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Interface-Enrichment-Induced Instability and Drug-Loading-Enhanced Stability in Inhalable Delivery of Supramolecular Filaments

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Abstract

Filamentous microorganisms traveling in aerosol particles display enhanced deposition and retention in the lungs. Inspired by this shape-related biological effect, we report here on the use of supramolecular filaments as potential inhalable drug carriers within aerosols via jet nebulization. We found that the peptide design and supramolecular stability play a crucial role in the interfacial stability and aerosolization properties of the supramolecular filaments. Monomeric units with a positively charged C-terminus produced filaments with reduced aerosol stability, promoting morphological changes after nebulization. Conversely, having a neutral or negatively charged terminus yielded filaments with enhanced stability, where supramolecular integrity is maintained with only reduced length. Our results suggest that molecular enrichment at the air–liquid interface during nebulization is the primary factor to deplete the monomeric peptide amphiphiles in solution, accounting for the observed morphological disruption/transitions. Importantly, encapsulation of drugs and dyes within filaments notably stabilize their supramolecular structure during nebulization, and the loaded filaments exhibit a linear release profile from a nebulizer device. We envision the use of this supramolecular carrier system as an effective platform for the inhalation-based treatment of many lung diseases.

Graphical Abstract

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Supporting Information

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Details of molecular synthesis and characterization, additional TEM images of the supramolecular filaments before and after nebulization, zeta potential measurements, full transition curves of fluorescence spectra for CAC measurements, and fluid and drug/dye release curves from a jet nebulizer (PDF)

The authors declare no competing financial interest.

Keywords

molecular assembly; supramolecular filaments; peptide amphiphiles; nebulization; aerosol delivery; drug delivery

Filamentous nanomaterials have been extensively explored for the delivery of therapeutic and/or imaging agents, where their shape can aid in enhancing cellular internalization,¹ extending circulation times,² inducing cell death for disease treatment,³ and improving the therapeutic potential of inhalable materials.⁴ Nature's own filamentous carriers of proteins and genetic material, bacteria and viruses (like *Mycobacterium tuberculosis* and influenza), exhibit effective transport through the air in aerosols with enhanced deposition and retention within the lungs as afforded by their shape.⁵ In this sense, filamentous systems may provide an additional benefit of enhanced retention within the lungs through avoidance of their clearance mechanisms, like the mucociliary escalator and phagocytosis by alveolar macrophages,^{6–11} which could improve therapeutic efficacy and mitigate off-target effects compared to aerosolized small molecule drugs.^{12–18} This shape-promoted effect has been demonstrated by Hanes and colleagues, where pulmonary delivery of their polymeric rodlike DNA nanoparticles provided long-term relief of allergic asthma symptoms in mice with a single dose.⁴ This effect has also been manifested with peptide-based systems, where Collier and co-workers showcased that intranasal delivery of self-assembling influenza epitope-bearing peptide nanofibers elicited greater CD8+ T cell responses in mice for flu vaccination.¹⁹ Additionally, spray and nebulization stability has been assessed by Discher and co-workers for filomicelles formed from amphiphilic block copolymers, demonstrating their ability to maintain their structure and deliver loaded pesticides within aerosol droplets.²⁰ These studies demonstrate the advantages of filamentous carriers for aerosol-based delivery, much like the filamentous microorganisms that inspired them.

Self-assembling peptides and peptide amphiphiles represent a class of molecular building units for filamentous nanomaterials whose assembly into one-dimensional nanostructures has been extensively explored for the development of supramolecular biomaterials.^{21–34} These supramolecular systems have been used for a wide variety of biomedical applications, such as tissue engineering,^{35–40} regenerative medicine^{41–45}, molecular imaging,^{46–49} and drug delivery.^{50–59} The increasing popularity of peptide-based supramolecular systems is partially due to their high biocompatibility, facile synthesis, and easily tunable bioactivity and assembly properties.^{41,44,58,60} Though therapeutic peptides have been studied for

pulmonary delivery within aerosols,⁶¹ the ability of peptide-based supramolecular nanostructures to travel within aerosols and the use of supramolecular filaments as inhalable carriers of therapeutic and imaging agents has yet to be studied. The distinct shape-afforded advantage of peptide amphiphiles could be potentially used to create filamentous carriers to enhance aerosol-based delivery. However, the most important aspect to consider is the stability of these filamentous assemblies, since mechanical forces present during aerosol formation may disrupt supramolecular structures due to the noncovalent nature of their assemblies.^{62–64}

In this context, we designed and synthesized peptide amphiphiles with varying surface charges and supramolecular stabilities that all spontaneously associate in aqueous solutions to form supramolecular filaments capable of encapsulating lung-disease-relevant drugs and dyes and report on their pioneering use as inhalable drug carriers within aerosols. The loaded supramolecular filaments are able to maintain their shape during aerosol formation using a jet nebulizer, and the stability of these systems within aerosols can be tuned through molecular engineering of the peptide units. Moreover, the drug-loaded supramolecular filaments with adjustable loading capacities are linearly released from a nebulizer, allowing for a controlled and sustainable delivery as therapeutic aerosols.

RESULTS AND DISCUSSION

Molecular Design.

Three peptide amphiphile (PA) molecules, each composed of a hydrophilic peptide segment and a hydrophobic alkyl chain, were designed to self-assemble into filamentous nanostructures. By manipulating the amino acid sequence at the C-terminus, we can influence the overall surface charge of the resulting supramolecular structures, with the aim of creating systems of varying surface charge to compare their aerosolization properties. As shown in Figure 1A, each molecule differs with respect to the charged or neutral amino acids at the C-terminus, which are separated by a double glycine (GG) spacer and help enhance the overall solubility and amphiphilicity of the PA molecule. The amphiphile EPA contains three glutamic acid residues (EEE) for a negative surface charge; OPA contains two lysine residues decorated with short oligoethylene glycol (OEG) chains for a neutral surface charge; and KPA contains three lysine residues (KKK) for a positive surface charge. Each PA contains a dodecyl chain (C₁₂ alkyl group) to act as the hydrophobic domain of the amphiphiles to promote hydrophobic interactions during self-assembly. Moreover, each molecule contains the same intermolecular hydrogen bond-regulating peptide sequence (YVVV), where the tyrosine residue (whose absorbance is also used for concentration calibration) can partake in π – π interactions and the valine residues can promote β -sheet formation to provide directionality to the supramolecular structures.^{65–67} All peptide amphiphiles were synthesized following standard Fmoc solid-phase peptide synthesis protocols. After synthesis, the molecules were purified using reverse-phase high-performance liquid chromatography (RP-HPLC) and their molecular weights confirmed using MALDI-TOF mass spectroscopy (Figures S1–S3). Upon dissolution in water and aging for 1 day, these peptide amphiphiles can spontaneously arrange into supramolecular filaments of several micrometers in length. The resulting filament solution could then be

transferred to a jet nebulizer to study their aerosolization properties within liquid droplets, as depicted in Figure 1B.

Molecular Assembly and Filament Characterization Post-Nebulization.

To understand the behavior of these filaments during aerosol delivery, we first compared any observable structural changes to their morphology during jet nebulization. All PAs were dissolved at 2 mM in water and aged for 24 h at room temperature before observing their assemblies. Figure 2A, D, and G depicts transmission electron microscopy (TEM) images of EPA, OPA, and KPA, respectively, where all three PAs are observed to form filamentous nanostructures over several micrometers in length. Our analysis revealed the tendency for EPA and OPA to produce ribbonlike structures with varying degrees of twisting while KPA favored straight fibers. Moreover, zeta potential measurements were conducted on the filament systems, which confirmed the surface charge of the assemblies was aptly conferred by the C-terminal amino acid design (Figure S4).

Since the supramolecular filaments are several micrometers long and are formed through noncovalent interactions, their structural integrity may be influenced by a number of factors during nebulization, such as agitation and aerosol formation. Thus, we first assessed the structural stability of these systems while traveling in aerosol droplets generated by a jet nebulizer. Nebulizers are commonly employed medical devices for producing liquid aerosol droplets containing therapeutics via high velocity air flow through the device nozzle. Droplets very large in diameter impact baffles and/or the walls of the nebulizer and are recycled back into the device's reservoir, but droplets small enough to circumvent these barriers (typically ranging in diameter from 1 to 10 μm) exit the device as a respirable aerosol.⁶⁸ Therefore, we characterized two groups of structures after nebulization: the exiting mist and the leftover solution in the reservoir. Filament solutions diluted down to 500 μM of the different PAs were loaded into a jet nebulizer and nebulized for 10 min, and the mist and leftover reservoir solutions were collected and subsequently analyzed with TEM. The EPA filaments after nebulization both in the mist (Figure 2B) and reservoir (Figure 2C) maintained their filamentous structure but a notable reduction in length was observed. This same observation is made for the OPA filaments for the nebulized (Figure 2E) and reservoir populations (Figure 2F), where shorter filaments are produced. However, a striking difference is observed for the KPA filaments; while a length reduction is noted, we also observed the formation of different morphologies in both populations (Figure 2H and I). For both the nebulized and reservoir populations, we see the introduction of short bundled fibrils, wormlike micelles, and spherical micelles (spherical micelles depicted in Figure S5). These resultant structures are reminiscent of a dilution effect, as the wormlike micelles and spherical micelles were observed for KPA after aging at 100 μM (Figure S6).

Given that a length reduction was observed for all three filament systems, we confirmed this phenomenon by analyzing the contour length of the filaments by tracing at least 300 structures in TEM images. Representative low-magnification TEM images of EPA, OPA, and KPA filaments before and after nebulization are provided in Figures S7–S9. Filaments before nebulization were not measured, as accurate contour lengths could not be determined, since the filaments are over several micrometers long. Size distributions of the filaments for

their nebulized and reservoir populations are shown in Figure 3A–C. For all three systems, the nebulized population was measured to be on average shorter than its respective reservoir population. This suggests factors in play during aerosol droplet formation and delivery have a greater influence on filament size reduction compared to recycling in the reservoir. The EPA, OPA, and KPA filaments averaged around 287 ± 147 , 174 ± 103 , and 133 ± 108 nm, respectively, exiting the nebulizer as a mist and around 360 ± 177 , 206 ± 88 , and 196 ± 113 nm, respectively, leftover in the reservoir. For the KPA system, spherical micelles were omitted from this analysis, as they bias the distribution representation toward nonfilamentous structures. Additionally, we observe a correlation between filament breakdown and a transition from the negative to positive surface charge assemblies, where EPA filaments were longest in comparison to KPA filaments after nebulization, which suggests molecular design may play a role in filament breakdown.

We next investigated whether this filament fragmentation was consistent over the course of a nebulization event. We measured the molar scattering rate of the filaments with dynamic light scattering (DLS) every 2 min for a total 10 min as a measure of assembly size, as larger filamentous assemblies will scatter light more strongly than smaller ones. The molar scattering rate represents the scattering rate normalized to the sample concentration and is used here, since the DLS software uses models for fitting spherical particles and our structures are anisotropic. The EPA, OPA, and KPA filaments, as shown in Figure 3D–F, respectively, showed consistent breakdown over the course of 10 min, where the molar scattering rate did not significantly vary over time. Moreover, the drop in molar scattering was consistent among the different filament systems and did not vary in value relative to the filaments before nebulization. In terms of delivering supramolecular filament systems with a jet nebulizer for treatment administration, the consistent filament breakdown over the course of a nebulization event is promising in regard to aerosol delivery, as the filaments are released reliably with a relatively uniform size drop over time from the device.

Nebulization Impact on Molecular Packing.

While we were able to directly observe filament breakdown in terms of length, we investigated further the impact nebulization may have on the molecular packing of the PA monomers within the supramolecular filaments. Figure 4 contains the representative circular dichroism (CD) spectra of the three filament systems before and after a 10 min nebulization event of both filaments that exit as mist and those leftover in the reservoir. EPA, OPA, and KPA filaments before nebulization show primarily β -sheet characteristic hydrogen bonding, as evidenced by a negative peak around 220 nm and a positive peak around 197 nm for each system. Immediately after nebulization, the EPA filaments collected in the mist exhibit a slightly increased β -sheet signal, while EPA filaments leftover in the reservoir show no change to their hydrogen bonding, as seen in Figure 4A. As depicted in Figure 4B, OPA filaments in both the nebulized and reservoir populations show an increased β -sheet signals, which suggests the nebulization process likely enhances the hydrogen bonding of the OPA monomers within the supramolecular filaments. This slightly contrasts from the behavior observed for the EPA filaments and is likely attributed to the potential alignment of OEG chains of OPA from aerosol formation forces, which reduces steric repulsion between OPA monomers. Conversely, the KPA filaments, as detailed in Figure 4C, exhibit a secondary

structure transition from β -sheet to random coil character in the nebulized group, as evidenced by a red-shifted negative band above 200 nm, whose shift is likely due to aromatic interactions between tyrosine residues.^{69,70} Moreover, the KPA reservoir group displays a weakened β -sheet signal compared to the filaments before nebulization. Altogether, the decrease in CD signal and transition to the unorganized random coil hydrogen bonding suggest partial dissociation of the KPA supramolecular filaments into smaller aggregates and/or PA monomers. This is corroborated by the morphologies observed for the KPA filaments after nebulization, as smaller worm-like micelles and spherical micelles were generated. The strength of the hydrogen bonding network within the supramolecular filaments is evidently important in the maintenance and stabilization of β -sheet character after nebulization.

To gain insight into the thermodynamic stability of the assembly states induced by nebulization, we analyzed the CD signal of the same filament solutions after nebulization after aging for an additional 2 weeks in solution at room temperature. As shown in Figure 4A and B, the EPA and OPA filaments maintained their β -sheet character with negligible variability in molar ellipticity between the before and after nebulization groups. Moreover, the OPA filaments, which originally showed a sharp increase in β -sheet signal, returned to levels comparable to the before control filaments, suggesting the enhanced hydrogen bonding was temporary and not reflective of a more thermodynamically stable state. Additionally, the shorter EPA and OPA filaments are still observed in the nebulized and reservoir groups with TEM imaging after 2 weeks (Figures S10 and S11). As depicted in Figure 4C, the KPA filaments 2 weeks later continued to display the weakened β -sheet signal in the reservoir group and the random coil character of the nebulized group. Since a transition back to β -sheet character is not observed for the nebulized group, it is likely that nebulization induces the formation of metastable assemblies of the KPA monomers.^{71–73} This is corroborated by TEM images of the KPA filaments 2 weeks after nebulization, as the fibril, wormlike micelle, and spherical micelle structures are still observed (Figure S12). Collectively, these results give insight into the impact nebulization induces on the hydrogen bonding within peptide-based supramolecular filaments and suggest that nebulization into aerosol droplets has greater influence on molecular packing compared to repeated recycling within the reservoir of the nebulizer device.

Mechanism of Filament Breakdown in Aerosols.

Based on our observations from our TEM and CD studies, we sought to elucidate the aspects of the molecular design and self-assembly of the supramolecular filaments that influences their behavior during aerosol delivery, as the KPA filaments exhibited distinctly different morphological transitions and hydrogen bonding disruption after nebulization compared to the EPA and OPA filaments. Therefore, we assessed the thermodynamic stability of each supramolecular filament system by measuring their critical assembly concentration (CAC) using a Nile Red assay. As shown in Figure 5, the CAC values of EPA, OPA, and KPA were measured to be 1, 1.9, and 40 μ M, respectively. Data from the Nile Red assay are given in full in Figure S13. The differences in CAC between the different PAs are likely reflective of differences in electrostatic repulsion between the charged groups of EPA and KPA and steric effects with respect to the size of the OEG groups of the OPA molecule.⁷⁴ We find a direct

correlation between the CAC value and nebulization stability of the supramolecular filaments with respect to nanostructure integrity and hydrogen bonding interactions. The EPA and OPA filaments with low CAC values exhibited morphological retention with reduced length and with unaltered to increased hydrogen bonding; in contrast, the KPA filaments with a high CAC value underwent notable filament breakdown with a morphological transition and disruption of the hydrogen bonding network within the supramolecular structures. As the CAC value represents the propensity of the monomer units to aggregate in solution, increasing the strength of hydrophobic interactions alongside other noncovalent interactions through molecular engineering can result in lower CAC systems, and thus create supramolecular filament systems with enhanced stability during aerosol formation.

Previous research has been conducted to assess protein and peptide stability during aerosolization for therapeutic applications, which concludes that air-liquid interface (ALI) interactions are the major contributing driving force for their denaturation and aggregation with some influence from shear stress.^{25,75,76} The formation of liquid aerosols creates droplets with very high surface area-to-volume ratios and thus exposes molecules within the droplets to a highly hydrophobic ALI; this hydrophobic interaction leads to enrichment of surface active and amphiphilic moieties at the interface. Our results suggest that enrichment at the ALI is the major driving force for PA monomer depletion and their respective supramolecular filament degradation during nebulization. This conclusion is particularly corroborated by CD measurements between KPA filaments within the nebulized and reservoir groups (Figure 4C). The nebulized group, which experiences the greatest ALI interactions, exhibits complete disruption of its β -sheet character, whereas the reservoir group, which experiences lesser ALI interactions and more agitation from repeated recycling within the device, exhibits only weakened β -sheet hydrogen bonding. While factors like agitation and shear stress may also contribute to the fragmentation of these systems,⁶²⁻⁶⁴ these are less significant compared to the ALI, as the TEM analysis of the contour lengths of the filaments would likely be similar for the nebulized and reservoir populations if these factors played a major role. As depicted in Scheme 1, we propose that peptidic supramolecular filaments interact with the ALI through a two-step mechanism: filament disassembly and subsequent partitioning at the ALI. Peptide amphiphiles exchange frequently between their assembled state and the ALI through their monomeric form in solution, where the strength of hydrophobic and noncovalent interactions within the supramolecular filament compared to the hydrophobic interactions at the ALI influences the partitioning fate of monomeric units during aerosolization. Together, these steps account for the observed filament fragmentation and disruptions to the internal hydrogen bonding of the PA monomers within the filaments: monomers are depleted from the bulk solution to subsequently enrich the ALI, which lowers their concentration in the bulk, and as the CAC is approached, filaments will fragment to replenish the monomer concentration within in the bulk. The surface activity of these molecules at the ALI is further corroborated by the observed foam generation during a nebulization event, suggesting enrichment of monomeric units at the interface. Therefore, through tuning of the molecular design of peptide amphiphile units, particularly through modifications of the C-terminal charge-bearing residues of the peptide sequence, we can influence the degree of filament disruption during

nebulization by constructing systems with high stability, as reflected through their CAC value.

Drug Encapsulation and Nebulizer Release.

With better insight into nebulization stability and the mechanism of supramolecular filament breakdown, we next sought to assess whether these systems can successfully carry drugs and dyes within aerosol droplets for potential inhalation-based therapies. Additionally, we investigated if encapsulation of drugs would influence the stability of the supramolecular structures during nebulization. We chose paclitaxel (PTX), an anticancer drug approved for lung cancer treatment, coumarin 6 (Cou6), a green fluorescent dye, and budesonide (BUD), a corticosteroid used to alleviate symptoms of asthma and chronic obstructive pulmonary disease, to encapsulate within KPA filaments, as influences on structural stability would be most obvious with the least stable system. Figure 6A depicts the molecular structure of these three drugs and dye. As depicted in Figure 6B, we dissolved PA monomers with these lung-disease-relevant drugs and fluorescent dye in water to encapsulate them within the hydrophobic core of the resulting supramolecular structures. Figure 6B contains the loading capacities (% by mass or mole) of each drug and dye within the KPA filaments with 1.5%, 3.7%, and 4.4% loading by mass for PTX, Cou6, and BUD, respectively. The increase in loading capacity for Cou6 and BUD compared to PTX is likely a result of their relatively smaller size/molecular weight.

After encapsulation, we used TEM imaging to confirm self-assembly of the system into filamentous nanostructures. As shown in Figure 6C, all three encapsulated molecules within KPA assemblies resulted in the formation of filaments over several micrometers long (Figure 6Ci, iii, and v), which measured slightly larger in diameter on average compared to unloaded KPA filaments (Figure 2G) likely as a result of drug/dye loading. The loaded filaments were then nebulized and representative TEM images of the resultant structures are shown in Figure 6Cii, iv, and vi. Low magnification images of the two groups for the three loaded drugs/dyes are displayed in Figures S14–S16. Notably, we observed a distinct difference compared to unloaded KPA filaments after nebulization (Figure 2H); the observed loaded filaments maintain their fiber morphology and reduce in length, but this size reduction is noticeably smaller in comparison to the unloaded filaments. Spherical micelles are still observed, but the fibril and wormlike structures are not. These results suggest that the encapsulation of drugs/dyes within the filaments enhances their structural stability and thus helps maintain their filamentous shape, likely due to strengthened hydrophobic interactions between the loaded moiety and surrounding PA molecules within the filament core. Moreover, these results give credence to our proposed mechanism of the ALI enrichment as the major driving force for filament fragmentation, as the same level of fragmentation should be observed for the loaded and empty KPA filaments if shear forces were the main factor. In terms of delivering therapeutics in an aerosol, this presents an effective strategy for improving structural stability of supramolecular peptide-based assemblies during nebulization.

We next monitored the release pattern of the supramolecular filaments in aerosol droplets from a jet nebulizer device alongside the output rate. First analyzing the blank EPA, OPA,

and KPA filaments, 3 mL solutions of the filaments were loaded into a jet nebulizer at varying concentrations and the remaining concentration of PA molecules and fluid weight loss were monitored over the course of a 10 min nebulization event. Figure 7A–C represents the measured release profile of the three filament systems at 10, 100, and 1000 μ M, respectively. We observed a linear release rate for all three systems over the course of 10 min. The total percentage of starting fluid released from the nebulizer is detailed in Figure S17, where regardless of filament system or starting concentration, roughly 60–70% of the fluid is emitted from the nebulizer over 10 min. This is consistent with typical dead volumes commonly reported for jet nebulizers and suggests that changes in surface tension and viscosity with the varying concentrations are not significant enough to heavily impact output.⁶⁸ Moreover, for each run, we observe a slight decrease in formulation fluid and total PA delivered after the 8 min time point, which corresponds to sputtering from the nebulizer as the dead volume is approached. Comparing the output rates of the filament systems, as shown in Figure 7D, we observe that the output rate of the PAs (μ g/min) scales linearly with the increasing formulation concentrations. For the same formulation concentration, we observe no statistically significant difference between the output rates of each system, suggesting that neither filament surface charge nor stability influences filament output and thus the nebulizer device itself is likely the more influential factor in output rate.

We also examined the release pattern and output rate of the loaded KPA filaments to ascertain if drug/dye encapsulation would influence the release pattern. We nebulized PTX-, Cou6-, and BUD-loaded KPA filaments (1 mM KPA, 3 mL) for 10 min and analyzed fluid loss and remaining drug concentration within the device over the course of a 10 min nebulization event. As shown in Figure 7E, the encapsulated drugs/dye are released linearly from the jet nebulizer. The total percentage of fluid released roughly estimated 70%, whereas the total percentage of drug/dye released averaged around 60–70%, as shown in Figure S18. While drug concentration is likely to increase with evaporation of water from aerosol droplets as they travel through air, the lower drug/dye output compared to fluid suggests that also the concentration of drug/dye (and thereby their PA carrier) varies from droplet to droplet,⁶⁸ and thus filament output deviates slightly from homogeneity over the course of a nebulization event. The output rates of the encapsulated molecules (μ g/min) are compared in Figure 7F. The rates of Cou6 and BUD are comparable but higher than those of PTX, which is reflective of their differences in loading capacity within the KPA filaments (Figure 6B). Therefore, by tuning the drug loading through adjustments in the molecular design of the PA molecules,⁵⁸ we can influence the delivery rate of encapsulated drugs and dyes in liquid aerosols from a nebulizer device.

CONCLUSION

In this work, we have established a proof of concept of peptide-based supramolecular filaments as inhalable drug carriers within liquid aerosol droplets generated by a jet nebulizer. The several micrometer long filaments formed by the self-assembly of peptide amphiphiles can travel within aerosol droplets, where nebulization reduces their contour length. Enrichment at the large air–liquid interface produced as a result of aerosol droplet formation was concluded to be the primary factor that leads to supramolecular filament breakdown. The impact of the interactions with the air–liquid interface is dependent on the

thermodynamic stability of the assembled state, which is reflected in the system's CAC values. Low stability systems (with a high CAC) show a higher degree of filament fragmentation and hydrogen bonding disruption, whereas high stability systems (low CAC) better maintain their filamentous shape and length. Therefore, through rational design of the peptidic units, particularly through alterations of the C-terminal amino acids, supramolecular filaments can be engineered with suitable stability within liquid aerosols. Regardless of the CAC of the system, however, the supramolecular structure stability can be further enhanced by encapsulating drugs and dyes within the core of the filaments, thereby increasing the strength of associative interactions to outweigh the influence of the air-liquid interface. Tuning of the loading capacity of the supramolecular filaments influences the drug/dye output rate from the nebulizer device, allowing for controllable linear release profiles.

We envision that the peptide-based supramolecular filament platform could be used for inhalation-based therapy and imaging applications of lung-resident diseases. The distinct shape and size of the filaments after nebulization may provide beneficial delivery properties of their loaded cargo into the lungs, where these systems may enhance therapeutic efficacy and retention through avoidance of the lungs' clearance mechanisms, particularly by exhibiting high aspect ratios that in turn mitigates the effects of the mucociliary escalator and alveolar macrophages. Moreover, through molecular design of C-terminal amino acids, the mucosal penetration properties can be influenced. With the insight gained in this study with respect to the influence design elements of the PA monomers play on supramolecular stability during nebulization, our future work involves evaluating how the interplay of these design parameters, such as the strength/size of the hydrophobic domain and hydrogen bond-regulating sequence, in turn impacts the therapeutic efficacy and retention of the platform against relevant lung disease models.

MATERIALS AND METHODS

Molecular Self-Assembly and Nebulization.

Peptide amphiphile self-assembly was promoted by directly dissolving EPA, OPA, and KPA into water at 2 mM and neutral pH. Vortexing and brief sonication (3 min) were used to aid dissolution; afterward, solutions were heated for 5 min at 80 °C in a water bath to accelerate the dissolution and assembly processes. All solutions were aged overnight at minimum and then used for further experiments. For studies involving nebulization, solutions (3 mL) were added to a disposable jet nebulizer (Neb Kit 500, Drive Medical, Port Washington, NY) which was then connected to a nebulizer compressor (Rite-Neb 4, ProBasics, Marlboro, NJ) for constant air flow supply (~6–10 lpm). Samples were collected from the reservoir of the device or the emitted mist. The outlet of the nebulizer was fitted with a 50 mL conical tube, and the liquid aerosol droplets were allowed to condense on the walls of the tube. After a nebulization event, the conical tubes were centrifuged at 4000 rpm for 3 min and the collected solution was removed for further analysis.

Transmission Electron Microscopy.

Solutions of the peptide amphiphiles prenebulization were diluted down to 500 μM and dropped (10 μL) onto a carbon film copper grid (400 square mesh, Electron Microscopy

Service, Hatfield, PA). After 1 min, the excess solution was wicked away with filter paper to leave a thin film of the sample on the grid. Then, 7 μ L of 2 wt % (in water) uranyl acetate was dropped on the grid (for negative staining), and excess solution was blotted off after 30 s. Grids were left to dry for at least 3 h before imaging. The same grid preparation was used for the solutions post nebulization, where peptide amphiphiles were nebulized at 500 μ M for 10 min, and grids were prepared of both the nebulized mist and remaining reservoir solution. Imaging was performed on a FEI Tecnai 12 TWIN transmission electron microscope operating at an acceleration voltage of 100 kV. All images were recorded using a SIS Megaview III wide-angle CCD camera. Filament diameters and contour lengths were measured using ImageJ software.

Dynamic Light Scattering.

Filament solutions of the three PAs were diluted to 500 μ M before nebulization, and then 3 mL of each PA was added to the nebulizer reservoir. During nebulization, solutions were collected at 2 min time intervals over the course of 10 min from the nebulized mist and reservoir to analyze. Samples were diluted to 100 μ M and placed in a UV-transparent disposable cuvettes ZEN0118 composed of polystyrene latex (Sarstedt, Germany) to then run on a Malvern ZEN3690 Zetasizer (Malvern Panalytical, Westborough, MA) at 25 °C. Since the software uses models for fitting of spherical particles and the structures in our samples are anisotropic, we used the molar scattering rate (scattering rate normalized to sample concentration, Mcounts/s/M) as a measure of assembly size.^{71,73} Three runs were collected for each sample (10 scans/measurement) and averaged. The molar scattering rates were normalized to the 0 min time point.

Circular Dichroism.

Solutions of the supramolecular filaments were diluted to 500 μ M for each system, and the 3 mL solutions were nebulized for 10 min with samples collected from the emitted mist and the leftover solution in the nebulizer reservoir. Measurements were performed on a Jasco J-710 spectropolarimeter (JASCO, Easton, MD) with samples immediately diluted to 100 μ M and added to a 1 mm path length quartz UV–vis absorption cell (Thermo Fisher Scientific, Pittsburgh, PA). Each sample was analyzed by three repeated scans from 190 to 300 nm. The high tension (HT) values for each spectrum ranged from 200 to 600 V to ensure no scattering artifacts were present in the representative spectra. A background spectrum of water was obtained and subtracted from sample spectra. Obtained spectra were averaged over the three scans and converted from ellipticity (mdeg) to molar ellipticity (deg cm² dmol⁻¹). As nebulization can lead to solvent evaporation during aerosol formation, sample concentration post nebulization was determined using a standard curve derived from analytical HPLC runs.

Critical Assembly Concentration (CAC) Measurements.

The CAC of the peptide amphiphile monomers were determined using the Nile Red assay, where Nile Red dye fluoresces intensely within hydrophobic environments but is strongly quenched and red-shifted in aqueous environments. Thus, when the concentration of PA monomers exceeds the CAC, Nile Red will embed within the hydrophobic core and emit a strong fluorescence signal. A 500 μ M stock solution of Nile Red was prepared in acetone,

and 10 μL of stock was added to microcentrifuge tubes. Acetone was evaporated off in the dark. Sample solutions of 500 μL of each PA were added to the tubes at various concentrations. Solutions were aged for 2 days in the dark at room temperature. Using a Fluorolog spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ), three parallel emission spectra recorded from wavelengths of 580 to 720 nm at an excitation wavelength of 550 nm. The intensity ratio for each run of the emission intensity at 635 nm (emission maximum of Nile Red in a hydrophobic environment) to 660 nm (emission maximum of Nile Red in an aqueous environment) was plotted against the tested concentrations to obtain a transition curve.

Drug/Dye Encapsulation.

Free drug/dye and PA were dissolved in 100 μL of hexafluoro-2-propanol (HFIP) at a molar ratio of 2:1 in a microcentrifuge tube to disrupt any preassembled structures. Tubes were vortexed and sonicated for 3 min to aid dissolution and mixing. HFIP was evaporated off and samples were left open under a fume hood overnight to remove any trace amount of HFIP. Next, 1 mL of water was added to each tube, which was then vortexed and sonicated for 1 min. The solutions were then heated at 80 $^{\circ}\text{C}$ in a water bath for 1 h to accelerate dissolution and assembly and then aged at room temperature overnight. Precipitated free drug/dye was then removed by centrifugation (13 400 rpm, 3 min), and the supernatant was removed. The encapsulated drug/dye content was determined using analytical HPLC. The drug loading capacity was calculated as the percentage of recovered drug/dye to the sum of recovered drug/dye and added PA (by either mass or mole).

Nebulizer Release Studies.

Solutions of the different filaments systems at various concentrations were added to a jet nebulizer device for a total 3 mL in the reservoir. The nebulizer device was weighed before and after adding the filament solutions. At every time point over the course of 10 min at 2 min intervals starting at 0 min, 50 μL of the reservoir solution was removed and collected, and the nebulizer and remaining solution were weighed with mass recorded. The collected reservoir samples were analyzed using analytical HPLC to determine the concentration of PA and/or drug/dye at each time point. Using gravimetric data and calculated concentrations, release profiles of the filament systems were determined over time through mass balance calculations around the nebulizer device.

Supplementary Material

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Figure 1.

Molecular design and assembly of three peptide amphiphiles and their potential use as inhalable drug carriers. (A) Chemical structures of the three peptide amphiphiles studied, where each contains a dodecyl alkyl chain (black) and the same hydrogen bond-regulating peptide sequence with a glycine spacer (blue) but varies by surface charge. EPA contains three glutamic acid residues (light blue) for negative charge; OPA contains two oligoethylene glycol-decorated lysine residues (green) for neutral charge; and KPA contains three lysine residues for positive charge (red). (B) Peptide amphiphile monomers, composed of a hydrophobic alkyl chain (green) and a hydrophilic peptide segment (blue) spontaneously assemble in aqueous solutions into supramolecular filaments. Solutions of these filaments are then loaded into a jet nebulizer to produce filament-bearing liquid aerosol droplets.

Figure 2.

Representative transmission electron microscopy (TEM) images of the supramolecular filaments formed from (A) EPA, (D) OPA, and (G) KPA at 500 μM after dissolving in water and aging for 24 h. Filaments are several micrometers in length, and a ribbonlike morphology with variable twisting is observed for EPA and OPA, whereas KPA formed fibers. Filament diameters are represented as mean $\pm$ SD ($n = 35$). TEM images of EPA filaments at 500 μM after jet nebulization for 10 min collected in the mist (B) and leftover in the reservoir (C), where filaments are still observed but with a reduced length. TEM images of OPA filaments at 500 μM after jet nebulization for 10 min collected in the mist (E) and leftover in the reservoir (F), where filaments are still observed with reduced length. TEM images of KPA filaments at 500 μM after jet nebulization for 10 min collected in the mist

(H) and leftover in the reservoir (I), resulting in reduced filament length and the formation of fibrils and wormlike micelles. Scale bars represent 200 nm.

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Figure 3.

Contour length reduction analysis of (A) EPA, (B) OPA, and (C) KPA filaments after nebulization, comparing the nebulized (red) and reservoir (blue) population size distributions (20 bins, 50 nm each). Nebulized populations measured shorter on average compared to the reservoir populations. Average contour lengths (μ) are given as mean $\pm$ SD alongside medians (M) for each population (**** $p < 0.0001$, two-tail unpaired t test; EPA: $n_N = 316$, $n_R = 316$; OPA: $n_N = 311$, $n_R = 339$; KPA: $n_N = 377$, $n_R = 369$). Dynamic light scattering measurements of (D) EPA, (E) OPA, and (F) KPA over the course of a 10 min nebulization event as collected every 2 min, showing consistent filament breakdown over time. Data are normalized to molar scattering rate of filaments at $t = 0$ min and given as mean $\pm$ SD ($p > 0.05$, two-way ANOVA, $n = 3$).

Figure 4.

Circular dichroism (CD) spectra of the supramolecular filament systems before and immediately after nebulization (solid lines) alongside the same solutions 2 weeks later (dashed lines). All filaments were nebulized at 500 μM for 10 min. Data given are mean spectra for each solution ($n = 3$). (A) EPA filaments before and after nebulization, showing preservation of β -sheet character during nebulization. (B) OPA filaments before and after nebulization, showing retention of β -sheet character with a temporary increase in CD signal. (C) KPA filaments before and after nebulization, showing a transition from β -sheet to random coil for the nebulization group and weakened β -sheet hydrogen bonding for the reservoir group.

Figure 5.

Critical assembly concentration (CAC) of (A) EPA, (B) OPA, and (C) KPA molecules in water as measured from a Nile Red assay. A fluorescence intensity maximum is shifted from a wavelength of 660 to 635 nm as Nile Red dye becomes encapsulated within the supramolecular structures of the PAs. Blue line represents the region of concentrations where Nile Red remains in solution; red line represents the region where Nile Red is encapsulated. Data are given as mean $\pm$ SD ($n = 3$).

Figure 6.

(A) Molecular structure of the lung-disease-relevant drugs and fluorescent dye used in loading within the supramolecular filaments: paclitaxel (PTX, MW = 853.92 g/mol), the anticancer drug; coumarin 6 (Cou6, MW = 350.44 g/mol), the green fluorescent dye; and budesonide (BUD, MW = 430.54 g/mol), the anti-inflammatory glucocorticoid used to treat symptoms of asthma and chronic obstructive pulmonary disease (top). (B) Encapsulation process of hydrophobic drugs and dyes within supramolecular peptide amphiphile (PA)-based filaments, where dissolution of PA monomers and drug/dye in aqueous solutions results in their self-assembly with the drug/dye loaded within the hydrophobic core (left). The loading capacity of PTX, Cou6, and BUD based on mass (left axis, solid bars) and mole (right axis, checkered bars) percent within KPA supramolecular filaments (right). Data are

given as mean $\pm$ SD ($n = 5$). (C) Representative transmission electron microscopy (TEM) images of KPA filaments loaded with PTX (i, ii), Cou6 (iii, iv), and BUD (v, vi) before and after nebulization at 500 μ M. Diameters are given as mean $\pm$ SD ($n = 35$). The before images (after aging for 24 h) show filamentous nanostructures over several micrometers in length; the nebulized images, representing filaments collected in aerosol mist released from a jet nebulizer immediately after a nebulization event, show a retained filament morphology shorter in length and the appearance of spherical micelles, indicating that loaded drugs/dyes help to stabilize the supramolecular structures during aerosol generation.

Figure 7.

Jet nebulizer release profiles of EPA, OPA, and KPA supramolecular filaments with starting formulation concentrations at (A) $10\ \mu\text{M}$, (B) $100\ \mu\text{M}$, and (C) $1000\ \mu\text{M}$, showing a linear release pattern for each system over the course of a 10 min nebulization event (3 mL solution). Data given represent mean $\pm$ SD ($n = 3$); dashed lines represent the line of best fit from linear regression analysis. (D) Aerosol output rate ($\mu\text{g}/\text{min}$) of the supramolecular filaments from a jet nebulizer at different formulation concentrations, showing no statistically significant difference (ns) between filaments at the same concentration and output rate scales linearly with increasing concentration. Data are given as mean $\pm$ SD ($p > 0.05$ among filament systems, **** $p < 0.0001$ among concentrations, two-way ANOVA, $n = 3$). (E) Drug and dye release profiles of paclitaxel (PTX), coumarin 6 (Cou6), and budesonide (BUD) loaded within KPA filaments (1 mM) from a jet nebulizer, showing a linear release profile for each encapsulated molecule over the course of a 10 min nebulization event (3 mL solution). Data are given as mean $\pm$ SD ($n = 3$); dashed lines represent the line of best fit from linear regression analysis. (F) Aerosol output rate of the encapsulated drugs and dye within KPA filaments (1 mM) from a jet nebulizer, where output rate reflects loading capacity. Data are given as mean $\pm$ SD (ns $p > 0.05$, * $p < 0.05$, two-tailed unpaired t test, $n = 3$).

Scheme 1. Proposed Mechanism of Supramolecular Filament Enrichment at the Air-Liquid Interface (ALI) during Liquid Aerosol Formation from a Jet Nebulizer^a

^aPAs, once exposed to the hydrophobic ALI, may shift the assembly–disassembly equilibrium from their supramolecular structure into the surrounding solution as monomers are drawn from the solution to the ALI. The PA monomers may reassemble at this stage into the filaments or partition and enrich the ALI, where their hydrophobic domain (green) is exposed to air and their hydrophilic domain (blue) is exposed to water. Each step of the proposed mechanism is reversible, and the tendency for PA monomers to remain within their assembled state is dictated by the strength of their noncovalent interactions, which correlates with their CAC value.