Interaction of Metal Ion Stabilized Oligomeric Dipeptide with Lipid Membrane: Concurrent Observations of Supported Lipid Membrane, Wetting and Uptake

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ABSTRACT. The interaction of biomolecules with cell membranes is crucial due to their important role in governing the cellular function and membrane dynamics. However, most of the studies predominantly focus on the interaction of monomeric forms of biomolecules with lipid membranes, leaving the effects of cytotoxic oligomeric self-aggregates largely unexplored. In this study, we present the first evidence that oligomeric intermediates of diphenylalanine exert diverse effects on lipid membranes, influenced by lipid phase and charge. Our findings reveal that the fibrillar morphology of diphenylalanine facilitates the formation of supported phospholipid membranes through liposome deformation. Importantly, metal ion stabilized oligomeric intermediates of diphenylalanine cause wetting, promote vesicle uptake, and induce coalescence, resulting in significant structural alterations of lipid membranes having different charges. These insights open new research directions with profound implications for biomedicine, nanotechnology, and help in fundamental biological understanding.

TOC GRAPHICS
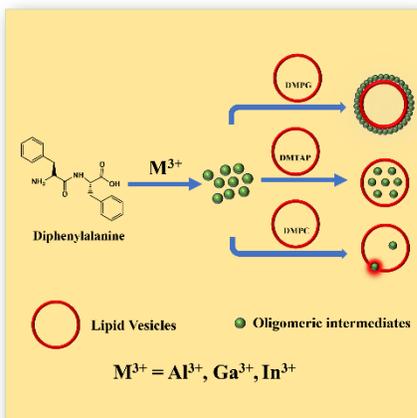
Self-assembly process of biomolecules is a fascinating phenomenon where simple building blocks, such as amino acids, peptides, proteins, and phospholipids, spontaneously organize into complex structures without any external direction.[1–5] This process is crucial in forming biological systems, including cells, tissues, and organs, where it plays a vital role in the development and function of living organisms. Additionally, the uncontrolled self-aggregation of proteins and peptides can result in the formation of amyloid fibrillar structures, responsible for various neurodegenerative diseases.[6–9] However, due to the structural complexity of proteins and peptides, the underlying mechanisms behind the self-assembly process remain elusive. Therefore, small dipeptides have garnered attention in recent years. The diphenylalanine (FF) peptides, which serve as core recognition motifs of amyloid plaques associated with neurological disorders like Alzheimer's and Parkinson's, are of particular interest.[10] FF shows diverse morphologies (nanotubes, nanowires, nanovesicles, nanofibers depending on specific conditions) during the self-assembly process, leading to extensive research into understanding the underlying mechanisms and factors that govern their self-assembly.[11–13] Different factors, such as the concentration of biomolecules, pH, and the presence of ions and nanoparticles, can affect the self-assembly process.[14–18] In this aspect, one of the major challenges is to capture the transient intermediates formed during the formation of self-assembly. Our group previously reported that certain bivalent and trivalent metal ions effectively decelerated the process of L-Phe fibril formation, which led to the creation of various unique intermediate structures with distinct morphologies.[19] These intermediates are oligomeric aggregates in nature, which subsequently evolved into fully developed fibrils, suggesting that the fibrillation process becomes more thermodynamically favorable over time. The oligomers can disrupt cell membranes, interfere with cellular function, and trigger cell death, contributing to neurodegeneration.[20,21]

While the aggregation and self-assembly of biomolecules and their interactions with cell membranes have been widely studied in recent years due to the crucial in understanding cellular toxicity, disease progression, and the regulation of extracellular matrix organization[22–24], the effects of small oligomeric intermediates on lipid membranes have been mostly overlooked. Proteins, as integral components of cell membranes, exert significant influence on diverse cellular mechanisms.[25,26] Therefore, elucidating their interactions with lipid membranes was imperative for advancing novel therapeutic strategies targeting a wide spectrum of human diseases. Amino acids and peptides, small building blocks of protein are known to affect the lipid
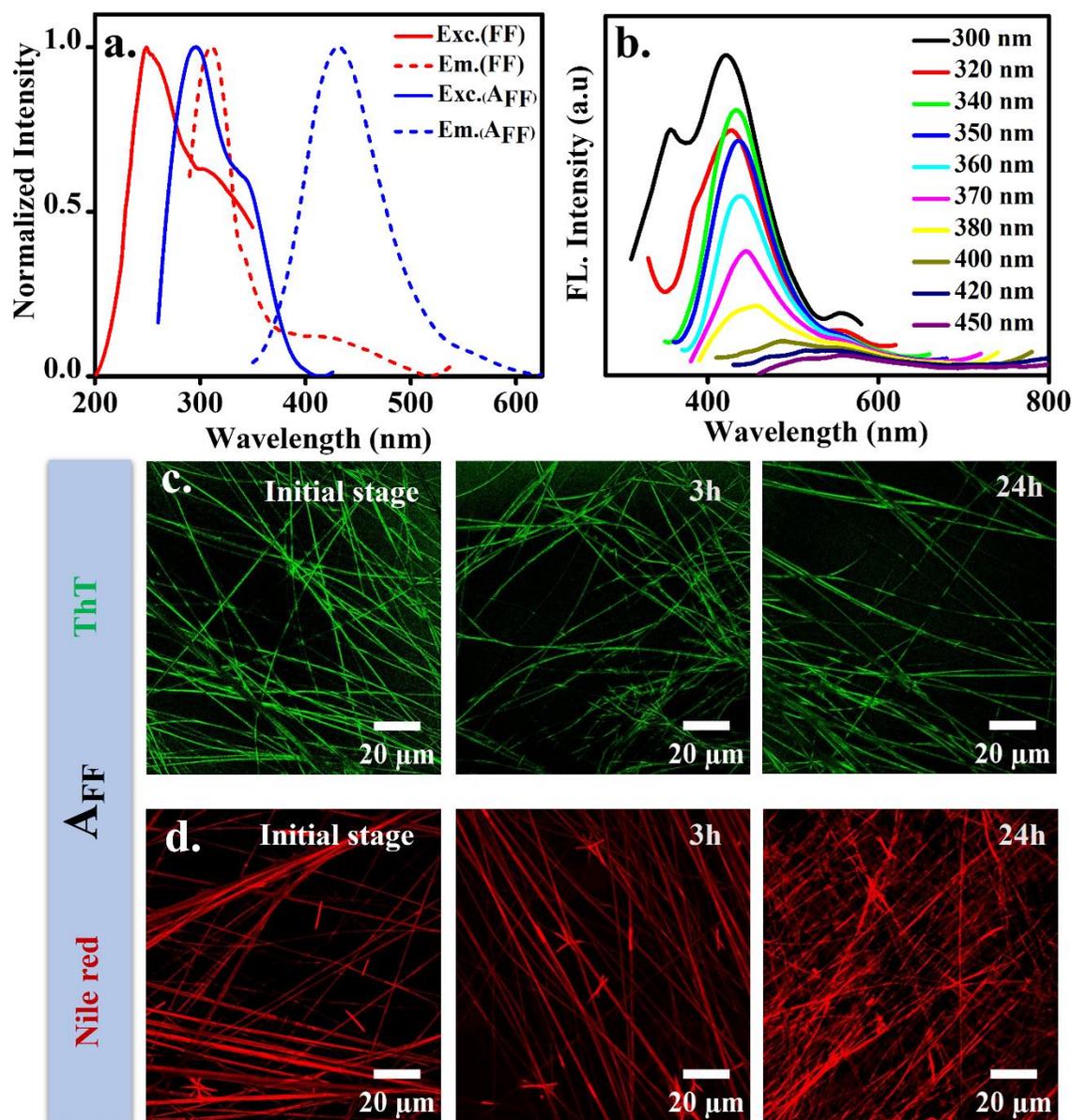
Figure 1. *Excitation-emission spectra of monomeric and self-assembly of FF (a) Excitation-dependent emission spectra of A_FF (b)Time-lapse CLSM images of A_FF using ThT(c) and Nile red(d). (FF concentration for self-assembly=5 mM). Scale bar: 20 µm.*

membrane properties. Amino acid can increase membrane permeability by aggregating within membranes.[27,28] Previously, we studied the interaction of model membranes with amyloid aggregates of self-assembled L-Phe and L-Phe functionalized gold nanoparticles.[29,30] Sarkar and co-workers studied how L-Phe creates harmful fibril structures in the blood that affect membrane flexibility, while D-Phe disrupts this formation, enhancing membrane rigidity and offering

potential therapeutic benefits.[31] It has been reported that under physiological conditions, the dissolution of FF crystals leads to the deformation and movement of the supported lipid membrane.[32] Interestingly, the literature consists of numerous studies on the interactions of coacervates with model membranes.[33–35] Different phenomena of interaction, like surface wetting and endocytosis of coacervates, have been proposed.[36,37] However, to our knowledge, there is currently no literature that investigates the impact of the oligomeric intermediate state of dipeptides on the bilayer lipid membrane.

In this study, we first examine the formation of the oligomeric intermediate of FF self-assembly in the presence of trivalent metal ions (Al^{3+}, Ga^{3+}, and In^{3+}). These intermediates gain stability due to surface charge inversion, as previously reported in the case of amino acids.[38] Then, we explore the potential interaction between the model membrane and the oligomeric intermediate state of diphenylalanine (FF). To unravel the interaction of fibrillar self-assembled FF and oligomeric unit of FF with the lipid membrane, we varied the lipid fluidity (based on different phase transition temperature and surface charge) by taking DOPC, DMPC, DPPC lipids and the charge of the lipid by mixing DMPC/DMTAP (positively charged lipid), and DMPC/DMPG (negatively charged lipid) as a model membrane. The spectroscopic and confocal imaging (CLSM) techniques were employed in a time dependent fashion to monitor the interaction. The zeta potential was used to measure the surface charge of the system.
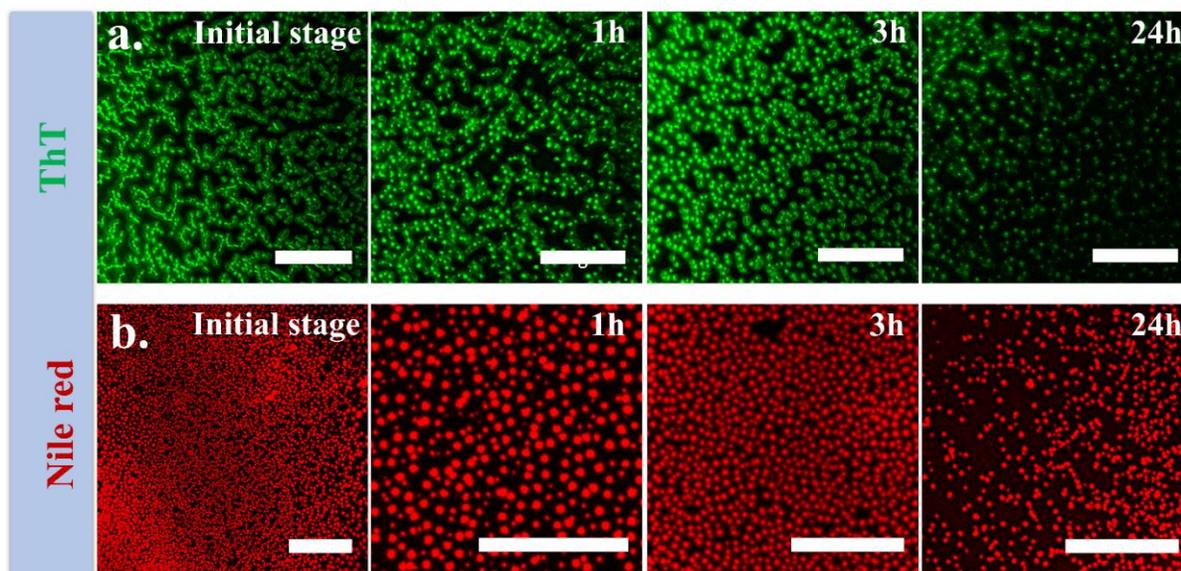
Figure 2. *Time-lapse CLSM images of the A_{FF}/Ga^{3+} assembly, captured by using ThT (a) and Nile red (b). (FF: Ga concentration=5 mM:10 mM). Scale bar: 20 µm.*

The study begins by examining the self-assembly mechanism of FF (Figure S1) through the initial dissolution of 5 mM FF in a HEPES buffer solution (pH~7.4, 10 mM). Following this, the solution undergoes heating at 90°C for a duration of 2 hours to confirm the monomeric condition and complete solubility of FF. Subsequently, the solution is slowly cooled to room temperature without any agitation, leading to the formation of an amyloid-like fibrillar structure. We examined the inherent blue fluorescence characteristics of diphenylalanine self-assemblies (A_{FF}) by using fluorescence spectroscopy. The monomeric FF is weakly fluorescent in nature and does not exhibit any emission spectra in visible region (Figure 1a). On the other hand, A_{FF} develops an emission band in the relatively longer wavelength and continuously shifts towards the red end from 425 to 570 nm as the excitation wavelength increases (Figure 1b). The excitation-dependent emission of the self-assembly of A_{FF} is attributed to the formation of diverse emissive entities in the course of the formation of self-assembly.[19] The excitation spectra reveal an additional band around 340-360 nm originating from the existence of the oligomeric dipeptide, and this band is responsible for the observed emission of A_{FF} in the visible range (Figure 1a). To validate the development of the self-assembly structure of diphenylalanine, CLSM (Confocal Laser Scanning Microscopy) was conducted using ThT and Nile Red (Figure 1c, 1d).[39–42] Strong signals were observed from the fibrillar structure of blank A_{FF}, suggesting that $\pi-\pi$ and hydrophobic interactions serve as the primary driving forces. It was observed that blank A_{FF} begins to develop fibrillar structures at the early stage without any external stimuli. We thus could not detect any intermediate nanostructures in the confocal images for A_{FF} as fibril formation emerged immediately. This indicates that the transition of monomeric FF to fibrillar structure is too fast to capture any intermediates. We next investigate the effects of various trivalent metal ions, specifically Al^{3+}, Ga^{3+}, and In^{3+}, on the self-assembly process of the FF. Importantly, the same series of metal ions are known to increase entropy by coordinating with amino acids.[43] Here, A_{FF} and the metal were mixed in a 1:2 molar ratio (5 mM:10 mM), before allowing A_{FF} solution to sit at room temperature. The progress of the formation of A_{FF} in the presence of metal ions was monitored by using time-lapse steady-state fluorescence spectroscopy (λ_{ex}~340 nm) and time resolved decay measurements (λ_{ex}~371 nm, decay collected at 440 nm). For blank A_{FF}, there was no significant change in fluorescence

intensity or lifetime decay with time (Figure S2a, d) was observed. However, in the presence of A_{FF}/Al^{3+} and A_{FF}/Ga^{3+}, there was a significant change in the fluorescence intensity and lifetime decay was observed. The addition of metal ions (Al^{3+} and Ga^{3+} ions) caused an enhancement in the fluorescence intensity in A_{FF} (Figure S2b, c). The unusual enhancement in intensity in the presence of metal ions was attributed to the formation of nanoaggregates, which cause structural changes and result in aggregation-induced emission (AIE) properties.[44] This observation is in accordance with previous reports that metal ions can induce the clustering-triggered emission of amino acids due to the formation of nano to micro-sized aggregates.[45] We observed that the time resolved decays significantly increased with time inintervalsor A_{FF}/Al^{3+} (Figure S2e) and A_{FF}/Ga^{3+} (Figure S2f) systems as compared to the blank A_{FF}. This suggests the formation of a more rigid conformation in the presence of these metal ions. However, in the case of A_{FF}/In^{3+}, turbidity was observed so no further spectroscopic study has been done.

As the trivalent metal ions can significantly attenuate the kinetics of the FF fibrillation process, it was essential to investigate the fibrillation formation process of FF in a time-dependent manner to reveal the intermediates that emerge throughout this process. Therefore, we conducted CLSM imaging to gain more insight into the morphological changes of A_{FF} in the presence of metal ions. In the case of A_{FF}/Al^{3+} (Figure S3a, b), FF formed spherical structures in the initial stage that remained stable for hours. The formation of a peptide-rich spherical-like structure was correlated to the clustering of peptides at the early stages of self-assembly. The intense emissions from both the ThT and Nile red indicate that enhanced hydrophobic interactions drove the clustering of peptides due to the presence of metal ions and π–π interactions, resulting from the coordination between metals and peptides. The initially obtained spherical aggregates of A_{FF}/Al^{3+}, fused and eventually developed into vesicle-like structures. The assembly of peptides in the interfacial region, away from the core, facilitated the formation of fibrillar structures and resulted in the core being undetectable by ThT. These vesicle-like structures subsequently transformed into mature fibrillar structures over time. Notably, it was observed that some microspheres exist along with the fibrils, indicating the moderate stability of the microspheres. But in the case of A_{FF}/Ga^{3+} (Figure 2a, b) and A_{FF}/In^{3+} (Figure S4a, b) systems, microspherical like structures and micro aggregates generated at an early stage remained stable for a long period of time owing to the more hydrophobicity in the system. The size of these micro spherical-like structures is almost 1-2 μm. Additionally, their small surface curvature contributes to greater stability against fusion in these

spherical structures. Furthermore, we observed that the zeta potential of the A_{FF} systems changed from negative (-20 mV) to positive value (almost +30 mV
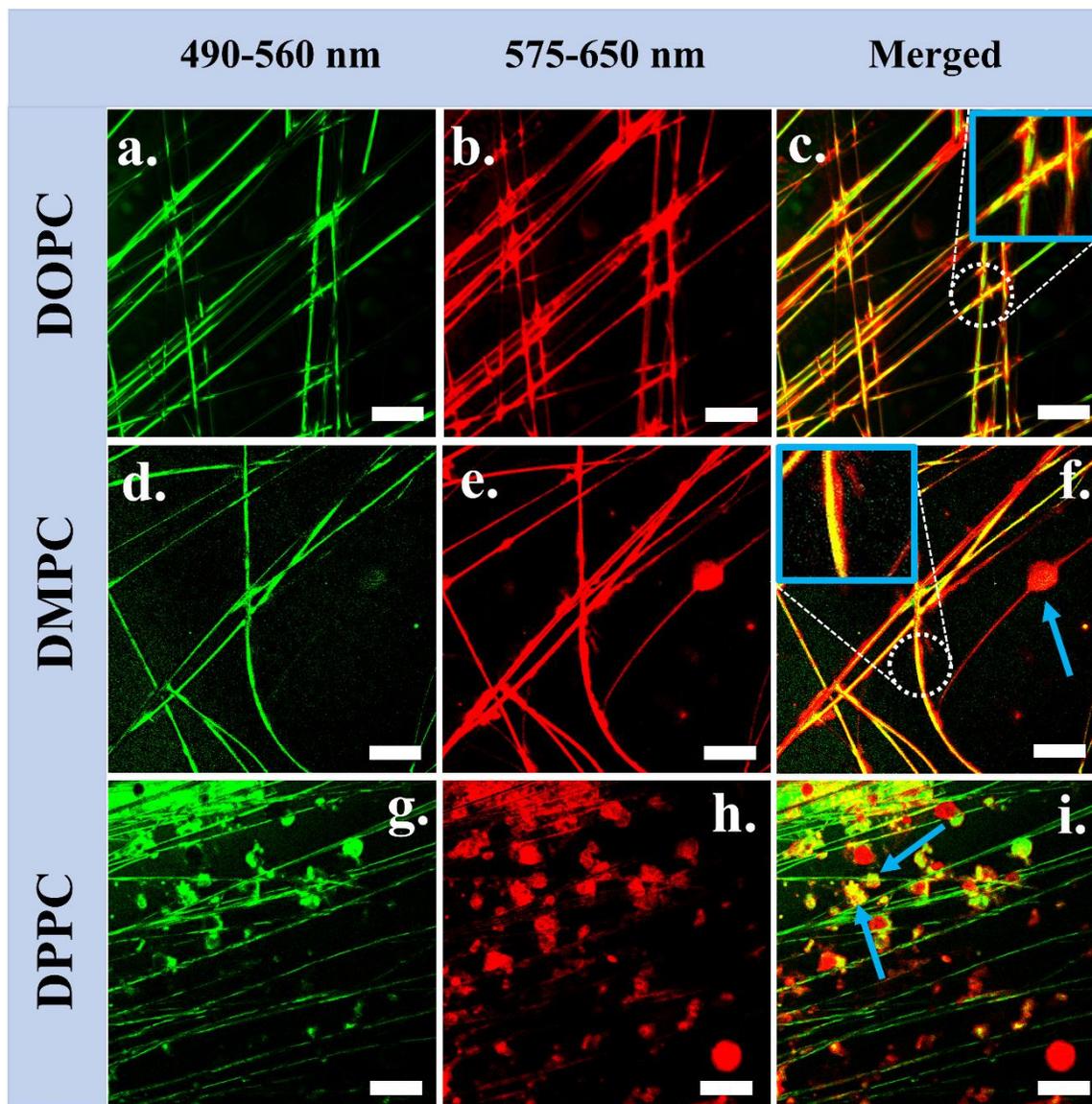
Figure 3. *CLSM images of DOPC (a-c), DMPC (d-f), and DPPC(g-i) vesicles in the presence of A_{FF}, where the assembly and vesicles were labeled with ThT and Rh-PE, respectively. Scale bar: 20 μm.*

to +40 mV) in the presence of metal ions (Figure S5). This charge inversion is in accordance with the previous report and imparts stability to the microsphere by strong mutual repulsion.[38]

After getting insights into the self-assembly process of A_{FF} and the formation of the metastable oligomeric intermediates, we investigated their interaction with the lipid membranes. Therefore, it was crucial to first study the interactions of fibrillar structures of FF (control systems) with lipid membrane. To unravel the interactions between A_{FF} and lipid membranes, we have chosen three zwitterionic lipid membranes (DOPC, DMPC, and DPPC) of different phase transition temperatures, which render different extents of fluidity. At room temperature, DOPC remains in the liquid crystalline phase (or disordered phase), while DMPC is in near the sol-gel to the liquid crystalline transition phase and DPPC remains in the sol-gel phase (or ordered phase). We conducted a dual-color CLSM imaging where ThT is co-assembled with A_{FF} (green), and Rh-PE is assembled with the lipid membrane (red). When the lipid membrane (Figure S6) was added to the A_{FF} solution, it was observed that the individual lipid membrane either disappeared or deformed (Figure 3). In the merged channel of the CLSM images, the strong red signals come from the outer edges of the A_{FF} fibrils and the green signal within the fibrillar structures firmly indicates the wrapping of lipid membranes around the FF fibrillar structures and the subsequent formation of supported lipid membranes.[32] Driven by the strong hydrophobic interactions between hydrophobic FF fibrils and phospholipid tails, the lipid membrane (DOPC and DMPC) was drawn towards the A_{FF} fibrils (Figure 3a-c, d-f). This can facilitate the formation of the A_{FF}-lipid complex and the resultant supported lipid membranes on the surface of A_{FF} (Figure 3c, f; inset). Moreover, no discrete or unreacted liposomes were observed on the surface of the FF fibrillar networks in A_{FF}/DOPC or A_{FF}/DMPC. On the contrary, we observed unreacted and deformed lipid vesicles in the case of A_{FF}/DPPC system (Figure 3g-i). The observation possibly stems from the fact that during the formation of the A_{FF}-lipid network, it is difficult to pull more rigid DPPC lipid membrane to form the A_{FF}/lipid co-assembly. This leads to the appearance of partially deformed DPPC lipid membrane on the surface of FF fibrils. In the A_{FF}/DMPC, a few tubule-like membrane structures were observed (Figure 3f, blue arrow).[32] However, we could not obtain such structures in A_{FF}/DPPC or A_{FF}/DOPC systems. The formation of a tubule-like structure may be attributed to the intermediate phase behavior of DMPC as compared to both DOPC and DPPC. Additionally, in A_{FF}/DPPC, the fluorescence signals were heterogeneously distributed, indicating the uneven organization of the lipid membrane. Whereas in the
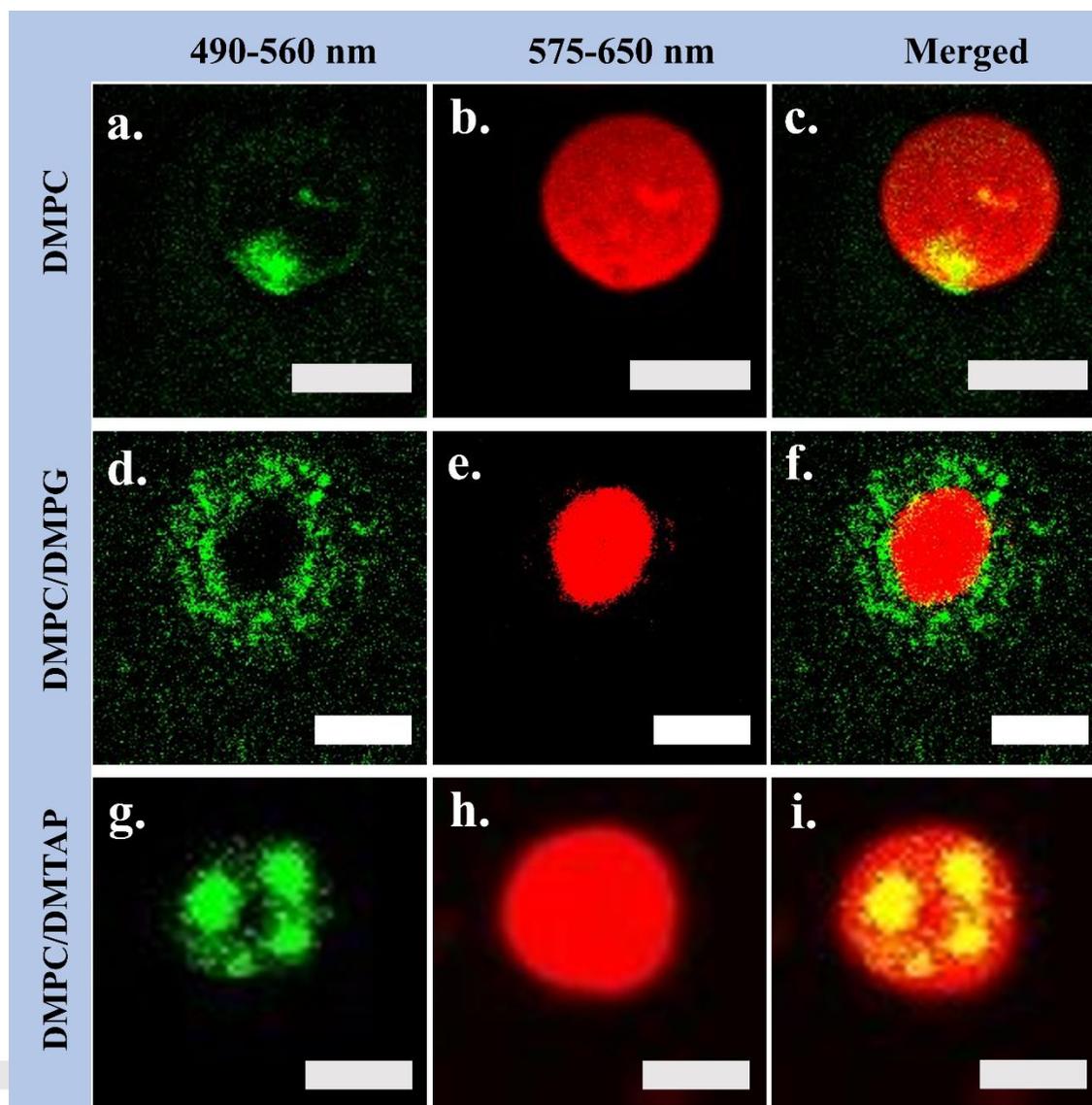
Figure 4. *CLSM images of DMPC (a-c), DMPC/DMPG(d-f), and DMPC/DMTAP vesicles (g-i), at the initial stage in the presence of A_{FF}/Ga^{3+}, where the assembly and vesicles were labeled with ThT and Rh-PE, respectively. Scale bar: 5 μm.*

A_{FF}/DMPC and A_{FF}/DOPC, the supported membranes were evenly formed on the hydrophobic side of the fibril, resulting in the homogeneous distribution of the fluorescence signals coming from the A_{FF}-lipid structures. Furthermore, in A_{FF}/DPPC, yellow signals (a mix of both green and red) were coming from the different aggregates (Figure 3i, blue arrow), indicating that the DPPC lipid membrane engulfed a large number of FF clusters, and distorted aggregates are visible.

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Next, we have done a critical investigation of how oligomeric FF intermediates interact with lipid membrane, revealing key insights that could transform our understanding of membrane dynamics. Therefore, we have taken A_{FF}/Ga^{3+} as the model system since the spherical intermediates formed were stable for a longer duration. Figure S5 reveals that the A_{FF}/Ga^{3+} has a net positive surface charge (+31 mV), so, along with the zwitterionic lipids (DOPC, DPPC and DMPC) having surface charge close to zero, we used negatively charged DMPC/DMPG (-73 mV) and positively charged DMPC/DMTAP (+76 mV) lipids for the investigation (Figure S7). Our study reveals that for DOPC membrane, the oligomers at the initial stage penetrated the membrane and remained attached to the inner surface, forming a ring-like structure (Figure S8a-c) due to electrostatic interaction. Eventually, they penetrated (Figure S8d-f) then coalesced and, as a result, deformed aggregates of oligomers and lipid membrane were obtained (Figure S8g-i). In contrast, the oligomers gathered on the surface of DPPC membrane and formed a ring-like structure (Figure S9a-c) before penetrating the lipid membrane without any coalescence (Figure S9d-f). However, neither coalescence nor gathering of oligomers was observed on the surface of DMPC membrane. Conversely, oligomers partially wetted the lipid membrane surface at the initial stage (Figure 4a-c) and then eventually penetrated the lipid membrane (Figure S10a-c). The phase-transition
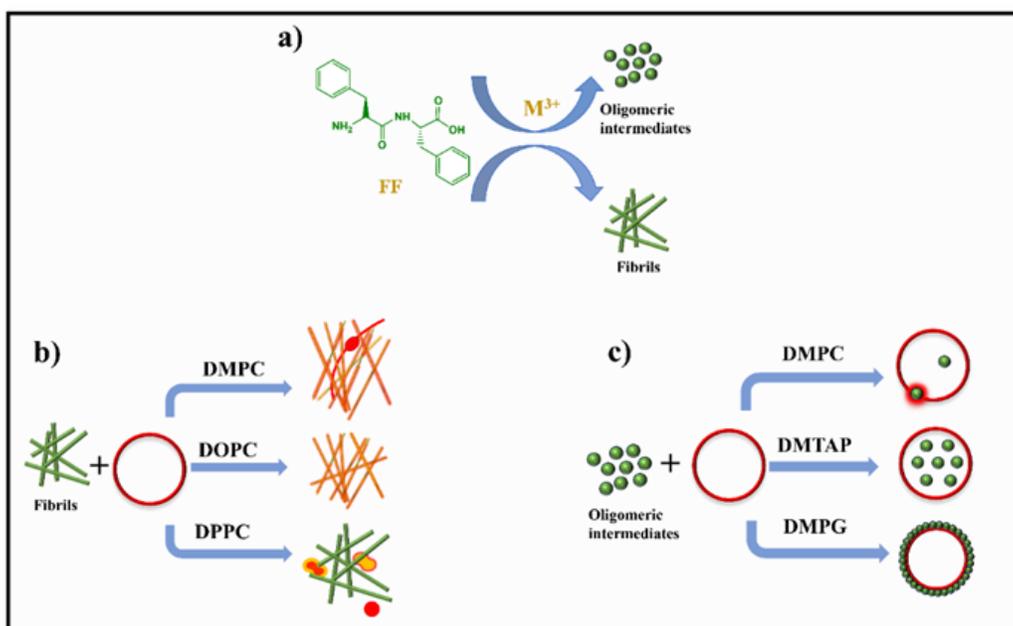
Scheme 1: *Schematic representation of self-assembly of diphenylalanine in the presence of trivalent metal ions and the interaction of fibrils and oligomeric intermediates with the different lipid membrane.*

temperatures of the DPPC (41 °C) or DMPC lipids (23 °C) are much higher than that of the DOPC (−21 °C). Due to the well-organized, condensed, and rigid arrangement of hydrocarbon chains, the DPPC and DMPC lipid membranes exhibit resistance to the deformation of shape. Further, due to the smaller head group area, DPPC most possibly accommodates more oligomers than DOPC and other lipid membranes. The ring-like formation in the inner leaflet and observation indicate that in zwitterionic lipids, the surface wetting of the membrane is the first step, followed by engulfment of the oligomeric FF.[37] Interestingly, for negatively charged lipid membrane (DMPC/DMPG) (Figure 4d-f), the uptake of oligomeric FF was not observed, instead the strong electrostatic interaction ruptured the oligomers into smaller pieces which finally adhered to the surface of the lipid membrane and wetted the lipid membrane. Our reports indicate that the moderate electrostatic interaction led to the initial contact and then facilitated the penetration of the oligomers inside the lipid membrane while the strong electrostatic interaction rendered the wetting of the membrane surface. Interestingly, a fascinating phenomenon was observed with positively charged lipid membrane (DMPC/DMTAP). The interaction with oligomers resulted in no surface wetting (Figure 4g- i). This is particularly important because it suggests that electrostatic repulsion played a critical role which prevents the surface wetting of lipid membrane. But here we observed that the oligomers crossed the membrane without being enclosed by the lipid membrane. This process indicates that in the case of DMPC/DMTAP lipid membrane, the oligomeric intermediate was able to overcome the electrostatic repulsion and penetrate inside the lipid membrane.[33] Therefore, these unique membrane-crossing properties of the oligomers open an avenue to build up a strategy for transfection of the materials into the cells.

In summary, we have explored the effect of trivalent metal ions on the self-assembly of FF and their interaction with lipid membranes of different fluidity and charges (Scheme 1). While fibrillation process of blank FF takes place very fast without capturing any intermediates, the trivalent metal ions stabilize the intermediate of the FF assembly by slowing the fibrillation process. In case of A_{FF}/Al^{3+}, early stage spherical-like morphology fused to form vesicle-like morphology, then converted into mature fibril. However, in the presence of A_{FF}/Ga^{3+} and A_{FF}/In^{3+}

systems, microspherical and micro aggregates formed initially remained stable for a long time (Scheme 1a). The observed stability of the intermediates in the presence of trivalent metal ions is attributed to the binding interactions of the metal ions, the unique effects associated with each type of metal ion, and the increased hydrophobicity resulting from the presence of trivalent metal ions. The interactions of lipid membranes with the fibrillar assembly of FF revealed that fibrillar FF transformed the liposomes into a supported lipid membrane (Scheme 1b). Conversely, the oligomeric intermediate can penetrate the zwitterionic and positively charged lipid membrane, while negatively charged lipids break the oligomers into smaller pieces that adhere to the lipid membrane (Scheme 1c). Overall, our findings indicated that the metastable intermediates of the metal-conjugated FF system can wet, penetrate, and change the shape of the lipid membrane based on their interactions. The uptake of peptide intermediates by lipid membrane offers insights into the mechanism of cellular functions within living organisms.

ASSOCIATED CONTENT

Supporting Information.

Further information on the materials and experimental methods, sample preparation, Instrumentation, Structure of different lipids, Chemical structure of FF, Spectroscopic study of A_{FF} and A_{FF}/M^{3+}, CLSM images of A_{FF}/Al^{3+} and A_{FF}/In^{3+} at various times, Surface charge measurement of A_{FF} and A_{FF}/M^{3+}, CLSM images and surface charge of multiple lipids, CLSM images of DOPC in presence of A_{FF}/Ga^{3+}, CLSM images of DPPC in presence of A_{FF}/Ga^{3+} CLSM images of DMPC in presence of A_{FF}/Ga^{3+} (**Scheme S1 and Supplementary figures S1-S10**).

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Notes

The authors declare no competing financial interests.

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