, cells were paraformaldehyde fixed for 20 mins and stained with DAPI. Images were taken using EVOS Cell Imaging System (Thermo Fisher Scientific).

2.12. Autophagic Induction Study

MEF-p62-Luc cells were plated in a white-walled, clear bottom 96-well plate at a density of 4000 cells/well in DMEM media (containing 10% fetal bovine serum (Corning), 5% Penicillin/Streptomycin (Corning), and 1 µg/mL doxycycline (Sigma-Aldrich)) and incubated at 37 °C for 24 hrs to allow cell growth and induction of p62-luciferase. Cells were dosed with serially diluted concentrations of free rapamycin, free peptide, or peptide-encapsulated rapamycin and incubate for 24 hrs at 37 °C. Measure cell viability and luciferase expression was measured using ONE-Glo + Tox Assay Kit (Promega).

2.13. In-vivo biodistribution

C57BL/6J mice (12 weeks old, Jackson Laboratory) were injected with 0.1mg/kg Cy7-conjugated 2F polymer via tail vein intravenous injection. The mice were euthanized and their organs (brain, heart, lungs, liver, spleen, and kidneys) were extracted 2 hours post injection. The organs were imaged using IVIS Spectrum in vivo imaging system (PerkinElmer) and images were processed using Living Image software (PerkinElmer). All animal experiments were conducted according to protocols approved by Institutional Animal Care and Use Committee (IACUC) and followed by OHSU, local, state and federal guidelines

2.14. MD simulation

The 2F-RT peptide sequence was built with a α -helical secondary structure, using the software Avogadro [33]. Bilayer aggregates were constructed by arranging 16 peptides with parallel orientation in a 4×4 squared fashion in both layers with an inter-peptide backbone distance of ~8 Å, which is the minimum separation at which side chains do not overlap or entangle. The two layers overlap only with their Fmoc residues, which are however ~5 Å separated from each other, that is roughly the optimal stacking interaction distance [34]. The peptide structure was solvated in water and a total of 256 of chloride ions were added to neutralize the positive charges on the Tat peptide regions. Overall, the system consists of ~120,000 atoms.

OPLS-AA parameters[35,36] were used for all amino acids with corresponding parameters for the Fmoc group recently derived by Mu et al[37] and the TIP3P model for water [38]. The simulations were performed and analyzed with the GROMACS software version 5.1.4

[39]. Long-range electrostatic interactions were calculated using the Particle-Mesh Ewald summation [40,41]. Short-range Coulomb and van der Waals (vdW) interactions were cutoff at 10 Å.

After energy-minimization, the system was heated to 300 K within 2 ns. All simulations were run in the NPT ensemble at the constant temperature of 300 K and a pressure of 1 bar. The temperature was maintained by velocity rescaling with a stochastic term to ensure proper sampling[42] and the Berendsen barostat was applied[43]. Bonds to hydrogen atoms were constrained using the LINCS algorithm[44] and the simulations were performed with a 2 fs time step.

An equilibration phase of 40 ns was performed. The average peptide RMSD increases within the first 25 ns of simulation to ~4 Å and remains fairly constant after that, confirming the convergence of the aggregate structural properties and peptide interactions. Subsequently, 150 ns of production MD were performed.

The peptide helix content is obtained by the DSSP program [45] within GROMACS assigning secondary structures to each amino acid. Their fraction assigned to 3_{10} -helix, α -helix, or π -helix is used to compute the helix content. The peptide salt bridge occupancy is obtained as the fraction of time either of the two ASP residues in the (RADA)₂ segment forms a salt bridge with any of the basic side chains, which is defined as a maximum distance of 4 Å between any of the relevant oxygen and nitrogen atoms. For each property, the average value over all 8 core peptides or 24 surface peptides, respectively, is reported and error bars are two standard errors of the mean.

2.15. Statistics

All the experimental measurements were done in triplicate, and the results were expressed as arithmetic mean $\pm$ standard deviation. Statistical analysis of the data was analysed by student's t-test. A value of significance " p " was denoted as 0.05 * p > 0.01, 0.01 ** p > 0.005, *** p > 0.005 for $n = 3$.

3. Results and Discussion

3.1. Development of CSPNs

Different numbers of Phe-substituted CSPNs were synthesized by solid phase peptide synthesis (SPPS) in high yield (>95%) (Scheme in Figure.S2). The purity of all CSPNs was profiled by LC-MS, although a single peak and tailing was observed for RT, 1F-RT and 2F-RT. The masses of all CSPNs were in agreement with the theoretical values, which further ensured the purity and synthetic efficacy of SPPS (Figure.S3–S7). Further, the intensity of aromatic C-H stretching was found to be in ascending order from RT to 4F-RT, which can be attributed to the increasing number of Phe,[46] as evident from the FT-IR spectral data (Figure.S8).

3.2. Observation of self assembly

The size of CSPNs in ultrapure water was measured by dynamic light scattering as a preliminary mode of analysis. All synthesized peptides were able to self assemble into sub

200 nm sized nanomaterials, irrespective of the number of Phe (Figure.S9.a–b, Table.S1). Both 2F-RT and 3F-RT formed two different kinds of populations with higher PDI values (2F-RT =0.431, 3F-RT=0.434) as compared to other CSPNs. This might be due to the heterogeneous population arising from high-aspect ratio nanostructures in case of 2F-RT and 3F-RT. To confirm this hypothesis, we performed detailed AFM analysis which is explained in Section.3.4. Since all the CSPNs are designed for delivery in physiological conditions, we also compared their particle size distributions in a mock intracellular, endosomal environment (pH 4.5) to a mock extracellular environment (pH 7.4) (Figure.S9.c). Among all the peptides, 2F-RT showed highest increment of size at endosomal pH in comparison with extracellular pH, implying probable aggregation behaviour at endosomal pH (Figure.S9.c, Table S1). On analyzing the zeta potential values of CSPNs; all candidates had net positive values of surface potential (+16.9 mV to +26.3mV, Table.S1), suggesting that the Tat peptides are located at the surface of these nanomaterials. This is important because the positively charged arginine residues on Tat exploit membrane glycoproteins to translocate into cells via endocytosis, and the acidic environment of the endosome may trigger aggregation of CSPNs, thereby facilitating endosomal escape [47].

3.3. Molecular basis of self assembly

The basis of CSPN self-assembly is the organization of individual peptides into different secondary structural motifs on addition of a hydrophobic amino acid. In order to understand the secondary structure of all CSPNs, we carried out circular dichroism (CD) spectral analysis to measure ellipticity between 190–260 nm in the far UV region(Figure.2.a)[48]. RT showed a characteristic negative band at 198 nm, indicating a random coiled structure without specific geometry (Figure.2.a). When one Phe was added to the N-terminal end of RT to form 1F-RT, the peptide maintained a random coiled structure, but the signal intensity decreased. This is suggestive of a few non-covalent interactions. Upon further addition of Phe to form 2F-RT, the CD spectra showed two negative bands at 206 nm and 217 nm, which are characteristic peaks for an α -helix structure. However, the absence of a positive band at 190–200 nm, another indicator of an α -helix, suggested that this particular CSPN is a distorted α -helix (Figure.2.a)[49]. Interestingly, further addition of Phe to form 3F-RT showed a positive peak at 200 nm and a negative peak at 218 nm, indicative of a β -sheeted arrangement (Figure.2.a). A red shift of the positive peak to 200 nm, from the characteristic positive peak of β -sheet (196 nm), was observed due to the interaction of the π - π^* transition of the amide I band with the Phe [50]. Further, FT-IR showed the presence of an amide I band at 1623 cm^{-1} , which suggests a parallel β -sheet formation (Figure.S10). Surprisingly, the spectra for 4F-RT showed two distinct negative peaks at 206 nm and 217 nm and a positive peak at 190–195 nm, thus demonstrating a transition to a pure α -helical structure (Figure 2. a).

These studies suggest that transition of the secondary structure is dependent on the number of consecutive, N-terminal Phe. Addition of a single Phe to RT decreased the disorder in the random coil structure, which may be attributed to minor non-covalent interactions. Upon introduction of a second Phe, the construct formed a distorted α -helix and conjugation of a third Phe resulted in formation of a pure β -sheet. Interestingly, conjugation of a fourth Phe (4F-RT) leads to the formation of a pure α -helix (Figure.2.a). These data suggest that the

presence of an even number of Phe results in the formation of α -helical structure (2F-RT and 4F-RT), whereas an odd number of Phe results in the transition or formation of β -sheets (1F-RT and 3F-RT) (Figure.2.b).

The reason for attaining α -helical structure for 2F-RT and 4F-RT can be established based on previously proposed helical wheel model[51] (Figure.S10.a). As per this model; the peptide forms α -helical coiled coils if and only if at least two chains interact with the each other by hydrophobic ($a-d'$ and $d-a'$) and ionic ($e-g'$ and $g-e'$) interaction (Figure.S11.a). On applying helical wheel model to all the peptides; only 2F-RT and 4F-RT showed the distinct formation of one heptad with necessary interactions per sequence to form parallel homodimer α -helical coils (Figure.2.c–d, Figure.S11.b–d). In both these peptides Arg and Asp of (RADA)₂ segment falls at position namely e and g which stabilises the α -helix through ionic interaction. The hydrophobic interacting positions ($a-d'$ and $d-a'$) in 2F-RT were occupied by two Phe and Ala residues each, while in 4F-RT ; four Phe falls at this region. This favours strong aromatic π - π stacking ($a-d'$ and $d-a'$) in addition to the hydrophobic interaction in 4F-RT resulting in more distinct α -helical structure than 2F-RT, which correlates well with the experimental CD data (Figure.2.a,c). In case of 3F-RT; a parallel β -sheeted structure is observed which might be due to the inherent β -sheeted nature of the (RADA)₂ segment. In contrast to this; two more non α -helical CSPNs, i.e. RT and 1F-RT exhibited the inherent random coil structure of native Tat_{48–59} segment. These factors lead us to speculate that in 3F-RT; the dominance of (RADA)₂ segment's secondary structure might be due to the serial introduction of three Phe residues forming a distinct hydrophobic region.

Based on these findings, we propose a scheme for the formation of α -helical structure as in Figure.2.e among the CSPNs. When CSPNs contains even number of Phe; the a and d positions get occupied by hydrophobic residues like Phe and Ala while at e and g ; the charged residues of (RADA)₂ like Arg and Asp reside. This facilitates the formation of α -helical coiled coils through specific interaction; i.e. in 2F-RT and 4F-RT (Figure.S10.a, Figure.2.e). When CSPNs have odd number of Phe; Arg falls to the hydrophobic region (position d) and Ala falls to hydrophilic region (position e and g). This results in the absence of sequence based necessary non-covalent interactions from the corresponding amino acids (Figure.S10.a,c–d) impeding the formation of α -helix for the CSPNs.

To further decipher the formation of hydrophobic regions in all the CSPNs by the introduction of Phe, we conducted spectrofluorimetric analysis using 8-anilino-1-naphthalene-sulfonic acid (ANS) as a probe (Figure.3.a–e)[52]. The probe binds at specific hydrophobic pockets of the self-assembled peptides, resulting in distinct increase in fluorescence intensity (hyperchromic shift) at 485–495 nm. A dose-dependent hyperchromic shift is evident in 2F-RT, 3F-RT and 4F-RT (Figure.3.d–e). A five-fold increase in the mean fluorescence intensity was observed in 3F-RT and 4F-RT compared to 2F-RT (Figure.3.f). Thus, onset of specific hydrophobic regions begins in 2F-RT, which becomes highly distinct in 3F-RT and 4F-RT due to formation of a densely packed hydrophobic core.

3.4. Morphological analysis of nanodrills

To visualize the morphology of the CSPNs, we carried out a series of atomic force microscopic (AFM) analyses. 2F-RT, 3F-RT and 4F-RT formed a variety of rod-shaped structures that were reminiscent of a “drill-bit” while we were unable to detect similar assembly in RT and 1F-RT (Figure.4.a–c, Figure.S12.a). RT and 1F-RT formed nanosized irregular aggregates without any distinct surface morphology. 2F-RT forms regular, coarsely-twisted and linearly-extending nanostructures that are 150 ± 111 nm with aspect ratio 8.8 ± 6.5 (Figure.S12.b). The lengthwise cross-sectional profile of a typical 2F-RT nanodrill shows a helical twist with a 150–170 nm periodicity in curve A (Figure.4.d, Figure. S13). The number of twists in a 2F-RT nanodrill was observed to be directly proportional to the length of the nanodrills. In addition to the twist; a distinct central groove of ~ 1 nm was visualized in 2F-RT nanodrills as per curve B (Figure. 4.d). Based on these results, we speculate that the individual peptide nanodrills self-assemble into a bilayer where the hydrophobic region forms the groove[53–55]. In order to assess the stability of these nanodrills, we continuously imaged 2F-RT (11 complete scans, 48 minutes) which delivers continuous electromechanical stimulus to the sample surface(Supplementary Video.1)[56,57]. During this test, only minor longitudinal shortening of the nanodrills was observed, which suggests that 2F-RT is highly mechanically stable[58].

3F-RT formed linear cuboidal nanodrills of 105 ± 67 nm with aspect ratio 6.2 ± 3.9 without twists and turns (Figure.4.b, Figure.S12.c). Surprisingly, the central groove visualized in 2F-RT was completely absent in 3F-RT and was independent of nanodrill orientation as measured by curve A-E (Figure.4.e, Figure.S13) To test the mechanical stability of the nanodrills and confirm the absence of any groove, we performed continuous imaging of the samples for 48 minutes (Supplementary Video.2) in which the AFM cantilever continuously imparts electromechanical stimulus by tapping the sample surface. A minor longitudinal decrease in size was observed, but we were unable to detect the presence of any grooves. Our previous spectrofluorometric experiment functionally demonstrated the formation of a very distinct hydrophobic core in 3F-RT compared to 2F-RT. As deeply buried hydrophobic pockets cannot be visualized directly through a surface technique such as AFM, we hypothesize that the encapsulation of ANS in 3F-RT will destabilize its hydrophobic core in response to the externally applied electromechanical stimuli of the AFM cantilever, thus allowing the structure to be interrogated by continuous imaging (11 complete scans for 48 minutes). We were able to view stepwise disassembly of the ANS-loaded 3F-RT nanodrills in layer-by-layer fashion (Supplementary Video 3, Figure. 5.a). A snapshot after 10 scans (39 min) of continuous imaging shows partial disassembly of 3F-RT with reduction in layer thickness as shown by curve A and B (Figure.5.b). Surprisingly, the diametric cross-section of ANS encapsulated 3F-RT nanodrills revealed a longitudinal groove (Figure.5.c).

To our surprise, 4F-RT displayed fine twisted nanodrills which extended linearly without any groove (Figure.4.c,e). The periodicity is roughly 55–80 nm, which is shorter than 2F-RT. The rapid twist in the nanodrills produced regular “inner notches”, which repeat periodically throughout the length, giving rise to a fine twist as observed by the cross-sectional profile (Figure.4.e). During continuous imaging for 11 scans (96 min); we observed as early as the third scan that the rods disassemble from the individual inner notch region and gets

separated into discrete segments (Supplementary Video.4). The occurrence of rapid fine twists with the minimal surface area for the continuous stimulus of the AFM cantilever results in the disruption of the nanodrill morphology. This suggests poorer mechanical stability of the 4F-RT nanodrills in comparison to 2F-RT and 3F-RT [58].

3.5. Intracellular delivery with nanodrills

Autophagy is a catabolic process that directs toxic cellular debris from the cytosol of the cell toward lysosomes for degradation [59]. Defects in autophagy lead to accumulation of toxic materials in various disease conditions ranging from infectious diseases to neurodegenerative disorders. Rapamycin, an inhibitor of mechanistic target of rapamycin (mTOR), mimics starvation that induces autophagy to promote catabolic activity within the cell for the maintenance of homeostasis. Since mTOR is a master regulator for cellular growth, inhibitors of this pathway have been shown to be effective anti-cancer agents [60]. Rapamycin is clinically used for prevention of organ transplant rejection, and treatment of a rare lung disorder, lymphangioliomyomatosis [61–64]. Since it is a highly non-polar macrolide, efficient delivery of rapamycin remains a challenge.

We hypothesize that because CSPNs have a distinct hydrophobic core, they can effectively encapsulate rapamycin, and that the presence of Tat on the CSPN surface can drive rapamycin into mammalian cells [59,65]. We packaged rapamycin in 2F-RT, 3F-RT and 4F-RT with >90% encapsulation (Figure.6.a). A study of the release kinetics of rapamycin showed more than 50% released from CSPNs within six hours (Figure.6.b). We selected 2F-RT and 3F-RT for further development as intracellular delivery platforms because 4F-RT showed gelation, thus making it unsuitable for subcellular delivery. Interestingly, *in vitro* cellular uptake studies in HeLa cells displayed internalization of Cy-7 labelled 2F-RT within 3 hours of exposure, while 3F-RT exhibited uptake after 24 hours (Figure.6.c). In the case of 2F-RT, the twists of the nanorod and the structure of the Tat region may be responsible for the expedited cellular internalization.

Due to rapid uptake, 2F-RT was selected to deliver rapamycin in cell lines that have stable transfection of p62-luciferase, a marker of autophagy. Induction of autophagy was characterized by the reduction in luciferase expression due to degradation of autophagy substrate p62. We found that rapamycin packaged in 2F-RT can induce autophagy in this cell line without compromising its activity. The normalized p62 luciferase expression also demonstrated limited toxicity of the free 2F-RT nanodrills irrespective of the dose administered. (Figure.6.d). Furthermore, we injected mice with Cy7-labeled 2F-RT and were able to visualize their biodistribution. 2F-RT showed accumulation in the liver within 2 hours of injection, while the other organs, including the lung, heart, brain, kidney and spleen showed little or no uptake (Figure. 6.e). These studies suggest that 2F-RT nanodrills might serve as liver delivery vehicles for autophagy inducers.

3.6. Proposed model for self assembly

Based on our observations; we propose a model for the self-assembly of the three CSPNs as shown in Figure. 7.a–c. The distorted α -helical peptides of 2F-RT assemble as pseudo bi-layers where in addition to the hydrophobic interactions, Phe forms intermolecular and

Fmoc forms inter-layer aromatic π - π stacking interactions predominantly (Figure.7.a)[66–68]. The weak hydrophobic core suggested by spectrofluorimetric studies in the bi-layered organization evidently forms a groove that extends in a helical manner throughout the nanodrill. The weak hydrophobic core along with the outer helical Tat region may be responsible for slight interlayer distortions during the process of self assembly resulting in gradual, coarse twists on the gross scale for 2F-RT nanodrills (Figure.7.a). For 3F-RT, the parallel β -sheeted arrangement of the peptides could sandwich the middle phenyl ring from one peptide between the two outer phenyl rings of the adjacent peptide. This type of Phe-Phe aromatic interaction along with interlayer Fmoc stacking possibly results in a strong hydrophobic core. This core along with complementary electrostatic attractions of the (RADA)₂ segment might hold the cationic Tat region in closer proximity than in 2F-RT, resulting in non-twisted 3F-RT nanodrills (Figure.7.b).

The 4F-RT forms fine-twisted nanodrills with a densely packed hydrophobic core (Figure. 7.c). The transition to pure α -helical structure from 2F to 4F-RT (Figure.2.a–d) and gain in hydrophobic interactions appears to increase the number of twists per unit length making it from a coarse to a fine-twisted nanodrills (Figure. 4.c,f, 7.c). All of these peptide amphiphiles form bilayers with a hydrophobic interior and a positively charged hydrophilic exterior that elongates lengthwise due to π - π stacking to form their corresponding supramolecular structure. The thickness as well as width of the nanodrill may be constrained due to the steric and electrostatic repulsion of the cationic surface, which likely promotes its unidirectional growth and monodispersity.

Overall, based on our data, we propose that the number of consecutive, *N*-terminal Phe residues is a critical determinant to manipulate the core architecture in these nanostructures, which ultimately dictates the surface charge, hydrophobicity, pattern of self-assembly and shape of CSPNs.

3.7. In-silico analysis of Tat in nanodrills

We were inquisitive about understanding the secondary structural behavior of Tat in 2F-RT nanodrills that could contribute to its overall morphology and promote its internalization. The bioactive sequence of native Tat (Tat_{48–59}) exhibits a random coiled structure which gains a distorted α -helical structure on engineering into these nanodrills (Figure.2.a–d, Figure. 7.b). To gain insights into the structural behavior of Tat, we performed all-atom molecular dynamics (MD) simulations of a 2F-RT peptide aggregate based on our proposed model of self assembly (Figure.7.b). During the simulation, the Tat segment loses its α -helical nature and forms random coils, while the amphiphilic (RADA)₂ retains its native helical structure for the most part (Figure.8.a–b). CPPs in their native secondary structure have been suggested to have strong cell membrane interactions[69,70], which might explain the ability of 2F-RT to enter cells. Further, we also observed that the peptides in the core of the aggregate fluctuate significantly less than their surface counterparts which allows them to form intermolecular salt bridges enabling self assembly (Figure.8.c–d). These simulation results provide microscopic insights into the surface properties of nanodrills that enhance intracellular delivery.

4. Conclusions

We deployed a shape-defined nanodrill engineered through controlled supramolecular assembly of CPPs for effective intracellular delivery. Our studies utilize sequential addition of individual amino acids to precisely control the tuning of synthetic peptides to form a “nanodrill” architecture. This bottom-up approach provides control over the secondary structure of the resultant peptides, which may be essential for tuning the efficacy of intracellular cargo delivery. The high aspect ratio, size and flexibility exhibited by nanodrills has been shown, for other materials, to be responsible for long-term circulation and rapid internalization with minimal interaction with macrophages [71,72]. We further show that serial introduction of Phe can enhance the hydrophobicity of the CSPNs, thus making it a versatile host that can accommodate guest molecules. The rapid cellular uptake of 2F-RT nanodrills further suggests that a coarse-twist morphology is required for successful delivery both *in vitro* and *in vivo*.

Supramolecular assembly of Tat-based amphiphiles presents a new and robust platform that can vary in shape and flexibility. Minute changes in the hydrophobicity or charge of their internal core structure or the temperature, pH or ionic strength of their environment can direct the self-assembly into a myriad of defined nanostructures. These tailor-made nanostructures could serve as ideal hosts for different non-polar molecules and can serve as stimuli-sensitive drug delivery systems. Further by exploiting the stimuli-responsive self-assembly behavior of these nanostructures, bioorthogonal sensors can be developed to probe the intracellular environment. Ultimately, we envision that modular CSPNs could serve as a new platform to enable efficient subcellular delivery of molecules across biological barriers presently considered impenetrable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structure and schematic representation of different CSPNs

We have highlighted the hydrophilic Tat (blue), amphiphilic (RADA)₂ (pink) and hydrophobic Fmoc and Phe (red). Within the hydrophobic region we show the aromatic regions in yellow. CSPNs that form drillbit like supramolecular assembly after sequential addition of Phe include (a). 2F-RT (coarse-twisted) (b). 3F-RT (non-twisted) and (c). 4F-RT (fine-twisted). These assemblies were visualized using tapping mode AFM (red insert shows a schematic of the nanodrills).

(a). CD spectral analysis of different CSPNs was performed to elucidate secondary structure. The transition of secondary structure was observed as the number of Phe was increased from zero to four (b). The transition of secondary structure was from a highly random coil (RT) to a moderate random coil (1F-RT) followed by formation of a pure β -sheet on addition of odd number of Phe. On the other hand, even number of Phe changed the random coil to distorted α -helical (2F-RT) and formed pure α -helical (4F-RT) structure. (c) Different amino acids of 2F-RT from N-terminal was designated as *abcdefg* which constitutes a heptad making a helical wheel (Chain I). The condition for the formation of coiled coils (α -helical structure) is “the heptad should interact with another chain (i.e. Chain II with amino acids *a'b'c'd'e'f'g'*) by the hydrophobic interactions (*a-a'* and *d-d'*) and ionic interactions (*e-g'* and *g-e'*).” Illustration of helical wheel diagram model of heptad interaction in two chains of 2F-RT from the N-terminal amino acid and shows hydrophobic interaction (Phe-Phe and Ala-Ala) and ionic interaction (Asp-Arg and Arg-Asp) suggesting the formation of a parallel homodimer. (d) Illustration of helical wheel diagram model of heptad interaction in two chains of 4F-RT from the N-terminal amino acid and shows hydrophobic interaction (Phe-Phe and Phe-Phe) and ionic interaction (Asp-Arg and Arg-Asp) suggesting the formation of a parallel homodimer (e) Scheme of secondary structural transition in different CSPNs based

on Phe number. The regions of the CSPNs such as hydrophobic (red), hydrophilic (dark blue), cationic (light blue), anionic (green), amphiphilic (pink) and charge per residue was also highlighted. The structural transition was dependent on the number of Phe residue per sequence. The even favours α -helical structure as depicted in the scheme.

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Figure 3. Molecular basis of hydrophobicity

(a–e) Spectrofluorimetric analysis of synthetic peptides at various concentrations (2–16 μM) followed by ANS (0.2 μM) encapsulation. Blank and ANS alone served as controls. Hyperchromic shift in intensity ANS was observed for (c–e). (f). Shows comparative analysis of mean fluorescence intensity of different peptides

Figure 4. Morphological analysis of self assembly

AFM images of (a, d) 2F-RT (b,e) 3F-RT and (c, f) 4F-RT were acquired in tapping mode. The primary images in panels a, b and c show the full height of the samples, whereas the color scale has been adjusted for inserts a, b and c as well as panels d, e and f in order to accentuate the unique, differentiating features of the samples. Insert (red-dotted line, a–c) shows higher magnification of CSPNs that assemble as coarse-twisted (a), non-twisted (b) and fine-twisted (c) nanodrills. Cross-sectional profile along the selected length (Top-panel) (d). A, (e) A, B, (f) A and diameter (Bottom Panel) (d) B, (e) C,D,E, (f) B, C is indicated. The cross sectional profile was used to develop schematics that show coarse twisted nanodrills with central groove (d), non-twisted nanodrill without any groove (e), fine-twisted nanodrills (f)

Figure 5. AFM analysis of ANS encapsulated 3F-RT

(a) Scans of blank 3F-RT and ANS encapsulated 3F-RT nanodrills under the similar conditions taken at different time points while continuously scanning. This demonstrates the relative mechanical instability of nanodrills loaded with hydrophobic molecules. The ANS encapsulated 3F-RT nanodrills (39 min, red insert) were subjected to cross sectional analysis given in (b) and (c). In (b–c), curves A–E represent two different orientations of the ANS encapsulated 3F-RT nanodrills. (b) Longitudinal height measurements (curves A and B) show partial dissociation of the ANS encapsulated 3F-RT nanodrills. (c) Cross-sectional measurements of 3F-RT shows nanodrills with a central groove (curve C) and without a

central groove (curve D and E). This is due to different orientations of the nanodrills on the mica surface. Each nanodrill is shown with a schematic model to explain their relative orientations. The yellow circles represent ANS molecules loaded within the hydrophobic core.

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Figure 6. Intracellular delivery of autophagy inducer by CSPNs

(a) Encapsulation efficiency of CSPNs to package rapamycin, an autophagy inducer (b) Kinetics of rapamycin release from CSPNs (c) The cellular uptake of Cy7 labeled 2F-RT and 3F-RT in mammalian cells (d) Mouse Embryonic fibroblasts that stably express p62-Luc were exposed to different concentrations of rapamycin loaded 2F-RT (7–250 nM) and the luciferase activity was measured normalized to cell viability. Rapamycin and 2F-RT serve as controls (0.05 $p > 0.01$, 0.01 $p > 0.005$, $p > 0.005$) (e) Cy7-labeled 2F-RT (0.1 mg/kg) was injected in mice. The animals were sacrificed at 2 hours, the organs harvested and imaged using IVIS. PBS alone served as a negative control.

Figure 7. Proposed model for self assembly

Schematic representation of the self-assembly pattern observed in nanodrills (a) 2F-RT self-assembled through aromatic π - π stacking into a distorted α -helical structure which forms coarse-twisted nanodrills, (b) 3F-RT self-assembled through aromatic π - π stacking into a parallel β -sheet that forms non-twisted nanodrills (c) 4F-RT self-assembled through aromatic π - π stacking into a pure- α -helical structure which forms fine twisted nanodrills. Inserts show AFM images of the nanodrills.

Figure 8. Molecular Dynamics (MD) simulation of 2F-RT nanodrills

(a) Snapshots of the 2F-RT nanodrill starting structure (left) and after 150 ns of simulation (right). Individual peptides are colored based on their segment (top) and on their starting position in the assembly (bottom) (b). Average helix content of different segments in core and surface peptides (c). Average percentage occupancy of intermolecular salt bridges among core and surface peptides (d). Average root mean square fluctuation (RMSF) of core and surface peptides.