Published in final edited form as:

Proteins. 2013 April ; 81(4): 690–703. doi:10.1002/prot.24229.

Evidence of π -stacking Interactions in the Self-Assembly of hIAPP_{22–29}[†]

Adam A. Profit^{*}, Valentina Felsen, Justina Chinwong, Elmer-Rico E. Mojica, and Ruel Z. B. Desamero^{*}

York College, Institute for Macromolecular Assemblies and the Graduate Center of the City University of New York, Jamaica, New York, 11451

Abstract

The role aromatic amino acids play in the formation of amyloid is a subject of controversy. In an effort to clarify the contribution of aromaticity to the self-assembly of hIAPP_{22–29}, peptide analogs containing electron donating groups (EDGs) or electron withdrawing groups (EWGs) as substituents on the aromatic ring of Phe-23 at the *para* position have been synthesized and characterized using turbidity measurements in conjunction with Raman, and fluorescence spectroscopy. Results indicate the incorporation of EDGs on the aromatic ring of Phe-23 virtually abolish the ability of hIAPP_{22–29} to form amyloid. Peptides containing EWGs were still capable of forming aggregates. These aggregates were found to be rich in β -sheet secondary structure. TEM images of the aggregates confirm the presence of amyloid fibrils. The observed difference in amyloidogenic propensity between peptides containing EDGs and those with EWGs appears not to be based on differences in peptide hydrophobicity. Fluorescence and Raman spectroscopic investigations reveal that the environment surrounding the aromatic ring becomes more hydrophobic and ordered upon aggregation. Furthermore, Raman measurements of peptide analogs containing EWGs, conclusively demonstrate a distinct downshift in the $\text{C}=\text{C}$ -ring mode (ca. 1600 cm^{-1}) upon aggregation that has previously been shown to be indicative of π -stacking. While previous work has demonstrated that π -stacking is not an absolute requirement for fibrillization, our findings indicate that Phe-23 also contributes to fibril formation through π -stacking interactions and that it is not only the hydrophobic nature of this residue that is relevant in the self-assembly of hIAPP_{22–29}.

Keywords

islet amyloid polypeptide; self-assembly; phenylalanine; pentafluorophenylalanine; Raman spectroscopy; fluorescence; π -stacking

INTRODUCTION

Human islet amyloid polypeptide (hIAPP), also known as amylin, is a 37 residue peptide that is co-secreted with insulin from pancreatic β -cells.^{1–3} In soluble form, hIAPP is believed to function as a regulator of glucose homeostasis. However, in type 2 diabetes, hIAPP forms insoluble amyloid deposits in the islets of Langerhans. These amyloid deposits can be found in over 95% of all type 2 diabetes patients and were thought to be cytotoxic to

[†]This work was supported by the Institute of General Medicine of the National Institutes of Health, grant no. 5SC3GM89624-2.

^{*}Corresponding Authors: Ruel Z. B. Desamero, Department of Chemistry, York College and The Graduate Center of the City University of New York, 94-20 Guy R. Brewer Blvd., Jamaica, New York 11451, rdesamero@york.cuny.edu; and Adam A. Profit, Department of Chemistry, York College and The Graduate Center of the City University of New York, 94-20 Guy R. Brewer Blvd., Jamaica, New York 11451, aprofit@york.cuny.edu.

β -cells.^{1,2,4,5} More recent studies have implicated soluble hIAPP oligomers rather than amyloid deposits as being the causative agents responsible for β -cell death.^{6,7} The resulting decrease in insulin secretion due to the depletion of β -cells serves to advance the pathology of the disease and is associated with hyperglycemia.

Soluble hIAPP is believed to exist in equilibrium between unstructured aggregates, helical and random coil conformations, whereas hIAPP in amyloid deposits is organized into a β -sheet secondary structure.^{8–10} The fact that the primary structure of hIAPP in both diabetic and healthy individuals is identical suggests that changes in the peptides processing or environment may be responsible for misfolding and the formation of amyloid deposits.

While the transformation of soluble hIAPP into amyloid has yet to be clarified on a molecular level, several short amyloidogenic sequences have been identified. These peptide sequences have been shown to aggregate and form β -sheet containing amyloid fibrils similar to full length hIAPP. Such amyloidogenic peptides include, but are not limited to, hIAPP_{8–20}, hIAPP_{14–19}, hIAPP_{20–29} and hIAPP_{30–37}.^{11–15} In some cases even shorter peptides derived from these sequences demonstrate the ability to form amyloid fibrils. For example the hexapeptide NFGAIL corresponding to hIAPP_{22–27} readily forms fibrils.¹⁵

Alanine scanning of hIAPP_{22–29} (NFGAILSS) seemed to reveal the importance of Phe-23 in amyloid formation.¹⁶ When Phe-23 was replaced with alanine, the peptide failed to aggregate and form amyloid fibrils. In contrast, replacement of Phe-23 with the aromatic amino acid tryptophan resulted in a peptide that not only formed fibrils but formed them at rate much higher than the corresponding phenylalanine containing peptide.¹⁷ Likewise, Marshall *et al.* have demonstrated that all the phenylalanine residues in the peptide KFFEAAKKFFE are essential for peptide aggregation.¹⁸ These findings suggest a role for aromatic amino acids and aromatic-aromatic interactions in the formation of amyloid.

Further evidence that aromatic-aromatic interactions may play a role in amyloid formation came about when Porat *et al.* demonstrated that substitution of Phe-23 with Tyr in NFGAILSS resulted in a peptide that did not undergo fibrillization.¹⁷ Since the aromatic ring of Tyr has an altered electronic structure due to the electron donating phenolic hydroxyl, its π -stacking mode may be different than that of Phe. Based on calculations and modeling studies, it was proposed that π -stacking in the Tyr containing peptide would involve a T-shaped geometry of the aromatic rings as opposed to the parallel geometry associated with Phe.^{17,19} This T-shaped stacking would not be spatially conducive to fibril formation and may explain the lack of amyloid formation. The fact that many small molecule inhibitors or reagents known to bind to amyloid fibrils are aromatic compounds and/or polyphenols also seems to suggest a potential role for aromatic-aromatic interactions.^{20–22} However, not all aromatic amyloid inhibitors may prevent aggregation through a mechanism that involves π - π stacking interactions.²³ Recent evidence indicates that the inhibition of hIAPP aggregates by (–)-epigallocatechin-3-gallate (EGCG) does not necessitate π -stacking.²⁴

In an apparent contradiction to the findings of Porat *et al.*,²⁰ Tracz and coworkers demonstrated that replacement of Phe-23 with Leu did not inhibit fibrillization.²⁵ Based on these results, it was concluded that Phe-23 is not required for amyloid formation in peptides derived from the hIAPP_{22–29} region. It has also been established that peptides derived from the amyloidogenic sequence hIAPP_{10–19} do not require Phe-15 for amyloid formation.^{25,26}

Experiments involving full length hIAPP corroborate these findings. Marek *et al.* prepared a hIAPP mutant in which all three aromatic residues in the peptide were substituted with Leu.²⁷ This triple mutant readily formed amyloid, although, at a slower rate than the wild type peptide. Thus, it was concluded that while aromatic amino acids are not an absolute

requirement for amyloid formation, they do play some role and may affect the kinetics of aggregation. These studies seem to indicate that it is the size of the amino acid side chain along with β -sheet propensity and hydrophobicity and not aromaticity that appear to be the important factors for amyloid formation. Studies conducted on human muscle acylphosphatase also support these conclusions.²⁸

While the work of Tracz *et al.* clearly demonstrates that Phe-23 is not required for fibril assembly,²⁵ it does not rule out the possibility that when Phe-23 is present in short peptides that there are π -stacking interactions between the aromatic rings. Furthermore, these findings do not readily explain the lack of fibril formation by NYGAILSS. While it is possible that the phenolic hydroxyl can form hydrogen bonds that may spatially orient the Tyr residue in a manner that does not favor fibril formation, the electronic characteristics of the ring may also play a role.

In an effort to directly interrogate the role of the electronic nature of the aromatic ring in the fibrillization process, we have synthesized a small library of peptides based on NFGAILSS in which Phe-23 contains *para*-substituted electron donating groups (EDGs) or electron withdrawing groups (EWGs) that alter the electronic structure of the π system (Fig. 1). Here we report the effects of these substitutions on the amyloidogenic properties of hIAPP₂₂₋₂₉ and provide experimental evidence of π -stacking interactions during amyloid formation.

MATERIALS AND METHODS

Fmoc-Ser(tBu)-Wang resin, Fmoc-(CH₃-O)Tyr-OH and Boc-Asn(Trt)-OH were purchased from EMD Chemicals, Inc (Gibbstown, NJ). Fmoc-4-cyano-Phe-OH was purchased from Bachem Americas, Inc (Torrance, CA). All other peptide synthesis reagents and Fmoc-protected amino acids were obtained from Advanced ChemTech (Louisville, KY). *N,N*-dimethylformamide (DMF) and dichloromethane (DCM) were purchased from Pharmco-AAPER (Brookfield, CT). All other reagents were from Sigma-Aldrich Co. (St. Louis, MO).

Peptide Synthesis

The resin-bound peptide GAILSS was synthesized manually on Fmoc-Ser(tBu)-Wang resin (substitution level 0.22 mmol/g). Fmoc removal was accomplished using 20% piperidine (v/v) in DMF. Couplings were conducted using three equivalents each of Fmoc-protected amino acid, *N,N,N',N'*-Tetramethyl-*O*-(1 *H*-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU), 1-Hydroxy-benzotriazole (HOBt) and nine equivalents of *N*-methylmorpholine (NMM) relative to resin-bound amine. Fmoc removal and amino acid couplings were monitored by Kaiser ninhydrin test. After the coupling of Fmoc-Gly-OH, the peptidyl resin was aliquoted and the appropriate phenylalanine analog coupled followed by Boc-Asn(Trt)-OH. The Boc protected derivative of Asn was incorporated at the N-terminus to avoid difficulties associated with Fmoc removal that have been previously reported for peptides based on this sequence.¹⁷

Cleavage of peptides from the resin with simultaneous removal of all side chain protecting groups was accomplished using a cocktail composed of 95% trifluoroacetic acid, 2.5% water and 2.5% triisopropylsilane (TFA/H₂O/TIS). After concentrating *in vacuo*, crude peptides were isolated by precipitation with cold diethylether. Peptides were purified by reverse-phase HPLC (Varian ProStar, Palo Alto, CA) on a Vydac C₁₈ column (22 × 250 mm) using a linear gradient of CH₃CN/H₂O containing 0.1% TFA. Column effluent was monitored at 218 nm. Appropriate fractions were pooled and lyophilized. Peptides were characterized by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Waters Corporation, Milford, MA). Peptide purity was confirmed by reverse phase analytical HPLC on a Vydac C₁₈ column (4.6 × 250 mm).

Kinetic Aggregation Assay

Lyophilized peptides were dissolved in 100% 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) to prepare concentrated stock solutions. Known molar absorptivities of Phe or its derivative, whenever available, were used to determine concentrations of representative samples. The concentrations determined using this method varied by 10 – 20 % to that based on mass hence, the final relative concentrations of each of the samples were similar. Stock solutions were sonicated immediately prior to use. Aliquots of the concentrated stocks were diluted into 10 mM Tris-HCl pH 7.5 to a final peptide concentration of 1 mM for peptide aggregation studies. The final concentration of HFIP ranged from 2 to 4%. Turbidity was measured at 405 nm at room temperature as a function of time on a Jasco V-570 spectrophotometer (Easton, MD).

Fluorescence Spectroscopy

Fluorescence kinetic measurements were made on a FluoroMax-4 Spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ) using 1 nm slits, time increments of 0.1s, and continuous stirring. The experimental conditions employed were identical to those described for the kinetic aggregation assay with the exception that the fluorescence was monitored over time instead of absorbance at 405 nm. Each sample was excited at its absorption λ_{max} and the emission detected at its emission λ_{max} . The excitation and emission wavelengths used for the fluorescence kinetics of the peptides were as follows: 257 and 287 nm, respectively, for peptide **1**; 275 and 303 nm for **2**; 275 and 300 nm for **3**; 238 and 352 nm for **4**; 275 and 300 nm for **5**; 279 and 404 nm for **6**; 260 and 373 nm for **7**; 240 and 378 nm for **8**; and 260 and 380 nm for **9**.

Raman Spectroscopy

All Raman spectra were obtained on a Jasco NRS-3100 confocal dispersive Raman spectrometer equipped with a macro-Raman measurement accessory (Easton, MD). Raman scattering was induced by a 12 mW 488 nm laser and collected on a thermoelectrically cooled CCD detector. The macro-Raman assembly permitted direct measurements of solution in quartz cuvettes. Raman spectra were taken of each peptide dissolved in HFIP, before the aggregation process was initiated, and immediately after aliquots of the concentrated stocks were diluted into 10 mM Tris-HCl. The aggregate forming peptides yielded the Raman spectra of the fibrils formed. Aggregates were collected, layered on to a quartz slide, and its spectra measured using the confocal Raman set-up. Aggregates layered on to a slide yielded stronger Raman signals free of interfering solvent signals. Samples were prepared under the same experimental conditions as described for the kinetic aggregation assay. Measurements were done at room temperature and solutions were at 1 mM.

Simulation Studies

To help analyze the Raman data, computational studies were performed to aid in the peak assignments and model the interactions occurring in the vicinity of the ring as the aggregate forms. The geometry of each model compound was optimized in the gas phase, in water and in other solvents consistent with the experimental data. We also modeled different structural motifs that mimic interactions with the ring moiety. All calculations were carried out using Gaussian 09W density functional theory (DFT) approximation implementing the Becke's three-parameter exchange functional in combination with the Lee, Yang, and Parr correlation function (or B3LYP).^{29–31} As a compromise between accuracy and applicability to large molecules, the 6–31G(d) basis set was used.^{32,33} Smaller basis sets are enough in DFT based calculations because the basic functions do not have to describe correlating orbitals. After the compounds geometries are optimized their corresponding vibrational

frequency, scaled by a factor of 0.963,^{32,34} are calculated. The electrostatic potential (ESP) plots of substituted benzene analogs were also generated using the above method and basis set. GaussView05 was used to prepare the input file and analyze the results of the calculations.³⁵

Transmission Electron Microscopy

TEM images were obtained at Brooklyn College of CUNY. Aliquots of aged peptide solutions containing aggregates were placed on carbon coated Formvar copper grids and negatively stained with 2% uranyl acetate.

RESULTS AND DISCUSSION

The role of π -stacking in the aggregation of hIAPP_{22–29} was assessed by preparing a series of peptides in which Phe-23 was replaced with phenylalanine analogs containing EDGs or EWGs at the *para* position of the aromatic ring (Fig. 1). The Phe analogs incorporated into peptides containing EDGs were tyrosine, *O*-methyltyrosine [(CH₃-O)Tyr], 4-methylphenylalanine [(CH₃)Phe] and 4-aminophenylalanine [(NH₂)Phe]. For this series of substituents, the strength of the EDGs is NH₂ > OH > CH₃O > CH₃. The phenylalanine derivatives containing EWGs utilized were pentafluorophenylalanine [(F₅)Phe], 4-fluorophenylalanine [(F)Phe], 4-nitrophenylalanine [(NO₂)Phe] and 4-cyanophenylalanine [(C≡N)Phe]. While pentafluorophenylalanine possesses a highly deactivated aromatic ring, assessing its electronic effects on peptide self-assembly is likely to be exacerbated by the corresponding increase in hydrophobicity. On this basis, we focused on the remaining EWGs to provide more accurate insights as to how the strength of the functional group affects peptide aggregation. The electron withdrawing capability of these functionalities is NO₂ > C≡N > F.

In all the above compounds, the substituents on the aromatic ring affect the electronic structure of the π system. In the case of peptide **5**, which contains a methyl group at the *para* position, electron donation to the π system of the aromatic ring can occur through hyperconjugation. The ability of these peptides to form amyloid as well as their aggregation kinetics was investigated using turbidity measurements and fluorescence spectroscopy. Results were compared to those obtained for the native peptide NFGAILSS (**1**) and the previously reported non-amyloidogenic sequence NYGAILSS (**2**).^{17,36}

The results of turbidity measurements for peptides **1** – **9** are presented in Figure 2. Turbidity has been used extensively to assess the propensity to form aggregates by the NFGAILSS peptide and its derivatives. This technique allows for the observation of peptide aggregates without the use of external dyes which potentially may perturb the ability of this short peptide to self-assemble.^{15–17,36,37} From the data it can readily be seen that peptides **2** – **5**, which all contain EDGs on Phe-23, do not exhibit any significant amount of aggregation. These peptides were found not to aggregate even after a period of one week. In most cases, these compounds contain lone pairs of electrons which are conjugated to the aromatic ring of Phe-23. The behavior of NYGAILSS (**2**) and the other EDG containing peptides are consistent with the findings of Porat *et al.* and suggest that these compounds may adopt an alternative stacking mode that precludes amyloid formation.^{17,36} These observations also lend support to the idea that aromatic-aromatic interactions do play a role in the aggregation process.

In contrast, several peptides that contain EWGs on the aromatic ring of Phe-23 were found to be significantly more amyloidogenic than the native Phe-containing sequence NFGAILSS. Peptides **6**, **7** and **9**, which contain (NO₂)Phe, (F₅)Phe and (F)Phe respectively, display the fastest aggregation kinetics. In the case of **7** and **9**, significant amounts of

amyloid have formed in less than one minute. In the case of peptide **7**, the substantial increase in aggregation rate, can be attributed to the enhanced hydrophobic nature of pentafluorophenylalanine due to the high degree of fluorination. In addition, (F₅)Phe has been shown to possess a higher β -sheet forming propensity than phenylalanine.³⁸ These observations are in keeping with results obtained utilizing model peptides based on the amyloidogenic sequence Ac-(FKFE)₂-NH₂ that incorporate (F₅)Phe.³⁹ Peptides containing pentafluorophenylalanine were found to aggregate more readily than those incorporating phenylalanine.

Peptide **9**, which contains a single fluorine atom on the aromatic ring, displays kinetics similar to **7**. However, in both cases the formation of aggregates is so rapid that, it is difficult to determine which compound self-assembles more quickly. However, it is expected that **7** should display faster kinetics as it is more hydrophobic. This is consistent with the results of ⁴⁰ hydrogelation studies on (F₅)Phe and singly fluorinated phenylalanine derivatives.

In the case of peptide **7** turbidity reaches a maximum then drops off sharply unlike in the native NFGAILSS, (NO₂)Phe and (F)Phe based peptides where there is an initial increase in turbidity that then dissipates over time due to the gradual settling of aggregates. This rapid decrease in light scattering for **7** can be accounted for by the formation of flocculent aggregates that quickly precipitate out of solution.

Peptide **8**, which contains the (C $\equiv$ N)Phe moiety, displays interesting kinetics as the peptide very slowly forms amyloid and appears to have an extended lag phase. Aggregation of **8** was found to be complete after five days. These observations are markedly different from those obtained from Marek and co-workers using full-length hIAPP.⁴¹ These researchers characterized three separate analogs of hIAPP in which each aromatic residue was substituted with (C $\equiv$ N)Phe. Each of these variants was found to display aggregation kinetics similar to the wild-type polypeptide. In contrast, peptide **8** displays much slower kinetics when compared to the NFGAILSS (**1**) control. These conflicting observations are most likely due to the differences between the two systems under investigation. While Marek *et al.* employed full-length hIAPP for their investigation,⁴¹ we are working with an eight residue peptide where a single amino acid substitution is a much greater perturbation than in a 37 residue polypeptide.

The above findings demonstrate that the nature of the functional group appended to the aromatic ring of Phe-23 can dramatically affect the amyloidogenic propensity of hIAPP₂₂₋₂₉. Electron donating substituents clearly inhibit the formation of amyloid. However, the effect of EWGs on peptide self-assembly is more complex as these groups do not inhibit amyloid formation but appear to modulate the kinetics of fibrillization. Hirata *et al.* have observed that substitution of the phenolic hydroxyl in the peptide VQIVYK with different functionalities also alters the self-assembly kinetics.⁴² Of the peptides studied, all displayed more rapid aggregation kinetics than the native tyrosine-containing sequence. However, it is difficult to ascertain whether these alterations in kinetics are due to changes in hydrophobicity or to electronic factors since only a very limited number of compounds were interrogated.

While this is not a rigorous kinetic analysis, the relative order of amyloidogenicity for those peptides capable of forming aggregates appears to be **7** **9** > **6** > **1** > **8**. No relationship between the strength of the electron withdrawing substituent and amyloidogenic propensity was observed. The order of amyloidogenicity seems to indicate that EWGs still allow for the formation of aggregates but that steric constraints associated with the geometry of the functional group on the aromatic ring may also play a role. Evidence of this can be seen in

the fact that both peptides **6** and **9** are more amyloidogenic than **1**. However, the greater amyloidogenicity of **9** over **6** is the opposite of the relative strengths of the nitro and fluorine EWGs. Peptide **8** which contains the nitrile moiety is the slowest to aggregate and contains an EWG that is stronger than fluorine but not as strong as the NO₂ group. The fact that the fluorine containing peptide more readily forms aggregates implies that there may be more favorable steric interactions between the aromatic rings allowing for more efficient packing during the self-assembly process. This is consistent with the fact that a fluorine atom will occupy substantially less space than the nitro group. Likewise, the fact that peptide **8** is significantly less amyloidogenic than **1** may be due to steric effects associated with ring stacking and the nitrile functionality.

We also observed that there was no correlation between the hydrophobic nature of peptides **6**, **8** and **9** and their ability to form amyloid. Based on analytical HPLC retention times (Table 1), the hydrophobicity of **1**, **6**, **8** and **9** are virtually identical with only very subtle differences (~ 0.5 min.) between them. Thus, the overall hydrophobic nature of these peptides remains relatively unchanged by the addition the NO₂, F and C≡N groups. Only peptide **7** is more significantly hydrophobic than **1** and the other EWG containing peptides.

Is it possible that the drastically reduced amyloidogenic potential of peptides **2** – **5** is somehow related solely to hydrophobicity and not electronic effects? Table 1 indicates that peptides **3** and **5**, which contain methoxy and methyl groups, have HPLC retention times of 26.78 and 26.58 minutes respectively. These values are virtually identical to the NFGAILSS control (26.63 minutes) and are similar to the retention times of several EWG containing peptides. Despite being equal in hydrophobicity to NFGAILSS, peptides **3** and **5** fail to form aggregates. Thus, it can be concluded that electronic effects and not differences in hydrophobicity are more likely responsible for the dramatic reduction in amyloidogenicity exhibited by the EDG containing peptides.

To further assess the role of aromatic-aromatic interactions in amyloid formation by hIAPP_{22–29}, we monitored the aggregation kinetics of peptides **1** – **9** using fluorescence spectroscopy. Unlike turbidity, fluorescence allows for the direct observation of aromatic residues during the aggregation process.

The fluorescence data for the peptides are presented in Figure 3 and are consistent with results from turbidity measurements. Peptides **2** – **5** which contain EDGs do not show any increase in fluorescence over time as they display no significant aggregation. In contrast, the fluorescence spectra of the wild type sequence NFGAILSS (**1**) along with peptides **6** – **9** all exhibit an increase in fluorescence intensity during the self-assembly process. The fact that peptides containing EDGs do not show an increase in fluorescence while the native peptide and those with EWGs do, strongly suggests that Phe-23 is involved in π - π stacking during aggregation process as the environment of the aromatic ring changes over time.

Results from turbidity and fluorescence measurements taken on peptide **1** demonstrate that fluorescence intensity is directly related to the formation of the aggregates. Figure 4 indicates that the kinetics, as measured by fluorescence intensity, correlates with the data obtained using turbidity measurements. In some cases, slight differences in lag times between the fluorescence and turbidity data can be observed. We attribute this to the fact that for turbidity measurements the aggregation process was initiated by manual agitation of aqueous peptide solutions whereas fluorescence measurements were initiated and obtained under conditions of continuous magnetic stirring. Amyloid formation has been shown to be sensitive to solvent composition, the shape and volume of spectrophotometric cells and as to whether or not samples are stirred.²⁷

To further confirm that the observed increase in fluorescence is indeed due to aggregation, the fluorescence of the Src kinase inhibitory peptide EELL(F₅)Phe was measured and compared to the N(F₅)PheGAILSS peptide (**7**).⁴³ Although both peptides contain the (F₅)Phe moiety, they yield entirely different results (data not shown). The EELL(F₅)Phe peptide, which does not form aggregates, displays no increase in fluorescence over time in comparison to **7**, which forms aggregates. The fluorescence spectrum of EELL(F₅)Phe is similar to that obtained for all non-aggregating peptides containing electron donating groups. These findings unequivocally demonstrate that the increase in fluorescence intensity observed for the aggregating peptides (**1**, **6**, **7**, **8** and **9**) is due to the self-assembly process and involves the aromatic ring.

Transmission Electron Microscopy (TEM) was employed to sample the ultrastructure of several peptide aggregates. From Figure 5 it can be seen that **1** forms amyloid fibrils as previously described in the literature.^{16,17,36} Morphologically, the ultrastructure of fibrils formed by peptides **6** and **8** resemble those of the native NFGAILSS sequence. Strikingly, the Phe to (F₅)Phe substitution found in peptide **7** results in the formation of amyloid composed of what appears to be dense helical tapes.

Raman spectra of peptide aggregates demonstrate that in addition to a perturbation in the aromatic ring modes, there is also a significant shift to lower wavenumbers in the Raman modes associated with the substituent on Phe-23. Evidence of β -sheet formation is also clear from the Raman data. In contrast to peptide **2**, all EWG containing peptides (**6** – **9**) exhibit two major bands in the 1600 – 1700 cm⁻¹ region (Fig. 6). The peak at *ca.* 1670 cm⁻¹ corresponds to the amide I band which is due to C=O stretching with a small contribution from N-H in plane bending modes of the peptide bond.^{44–46} This vibrational mode indicates the presence of β -sheet structure in the resulting fibrils. Aggregates from peptides **1**, **6**, **8** and **9** have identical peaks at 1674 cm⁻¹, while the fibrils from **7** yielded a slightly shifted peak at 1671 cm⁻¹. This is perhaps due to differences in the nature of the β -sheet structure attributable to the ring hydrogens in **7** being replaced by fluorines. The distinct morphology of **7** (Fig. 5) correlates with the slightly shifted β -sheet amide mode (1671 cm⁻¹) observed in the Raman spectra obtained from aggregates of this peptide. IR spectra of the aggregated samples revealed a peak at 1628 cm⁻¹ confirming β -sheet conformation (Fig. S1).

Another peak in the 1600 – 1700 cm⁻¹ region is a vibrational mode associated with ring motion. This ring –C=C– stretching mode (ν_{8a}) at *ca.* 1600 cm⁻¹ is sensitive to the ring substituents.^{47,48} Aggregates from peptide **1**, with the unsubstituted Phe-23, display a peak at 1609 cm⁻¹. Para substitution of Phe-23 with F and NO₂ shifts the ring-stretching mode to 1607 and 1603 cm⁻¹, respectively; while a C $\equiv$ N substitution at the same position shifts the mode to a higher wavenumber, 1614 cm⁻¹ (Fig. 6). For the aggregates formed from peptide **7**, the mode shifts to 1617 cm⁻¹ as the ring hydrogen atoms were completely replaced by fluorine. The sensitivity of this mode to the type of ring substitution supports its assignment as a ring vibrational mode.

The observed shifts in the ring and associated functional group modes due to aggregation suggest that the environment near the ring changes and that π - π stacking is evidently formed. Figure 7 illustrates the Raman spectra of peptide **6** dissolved in HFIP, *i. e.* before the aggregation process was initiated, and after the sample was allowed to aggregate under aqueous conditions (10 mM Tris, pH 7.5). It should be noted that in HFIP we are measuring the signal of the dissolved peptide while in the Tris buffer we are essentially monitoring the solid aggregates thus explaining the difference in the noise level in the spectra. Since peptide **6** aggregates rapidly, we are only able to obtain Raman spectra of the species before and after the aggregation process and not during the transition period. Nonetheless, it is clear that the ring stretching mode at *ca.* 1600 cm⁻¹ and –NO₂ modes at *ca.* 1350 cm⁻¹ shifted as

a result of fibrillization. Results indicate that the ring mode at ca. 1600 cm^{-1} downshifts by 3 cm^{-1} while the $-\text{NO}_2$ mode downshifts, by 5 cm^{-1} , upon fibrillization. In HFIP, when peptide **6** was in the non-aggregated form, the ring and $-\text{NO}_2$ modes were located at 1608 and 1356 cm^{-1} , respectively. Once mixed with Tris buffer, aggregates formed, resulting in peaks at 1605 and 1351 cm^{-1} due to the ring and $-\text{NO}_2$ modes, respectively. The observed downshifts in the ring and $-\text{NO}_2$ modes clearly indicate significant interaction, most likely π - π interaction (see below), between the ring moieties as a consequence of aggregate formation. It should also be noted that the amide mode 1674 cm^{-1} , indicative of β structure, appears as the aggregate formed.

Peptide **8**, which contains $(\text{C}\equiv\text{N})\text{Phe}$, aggregates at a much slower rate than all the other amyloid-forming peptides. This allowed for monitoring of the nitrile mode at various stages of the aggregation process. Figure 8 displays the Raman spectra in the region $2200 - 2300\text{ cm}^{-1}$ of **8** dissolved in HFIP, immediately after initiating aggregation, and during the aggregation process until its completion 5 days after. Dissolved in HFIP, the $\text{C}\equiv\text{N}$ stretching mode is located at 2251 cm^{-1} . Once mixed with Tris buffer, the $\text{C}\equiv\text{N}$ mode shifts to 2239 cm^{-1} and continued down shifting to 2234 cm^{-1} after 5 days. The narrowing of the CN resulting from the aggregate formation is also quite obvious, the full width at half height (fwht) changed from 35 cm^{-1} in HFIP to 20 cm^{-1} in the aggregated sample.

We focused on the $\text{C}\equiv\text{N}$ mode of peptide **8** for it is more intense than its ring modes and it is one of the modes that exhibited a dramatic peak narrowing. The ring modes of peptide **8** as well as the other EWG containing peptides (Fig. 7 for peptide **6**) shift to lower wavenumbers as a result of aggregation. While peak narrowing was observed for the other EWG peptides (Fig. 7 for peptide **6**) it was not as extensive as that observed for peptide **8**.

Taken together, the data indicate that for the aggregating EWG containing peptides both the ring $-\text{C}=\text{C}-$ modes as well as the modes associated with the substituent on Phe-23 shift to lower frequency and that the fwht of the substituent specific peaks becomes narrower as aggregates are formed. Since both the ring and the substituent specific modes shift in the same direction and by about the same magnitude, both modes are most likely reporting on the same structural perturbation. In contrast, the non-aggregating EDG containing peptides exhibit no peak perturbation and do not form β -structure. The associated ring and substituent modes are inherently weaker in intensity for the non-aggregating peptides because these measurements are being made in solution whereas the signals from the EWG peptide aggregates are more intense since they are being measured in the solid phase.

These results convey a good deal of information regarding the nature of the environment surrounding the aromatic ring as the peptides form aggregates. Weeks *et al.* reported that the $\text{C}\equiv\text{N}$ mode of 4-cyanophenylalanine was strongly dependent on the polarity of its environment,^{49,50} the frequency ($\nu\text{C}\equiv\text{N}$) at ca. 2250 cm^{-1} of 4-cyanophenylalanine down shifts from 2237.9 cm^{-1} in water to 2229.5 cm^{-1} in tetrahydrofuran.⁵⁰ Similarly, the $-\text{NO}_2$ stretching mode (ca. 1350 cm^{-1}) in nitrophenylalanine up shifts as the solvent increased in polarity.^{51,52} It is clear from these earlier studies that increasing hydrophobic interactions downshifts the $\text{C}\equiv\text{N}$ and $-\text{NO}_2$ modes while hydrogen bonding of these functional groups results in an upshift of the corresponding Raman frequencies. Downshifts in the $\text{C}\equiv\text{N}$ and $-\text{NO}_2$ modes that result from the formation of aggregates are consistent with the formation of a hydrophobic environment around the ring and completely rules out hydrogen bonding involving the aromatic ring or its substituent as being a requirement for aggregation.

Further analysis reveals that the Raman frequencies associated with the functional groups of the aggregated peptides are even more downshifted than when dissolved in the hydrophobic environment of HFIP. This additional downshifting suggests that not only is the

environment around the aromatic ring in the aggregates more hydrophobic than when dissolved in HFIP but that other types of interactions, most likely π - π interactions, are at work. This finding is corroborated by the observed downshift in ring mode at *ca.* 1608 cm^{-1} concomitant with the formation of aggregates. The downshift in the ring mode at *ca.* 1608 cm^{-1} is consistent with results previously observed in the aggregation of amidated aromatic acids which was attributed to π -stacking.⁵³ Attributing the downshift observed in the Raman spectrum to an interaction such as π -stacking is further supported by another study involving tryptophan indole rings which indicate that weak electrostatic interactions, in this case a cation- π interaction, leads to a downshift in the corresponding ring stretch mode.⁵⁴ Further proof that it could not be hydrogen bonding is the report that weak hydrogen bonding involving the ring up shifts the ring modes.⁵⁵

Computer simulations using Gaussian 09 not only confirmed our mode assignments but also provided evidence that the shift in the ring mode is not due to formation of hydrogen bonds or hydrophobic interactions and that it must be a result of ring-ring interactions.²⁹ We modeled two possible interactions that arise from the formation of aggregates which involves β -sheet formation. First, we modeled an interaction that can occur between the ring and a nearby aliphatic group, such as in an anti-parallel β -sheet motif. Second, we modeled a ring-ring interaction that can occur in parallel β -sheet conformation involving a parallel shifted geometry of aromatic rings. Simulation data reveal that interaction between the ring and aliphatic chain do not yield the same peak shift observed in Figure 7. The observed peak shifts can only be replicated if we place several rings in a shifted-parallel arrangement. While the presence of aliphatic ring interaction cannot be completely ruled out, it is definitely a minor component. This finding does not conclusively indicate that an antiparallel arrangement is not possible but that it is not a major feature of the aggregated structure. Structural details of these interactions are still being investigated in our laboratory.⁵⁶

The observed narrowing of the $\text{C}\equiv\text{N}$ mode is also significant and suggests that the structure becomes more rigid, confined and ordered as aggregates form. This is the opposite of what occurs in solution where peaks broaden as a result of sampling various peptide-solvent structures that remain static during the time frame of the Raman measurement.⁴⁹

It is significant that no perturbations in the aromatic ring or functional group vibrational modes were observed for NYGAILSS (2) and the other EDG containing peptides. On this basis, we conclude that no π -stacking interactions are occurring in these compounds. This excludes the alternative T-stacking geometry as being responsible for the failure of NYGAILSS to aggregate as previously proposed. Ultimately, it may be the complete absence of π - π interactions that accounts for why NYGAILSS and the other EDG containing peptides fail to aggregate.

It has been hypothesized that failure of NYGAILSS to aggregate is due to the more hydrophilic nature of tyrosine compared to phenylalanine. While HPLC retention times confirm that NYGAILSS is more hydrophilic than its phenylalanine containing counterpart (Table 1), these observations once again do not account for the lack of amyloid formation by peptides 3 and 5 which both contain EDGs. Based on retention times, peptides 3 and 5 are just as hydrophobic as NFGAILSS (1), yet they fail to aggregate. This reveals that differences in hydrophobicity alone are not sufficient to account for the failure of NYGAILSS and the other EDG containing peptides to self-assemble.

Recently, studies involving analogs of hIAPP₂₀₋₂₉ (SNNFGAILSS) have been used to probe the role of amino acid aromaticity and hydrophobicity in the formation of amyloid.⁴⁴ Modification of the N- or C-terminal of hIAPP₂₀₋₂₉ by acetylation and/or amidation

respectively affects the secondary structure of aggregates formed from this sequence. Aggregates formed from Ac-SNNFGAILSS-NH₂ contain both anti-parallel and parallel β -sheet structures. Solid-state NMR and X-ray fiber diffraction studies place the aromatic rings of Phe-23 in close enough proximity to each other to form a series of π - π stacking interactions within each cross β -sheet layer in the parallel form of the aggregates.⁵⁷ Amyloid derived from SNNFGAILSS-NH₂ is composed exclusively of anti-parallel β -sheets that do not permit π -stacking interactions involving Phe-23.⁵⁸ Like hIAPP₂₂₋₂₉, replacement of Phe-23 with Tyr in C-terminal amidated hIAPP₂₀₋₂₉ yields a peptide incapable of undergoing self-assembly.⁵⁹ However the failure of SNNYGAILSS-NH₂ to aggregate may only indicate that, in this system, decreases in hydrophobicity alone are sufficient to inhibit amyloid formation. Our data indicate that this may not be the case for hIAPP₂₂₋₂₉ as peptide analogs more hydrophobic than NYGAILSS also fail to aggregate.

While investigations utilizing SNNYGAILSS-NH₂ may provide insight into the hydrophobic and steric constraints required in amyloid formation, the electronic, steric and hydrophobicity demands of this system may not exactly match those of hIAPP₂₂₋₂₉. Evidence of this can be seen when comparing Phe-23 to Leu-23 mutations in both the hIAPP₂₀₋₂₉ and hIAPP₂₂₋₂₉ systems. The peptide SNNLGAILSS-NH₂ fails to form aggregates whereas NLGAILSS readily forms amyloid.^{25,59} These observations clearly indicate that the steric requirements of the two systems are distinctly different. Substitution of Phe-23 with Trp in the amidated hIAPP₂₀₋₂₉ sequence also produces a peptide incapable of forming aggregates while NWGAILSS has been demonstrated to self-assemble with enhanced kinetics relative to NFGAILSS.^{17,25,36,59} This again implies that the two systems have different tolerances for changes in the aromatic, steric and hydrophobic character of the amino acids incorporated into position-23. This may be a consequence of the amidated hIAPP₂₀₋₂₉ sequence only having the ability to form anti-parallel β -sheets.

Since the EDG peptide data exhibits no correlation between decreased hydrophobicity and the inhibition of amyloid, we speculate that failure of NYGAILSS and peptides **3**, **4** and **5** to aggregate may simply be explained by the Hunter-Sanders model of aromatic-aromatic interactions.^{60,61} Hunter-Sanders rules state that aromatic rings with electron donating substituents will hinder or prevent stacking interactions due to repulsion between the electron rich π -systems. Hence, the propensity of the EWG containing peptides to self-assemble may be attributed to attractive forces between the electron deficient π -systems in these compounds. ESP plots of substituted benzenes analogous to the phenylalanine derivatives utilized in this study largely support this hypothesis (Figure S2). It is clear from these ESP plots that the electron densities of the π -systems in phenol, anisole and aniline are greater than that of benzene. Conjugation of the lone pair of electrons along with the inductive effects of the oxygen atoms in phenol and anisole are also apparent. In contrast, the electrostatic potential diagrams of nitrobenzene, hexafluorobenzene, benzonitrile and fluorobenzene all illustrate the highly electron deficient nature of the π -systems in these compounds.

From the ESP maps it is not readily apparent as to why peptide **5** fails to aggregate as the electrostatic potential of toluene appears quite similar to that of benzene. Indeed, toluene is only slightly more electron rich than benzene. However, it is possible that this subtle difference between the electron densities of these two systems may represent the threshold at which repulsive forces begin to inhibit the self-assembly process. It is also possible that some steric factors may be at work.

The fact that those peptides containing EWGs aggregate at different rates, that do not correspond to the strength of the EWG, suggests that the steric demands associated with the

geometry of each functional group on the aromatic ring may be an important factor in self-assembly.

Molecular dynamics studies reveal that Tyr – Phe π -stacking interactions involve an orientation between the aromatic rings that may not be geometrically favorable to fibril formation.¹⁹ Consistent with this, we have found that NYGAILSS is able to inhibit the formation of amyloid by hIAPP_{22–29} (Figure S3). This observation further corroborates the findings of Porat *et al.* that NYGAILSS inhibits the aggregation of hIAPP_{1–37}.³⁶

Taken together, the results from turbidity, fluorescence and Raman measurements strongly indicate that aromatic-aromatic interactions involving Phe-23 contribute to the aggregation of hIAPP_{22–29}. Gazit has hypothesized that specific patterns of π -stacking may account for the highly ordered structure of amyloid fibrils.⁶² Our findings imply that hIAPP_{22–29} self-assembles into a parallel β -sheet as an antiparallel conformation would not facilitate aromatic-aromatic interactions.⁵⁷ In the parallel β -sheet conformation, aromatic rings likely stack in a parallel displaced geometry. These π -stacking interactions may exert their effects by contributing to the directional organization of the fibrillization process and are likely to arise during events in the lag phase when soluble oligomers form to provide the template for nucleation and fibril growth. Such interactions may also enhance the growth rate and contribute to the overall thermodynamic stability of the amyloid fibrils.³⁷

While our results are consistent with those of Porat and coworkers,^{17,36} they do not necessarily contradict findings that have demonstrated Phe-23 is not required for amyloid formation. In the absence of Phe-23, hIAPP_{22–29} fibrils may adopt an alternative packing mode that can accommodate different hydrophobic amino acid side chains such as Leucine. Indeed, the extent of polymorphism that can be tolerated at a particular position in a peptide may not only be due to the local requirements in hydrophobicity, sterics and electronics at that position but may also be influenced by the overall character of the peptide sequence. Some sequences may be more tolerable to substitutions than others.

Tracz *et al.* have shown that replacement of Phe-23 with Leu does not prevent fibrillization.²⁵ However, these investigators do acknowledge that aromatic residues may play a role in the kinetics of fibril formation by short peptide fragments encompassing the hIAPP_{22–29} region. In addition, Marek *et al.* have shown that substitution of all three aromatic residues in full-length hIAPP with leucine does not preclude amyloid formation, but does lead to slower aggregation kinetics.²⁷

Our findings demonstrate that substituents on the aromatic ring of Phe-23 alter the kinetics of hIAPP_{22–29} self-assembly. Incorporation of EDGs on the aromatic ring leads to peptides with a drastically reduced amyloidogenic potential. Hence it appears that enrichment of the electronic structure of the aromatic ring is not conducive to fibril self-assembly. In contrast, the presence of EWGs facilitates aggregation. We are aware that hydrogen bonding may be a contributing factor to the reduced amyloidogenic potential of those peptides containing EDGs. The phenolic hydroxyl, amino and methoxy groups can all potentially participate in hydrogen bonding interactions. It cannot be completely ruled out that some hydrogen bonding interactions may orient these functionalities in a manner that is not productive for fibril formation. However, this is unlikely since the EWGs utilized in peptides **6** – **9** are also capable of participating in hydrogen bonding interactions yet these compounds still form aggregates albeit at different rates. Furthermore, results from Raman measurements demonstrate that in the case of the aggregate forming peptides no hydrogen bonding interactions are present that involve the functional groups. Therefore, the reduced propensity to form amyloid in compounds **2** – **5** is most likely attributable to electronic factors.

It is worth noting that the present work does not draw any conclusions as to whether aromatic-aromatic interactions play a role in the self-assembly of other short peptide fragments of hIAPP or in other amyloidogenic systems. The extent of the involvement of such interactions may vary from case to case and may be limited to particular peptide sequences. However, our findings do indicate that, in the specific case of hIAPP_{22–29}, aromatic-aromatic interactions involving Phe-23 do contribute to fibrillization.

CONCLUSION

The exact contribution of aromaticity to amyloid formation is controversial and remains under debate. Evidence from several sources suggests that it is the hydrophobic character of aromatic amino acids along with their β -sheet propensity and planar geometry that are the important factors in amyloid formation and not the ability of these residues to engage in π -stacking interactions. Here we have provided experimental evidence of π -stacking in the self-assembly of hIAPP_{22–29}. Our findings demonstrate that; (i) EDGs on Phe-23 inhibit amyloid formation whereas EWGs do not; (ii) the environment of the aromatic ring becomes increasingly more hydrophobic and ordered upon aggregation; (iii) the failure of NYGAILSS to form aggregates cannot solely be due the more hydrophilic nature of Tyr and it is also not attributable to an alternate T-stacking geometry; (iv) downshifts in the --C=C-- ring mode (*ca.* 1600 cm^{-1}) of the EWG peptides provides conclusive evidence of π -stacking interactions.

Based on the results of this study and others, it is apparent that both hydrophobicity *and* aromaticity play a role in the self-assembly of hIAPP_{22–29}. While aromaticity may not be the dominant driving force in fibril formation we have shown that it does contribute to the process. This contribution may manifest itself through alterations in aggregation kinetics, morphology and thermodynamic stability of the amyloid fibrils. These observations are consistent with the polymorphism observed in hIAPP_{22–29} and many amyloid forming systems. The precise role and the extent to which aromaticity contributes to the assembly of other peptide sequences requires further investigation. Clearly, alteration of the electronic structure of the aromatic ring of Phe-23 can have a profound effect on the amyloidogenic properties of hIAPP_{22–29}. Steric demands associated with the functional group on the aromatic ring may also play a role in modulating the rate of peptide assembly.

The incorporation of functional groups on the aromatic ring of Phe-23 offers a facile method by which the rate of assembly and ultrastructure of hIAPP_{22–29} fibrils can be controlled and modulated. These results may ultimately find applications in the development of potential amyloid inhibitors or in the design of amyloid-based materials for nanotechnology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Christine Tada from York College of CUNY for providing MALDI-TOF mass spectrometric analysis and Dr. Caleen Ramsook from Brooklyn College of CUNY for her assistance in obtaining TEM images. We are grateful to Ms. Jessica Desamero for careful reading of this manuscript.

ABBREVIATIONS

hIAAP	human islet amyloid polypeptide
EDG	electron donating group

EWG	electron withdrawing group
DMF	<i>N,N</i> -dimethylformamide
DCM	dichloromethane
GBTU	<i>N,N,N',N'</i> -Tetramethyl- <i>O</i> -(1 <i>H</i> -benzotriazol-1-yl)uranium hexafluorophosphate
EGCG	(-)-epigallo-catechin-3-gallate
HOBT	1-Hydroxy-benzotriazole
NMM	<i>N</i> -methylmorpholine
TFA	trifluoroacetic acid
TIS	triisopropylsilane
HFIP	1,1,1,3,3,3-hexafluoroisopropanol
MALDI-TOF	matrix-assisted laser desorption ionization time of flight
TEM	Transmission Electron Microscopy
CCD	charge coupled-device
fwht	full width at half height
DFT	density functional theory
ESP	electrostatic potential

References

- Westermarck P, Wernstedt C, Wilander E, Hayden DW, O'Brien TD, Johnson KH. Amyloid fibrils in human insulinoma and islets of Langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells. *Proceedings National Academy of Science USA*. 1987; 84:3881–3885.
- Cooper GJS, Willis AC, Clark A, Turner RC, Sim RB, Reid KBM. Purification and characterization of a peptide from amyloid-rich pancreases of type II diabetic patients. *Proc Natl Acad Sci U S A*. 1987; 84:8628–8632. [PubMed: 3317417]
- Clark A, Cooper GJ, Lewis CE, Morris JF, Willis AC, Reid KB, Turner RC. Islet amyloid formed from diabetes-associated peptide may be pathogenic in type-2 diabetes. *Lancet*. 1987; 2:231–234. [PubMed: 2441214]
- Jaikaran E, Clark A. Islet amyloid and type 2 diabetes: from molecular misfolding to islet pathophysiology. *Biochim Biophys Acta*. 2001; 1537:179–203. [PubMed: 11731221]
- Kapurniotu A. Amyloidogenicity and cytotoxicity of islet amyloid polypeptide. *Biopolymers*. 2001; 60:438–459. [PubMed: 12209476]
- Engel MF. Membrane permeabilization by islet amyloid polypeptide. *Chem Phys Lipids*. 2009; 160:1–10. [PubMed: 19501206]
- Lin CY, Gurlo T, Kaye R, Butler AE, Haataja L, Glabe CG, Butler PC. Toxic human islet amyloid polypeptide (h-IAPP) oligomers are intracellular, and vaccination to induce anti-toxic oligomer antibodies does not prevent h-IAPP-induced beta-cell apoptosis in h-IAPP transgenic mice. *Diabetes*. 2007; 56:1324–1332. [PubMed: 17353506]
- Kaye R, Bernhagen J, Greenfield N, Sweimeh K, Brunner H, Voelter W, Kapurniotu A. Conformational transitions of islet amyloid polypeptide (IAPP) in amyloid formation in vitro. *J Mol Biol*. 1999; 287:781–796. [PubMed: 10191146]
- Abedini A, Raleigh DP. A critical assessment of the role of helical intermediates in amyloid formation by natively unfolded proteins and polypeptides. *Protein Eng Des Sel*. 2009; 22:453–459. [PubMed: 19596696]

10. Soong R, Brender JR, Macdonald PM, Ramamoorthy A. Association of Highly Compact Type II Diabetes Related Islet Amyloid Polypeptide Intermediate Species at Physiological Temperature Revealed by Diffusion NMR Spectroscopy. *J Am Chem Soc.* 2009; 131:7079–7085. [PubMed: 19405534]
11. Westermark P, Engstrom U, Johnson KH, Westermark GT, Betsholtz C. Islet amyloid polypeptide: Pinpointing amino acid residues linked to amyloid fibril formation. *Proceedings National Academy of Science USA.* 1990; 87:5036–5040.
12. Jaikaran ET, Higham CE, Serpell LC, Zurdo J, Gross M, Clark A, Fraser PE. Identification of a novel human islet amyloid polypeptide β -sheet domain and factors influencing fibrillogenesis. *J Mol Biol.* 2001; 308:515–525. [PubMed: 11327784]
13. Nilsson MR, Raleigh DP. Analysis of amylin cleavage products provides new insights into the amyloidogenic region of human amylin. *J Mol Biol.* 1999; 294:1375–1385. [PubMed: 10600392]
14. Goldsbury C, Goldie K, Pellaud J, Seeling J, Frey P, Muller SA, Kistler J, Cooper GJS, Aepli U. Amyloid fibril formation from full-length and fragments of amylin. *J Struct Biol.* 2000; 130:352–362. [PubMed: 10940238]
15. Tenidis K, Waldner M, Bernhagen J, Fischle W, Bergmann M, Weber M, Merkle ML, Voelter W, Brunner H, Kapurniotu A. Identification of a penta- and hexapeptide of islet amyloid polypeptide (IAPP) with amyloidogenic and cytotoxic properties. *J Mol Biol.* 2000; 295:1055–1071. [PubMed: 10656810]
16. Azriel R, Gazit E. Analysis of the minimal amyloid-forming fragment of the islet amyloid polypeptide. *J Biol Chem.* 2001; 276:34156–34161. [PubMed: 11445568]
17. Porat Y, Stepensky A, Naider F, Gazit E. Completely different amyloidogenic potential of nearly identical peptide fragments. *Biopolymers.* 2003; 69:161–164. [PubMed: 12767119]
18. Marshall KE, Morris KL, Charlton D, O'Reilly N, Lewis L, Walden H, Serpell LC. Hydrophobic, aromatic, and electrostatic interactions play a central role in amyloid fibril formation and stability. *Biochemistry.* 2011; 50:2061–2071. [PubMed: 21288003]
19. Chelli R, Gervasio FL, Procacci P, Schettino V. Stacking and T-shape competition in aromatic-aromatic amino acid interactions. *J Am Chem Soc.* 2002; 124:6133–6143. [PubMed: 12022848]
20. Porat Y, Abramowitz A, Gazit E. Inhibition of amyloid fibril formation by polyphenols: Structural similarity and aromatic interactions as a common inhibition mechanism. *Chem Biol Drug Des.* 2006; 67:27–37. [PubMed: 16492146]
21. Meng F, Abedini A, Plesner A, Verchere CB, Raleigh DP. The flavanol (–) epigallocatechin 3-gallate inhibits amyloid formation by islet amyloid polypeptide, disaggregates amyloid fibrils and protects cultured cells against IAPP-induced toxicity. *Biochemistry.* 2010; 49:8127–8133. [PubMed: 20707388]
22. Levy-Saskin M, Shreberk M, Daniel Y, Gazit E. Targeting insulin amyloid assembly by small aromatic molecules. *Islets.* 2009; 1:210–215. [PubMed: 21099274]
23. Convertino M, Pellarin R, Catto M, Carotti A, Caflisch A. 9, 10-Anthraquinone hinders β -aggregation: How does a small molecule interfere with A β -peptide amyloid fibrillation? *Protein Sci.* 2009; 18:792–800. [PubMed: 19309732]
24. Cao P, Raleigh DP. Analysis of the Inhibition and Remodeling of Islet Amyloid Polypeptide Fibers by Flavanols. *Biochemistry.* 2012; 51:2670–2683. [PubMed: 22409724]
25. Tracz SM, Abedini A, Driscoll M, Raleigh DP. Role of aromatic interactions in amyloid formation by peptides derived from human amylin. *Biochemistry.* 2004; 43(50):15901–15908. [PubMed: 15595845]
26. Milardi D, Sciacca MFM, Pappalardo M, Grasso DM, La Rosa C. The role of aromatic side-chains in amyloid growth and membrane interaction of the islet amyloid polypeptide fragment LANFLVH. *Eur Biophys J.* 2011; 40:1–12. [PubMed: 20809197]
27. Marek P, Abedini A, Song BB, Kanungo M, Johnson ME, Gupta R, Zaman W, Wong SS, Raleigh DP. Aromatic interactions are not required for amyloid fibril formation by islet amyloid polypeptide but do influence the rate of fibril formation and fibril morphology. *Biochemistry.* 2007; 46:3255–3261. [PubMed: 17311418]

28. Bemporad F, Taddei N, Stefani M, Chiti F. Assessing the role of aromatic residues in the amyloid aggregation of human muscle acylphosphatase. *Protein Sci.* 2006; 15:862–870. [PubMed: 16600970]
29. Frisch, M.; Trucks, GW.; Schlegel, HB.; Scuseria, GE.; Robb, MA.; Cheeseman, JR.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, GA.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, HP.; Izmaylov, AF.; Bloino, J.; Zheng, G.; Sonnenberg, JL.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, JA., Jr; Peralta, JE.; Ogliaro, F.; Bearpark, M.; Heyd, JJ.; Brothers, E.; Kudin, KN.; Staroverov, VN.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A.; Burant, JC.; Iyengar, SS.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, JM.; Klene, M.; Knox, JE.; Cross, JB.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, RE.; Yazyev, O.; Austin, AJ.; Cammi, R.; Pomelli, C.; Ochterski, JW.; Martin, RL.; Morokuma, K.; Zakrzewski, VG.; Voth, GA.; Salvador, P.; Dannenberg, JJ.; Dapprich, S.; Daniels, AD.; Farkas, Ö.; Foresman, JB.; Ortiz, JV.; Cioslowski, J.; Fox, DJ. Gaussian 09, Revision A.1. Gaussian Inc; Wallingford, CT: 2009.
30. Becke AD. Density-functional thermochemistry. III. The role of exact exchange. *J Chem Phys.* 1993; 98:5648–5652.
31. Lee C, Yang W, Parr RG. Developemnt of the Colle-Salvetti correlation-energy formula into a functional of the electron density. *Phys Rev B.* 1988; 41:785–789.
32. Rauhut G, Pulay P. Transferable Scaling Factors for Density Functional Derived Vibrational Force Fields. *J Phys Chem.* 1995; 99:3093–3100.
33. Stephens PJ, Devlin FJ, Chabalowski CF, Frisch MJ. Ab initio Calculations of vibrational absorption and circular dichroism spectra using density functional force fields. *J Phys Chem.* 1994; 98:11623–11627.
34. Pulay P, Fogarasi G, Pongor G, Boggs JE, Vargha A. Combination of theoretical ab initio and experimental information to obtain reliable harmonic force constants. Scaled quantum mechanical (QM) force fields for glyoxal, acrolein, butadiene, formaldehyde, and ethylene. *J Am Chem Soc.* 1983; 105:7037–7047.
35. Dennington, R.; Keith, T.; Millam, J. GaussView, Version 5. Semichem, Inc., Shawnee Mission; KS: 2009.
36. Porat Y, Mazor Y, Efrat S, Gazit E. Inhibition of islet amyloid polypeptide fibril formation: A potential role for heteroaromatic interactions. *Biochemistry.* 2004; 43:14454–14462. [PubMed: 15533050]
37. Ashburn TT, Lansbury PT. Interspecies sequence variations affect kinetics and thermodynamics of amyloid formation: peptide models of pancreatic amyloid. *J Am Chem Soc.* 1993; 115:11012–11013.
38. Woll MG, Hadley EB, Mecozzi S, Gellman SH. Stabilizing and destabilizing effects of phenylalanine --> F5-phenylalanine mutations on the folding of a small protein. *J Am Chem Soc.* 2006; 128:15932–15933. [PubMed: 17165695]
39. Bowerman CJ, Liyanage W, Federation AJ, Nilsson BL. Tuning β -sheet peptide self-assembly and hydrogelation behavior by modification of sequence hydrophobicity and aromaticity. *Biomacromolecules.* 2011; 12:2735–2745. [PubMed: 21568346]
40. Ryan DM, Doran TM, Anderson SB, Nilsson BL. Effect of C-terminal modification on the self-assembly and hydrogelation of fluorinated Fmoc-Phe derivatives. *Langmuir.* 2011; 27:4029–4039. [PubMed: 21401045]
41. Marek P, Mukherjee S, Zanni MT, Raleigh DP. Residue-Specific, Real-Time Characterization of Lag-Phase Species and Fibril Growth During Amyloid Formation: A Combined Fluorescence and IR Study of p-Cyanophenylalanine Analogs of Islet Amyloid Polypeptide. *J Mol Biol.* 2010; 400:878–888. [PubMed: 20630475]
42. Hirata A, Sugimoto K, Konnob T, Morii T. Amyloid-forming propensity of the hydrophobic non-natural amino acid on the fibril-forming core peptide of human tau. *Bioorg Med Chem Lett.* 2007; 17:2971–2974. [PubMed: 17416523]
43. Profit AA, Lee TR, Niu J, Lawrence DS. Molecular Rulers: An Assessment of Distance and Spatial Relationships of Src Tyrosine Kinase SH2 and Active Site Regions. *J Biol Chem.* 2001; 276:9446–9451. [PubMed: 11118446]

44. Colthup, NB.; Daly, LH.; Wiberley, SE. Introduction to Infrared and Raman Spectroscopy. San Diego: Academic Press; 1990.
45. Lin-Vien, D.; Colthup, NB.; Fateley, WG.; Grasselli, JG. The Handbook of Infrared and Raman Characteristic Frequencies of Organic Molecules. San Diego: Academic Press; 1991.
46. Deng H, Schindler JF, Berst KB, Plapp BV, Callender R. A Raman Spectroscopic Characterization of Bonding in the Complex of Horse Liver Alcohol Dehydrogenase with NADH and N-Cyclohexylformamide. *Biochemistry*. 1998; 37:14267–14278. [PubMed: 9760265]
47. Fodor SPA, Copeland RA, Grygon CA, Spiro TG. Deep-Ultraviolet Raman Excitation Profiles and Vibronic Scattering Mechanisms of Phenylalanine, Tyrosine, and Tryptophan. *J Am Chem Soc*. 1989; 111:5509–5518.
48. Desamero RZB, Kang J, Dol C, Chinwong J, Walterd K, Sivarajah T, Profit AA. Spectroscopic characterization of the SH-2 and active site-directed peptide sequences of a bivalent Src kinase inhibitor. *Appl Spectrosc*. 2009; 63:767–774. [PubMed: 19589214]
49. Getahun Z, Huang C-Y, Wang T, De Leon B, DeGrado WF, Gai F. Using Nitrile-Derivatized Amino Acids as Infrared Probes of Local Environment. *J Am Chem Soc*. 2003; 125:405–411. [PubMed: 12517152]
50. Weeks CL, Polishchuk A, Getahun Z, DeGrado WF, Spiro TG. Investigation of an unnatural amino acid for use as a resonance Raman probe: detection limits and solvent and temperature dependence of the $\nu_{\text{C}\equiv\text{N}}$ band of 4-cyanophenylalanine. *J Raman Spectrosc*. 2008; 39:1606–1613. [PubMed: 20648227]
51. Schmid ED, Moschallski M, Peticolas WL. Solvent Effects on the Absorption and Raman Spectra of Aromatic Nitro Compounds. 1. Calculation of Preresonance Raman Intensities. *J Phys Chem*. 1986; 90:2340–2346.
52. Smith EE, Linderman BY, Luskin AC, Brewer SH. Probing Local Environments with the Infrared Probe: L-4-Nitrophenylalanine. *J Phys Chem B*. 2011; 115(10):2380–2385. [PubMed: 21344930]
53. Desamero RZB, Cheng H, Cahill S, Girvin M, Deng H, Callender R, Rath P, Variano B, Smart JE. Physical properties of compounds promoting oral delivery of macromolecular drugs. *Biopolymers (Biospectroscopy)*. 2002; 67:26–40. [PubMed: 11842411]
54. Takeuchi H. UV Raman Markers for Structural Analysis of Aromatic Side Chains in Proteins. *Anal Sci*. 2011; 27:1077–1086. [PubMed: 22076333]
55. Barnes AJ. Blue-shifting hydrogen bonds - are they proper or improper? *J Mol Struct*. 2004; 704:3–9.
56. Desamero, RZB.; Mojica, E-R.; Vedad, J.; Profit, AA. Structural details of the π -stacking interactions in the self-assembly of hIAPP. p. 22-29.(in preparation)
57. Madine J, Jack E, Stockley PG, Radford SE, Serpell LC, Middleton DA. Structural insights into the polymorphism of amyloid-like fibrils formed by region 20–29 of amylin revealed by solid-state NMR and X-ray fiber diffraction. *J Am Chem Soc*. 2008; 130:14990–15001. [PubMed: 18937465]
58. Nielsen JT, Bjerring M, Jeppesen MD, Pedersen RO, Pedersen JM, Hein KL, Vosegaard T, Skrydstrup T, Otzen DE, Nielsen NC. Unique identification of supramolecular structures in amyloid fibrils by solid-state NMR spectroscopy. *Angew Chem Int Ed*. 2009; 48:2118–2121.
59. Doran TM, Kamens AJ, Byrnes NK, Nilsson BL. Role of amino acid hydrophobicity, aromaticity, and molecular volume on IAPP(20–29) amyloid self-assembly. *Proteins*. 2012; 80:1053–1065. [PubMed: 22253015]
60. Hunter CA, Sanders JKM. The nature of π - π interactions. *J Am Chem Soc*. 1990; 112:5525–5534.
61. Cockroft SL, Hunter CA, Lawson KR, Perkins J, Urch CJ. Electrostatic control of aromatic stacking interactions. *J Am Chem Soc*. 2005; 127:8594–8595. [PubMed: 15954755]
62. Gazit E. A possible role for π -stacking in the self-assembly of amyloid fibrils. *FASEB J*. 2002; 16:77–83. [PubMed: 11772939]

Figure 1.

The structure of peptides synthesized based on hIAPP₂₂₋₂₉ containing electron withdrawing groups (EWGs) or electron donating groups (EDGs) on the aromatic ring of Phe-23.

Figure 2.

Turbidity (optical density reading at 405 nm) plots for the standard amyloidogenic peptide fragment (**1**) and analogues containing phenylalanine derivatives (**2 – 9**). Peptides were incubated in cuvettes containing 1 mM peptide in 10 mM Tris-HCl (pH 7.5) and 2 – 4% HFIP.

Figure 3.

Time dependent fluorescence emission spectra of peptides **1** – **9**. Peptide samples were excited at the absorbance λ_{max} and the changes in fluorescence emission were monitored at the corresponding emission λ_{max} . Samples were incubated in cuvettes containing 1 mM peptide in 10 mM Tris-HCl (pH 7.5) and 2 – 4% HFIP.

Figure 4.

Time dependent fluorescence emission measurements (markers only, y-axis on right) of peptide **1** overlaid on top of its turbidity plot (solid line, y-axis on left) probed at 405 nm. Sample fluorescence emission at 282 nm (emission λ_{max} of peptide **1**) was monitored, as a function of time, while excited at 260 nm (absorption λ_{max} of peptide **1**). Samples were incubated in cuvettes containing 1 mM peptide in 10 mM Tris-HCl (pH 7.5) and 2 – 4% HFIP.

Figure 5. TEM images of representative peptide aggregates: A) peptide **1**; B) peptide **6**; C) peptide **7**; and D) peptide **8**. Aliquots of aged peptide solutions containing aggregates were deposited on carbon coated Formavar copper grids and stained with uranyl acetate.

Figure 6.

Raman spectra of the peptides with amyloidogenic fragment. The peak at *ca.* 1670 cm⁻¹, common to all peptides that aggregated (1, 6, 7, 8, 9), indicates β -sheet formation. The peptides with non-amyloidogenic fragment have Raman spectra similar to that of peptide 2. Also evident are the peaks at *ca.* 1600 cm⁻¹, a Phe-ring mode, which is clearly influenced by the type of derivative on the phenylalanine ring. The ring modes for peptides 7 and 9 are very weak hence their spectra were multiplied by a factor of 3.

Figure 7.

Raman spectra of peptide **6** when dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (—) and its aggregates (- - -). Formation of aggregates not only lead to the appearance of the peak at the 1674 cm⁻¹, indicative of β -sheet formation, but also the perturbation of the Phe-ring (*ca.* 1600 cm⁻¹) and -NO₂ (*ca.* 1350 cm⁻¹) modes.

Figure 8.

Raman spectra of peptide **8** when dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (—), one day after the aggregation process was initiated (- -) and 6 days after when aggregation was complete (- · -). To initiate aggregation, samples dissolve in 1,1,1,3,3,3-hexafluoroisopropanol are added to a 10 mM Tris-HCl (pH 7.5) and 2–4% HFIP. Since peptide **8** aggregates the slowest, it was possible to obtain its Raman spectra at the start and end of the aggregation process. As peptide **8** aggregates, the $\text{--C}\equiv\text{N}$ mode shifts towards a more hydrophobic environment. Curve fits are overlaid on top of the raw spectra.

Table I

Analytical HPLC retention times of synthesized peptides. Retention times were obtained utilizing a Vydac C₁₈ column and a linear gradient of CH₃CN/H₂O (0 – 100% CH₃CN over 40 min.)

Peptide	Sequence	Retention Time (min.)
1	NFGAILSS	26.63
2	NYGAILSS	24.99
3	N(CH ₃ O)PheGAILSS	26.78
4	N(NH ₂)PheGAILSS	23.24
5	N(CH ₃)PheGAILSS	26.58
6	N(NO ₂)PheGAILSS	27.10
7	N(F ₅)PheGAILSS	28.41
8	N(C≡N)PheGAILSS	26.44
9	N(F)PheGAILSS	27.10