Identification of transmissible proteotoxic oligomer-like fibrils that expand conformational diversity of amyloid assemblies

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Protein misfolding and amyloid deposition are associated with numerous diseases. The detailed characterization of the proteospecies mediating cell death remains elusive owing to the (supra)structural polymorphism and transient nature of the assemblies populating the amyloid pathway. Here we describe the identification of toxic amyloid fibrils with oligomer-like characteristics, which were assembled from an islet amyloid polypeptide (IAPP) derivative containing an Asn-to-Gln substitution (N21Q). While N21Q filaments share structural properties with cytocompatible fibrils, including the 4.7 Å inter-strand distance and β-sheet-rich conformation, they concurrently display characteristics of oligomers, such as low thioflavin-T binding, high surface hydrophobicity and recognition by the A11 antibody, leading to high potency to disrupt membranes and cause cellular dysfunction. The toxic oligomer-like conformation of N21Q fibrils, which is preserved upon elongation, is transmissible to naïve IAPP. These stable fibrils expanding the conformational diversity of amyloid assemblies represent an opportunity to elucidate the structural basis of amyloid disorders.
Misfolding and aggregation of proteins into ordered cross-β amyloid assemblies are associated with over fifty human diseases, including the Alzheimer’s disease (AD), type II diabetes mellitus and systemic amyloidosis. Amyloid fibril formation and tissue deposition are initiated by the self-recognition of (partially) unfolded proteins and implicate an infinite array of off- and on-pathway prefibrillar intermediates. Compelling experimental and clinical evidence have indicated that the most cytotoxic proteospecies of the amyloid cascade are the oligomeric intermediates and that fibrils are generally non-toxic. For instance, the amyloid-β (Aβ) peptide impairs synaptic plasticity in absence of fibrils, through the formation of oligomeric intermediates, while the influx of Ca2+ across neuronal membranes correlates closely with binding of Aβ oligomers to the plasma membrane. Cytotoxicity, including persistent ability to disrupt cellular membranes, has been reported for oligomers assembled from numerous amyloidogenic proteins, including the islet amyloid polypeptide (IAPP), α-synuclein and transthyretin. Strikingly, cytotoxic oligomers have been generated from proteins that do not spontaneously self-assemble and that are not associated with amyloid disorders, suggesting that prefibrillar aggregates share physicochemical and/or structural properties mediating toxicity.

Although these studies have supported the oligomer hypothesis, i.e. that soluble aggregates are the main causative agents of cell death, prefibrillar assemblies are transient and heterogeneous, leading to major challenges for elucidating their structures and mechanisms of toxicity. Besides, thorough studies have periodically reported the cytotoxicity of well-defined fibrils, including those assembled from IAPP, huntington protein, β2-microglobulin and α-synuclein. Discrepancies among the relationships between toxicity and (supra)molecular architecture of amyloid proteospecies originate from their high structural diversity at the atomic and mesoscopic levels and/or from their dynamic nature. A given protein can misfold and aggregate into multiple populations of oligomers and fibrils, and each of these quaternary conformers can display divergent cytotoxicity. For instance, Aβ peptide oligomerization can lead either to stable non-toxic dimers/trimers, or to pentamers that are very toxic to neuronal cells. By varying self-assembly conditions, two populations of stabilized α-synuclein oligomers with similar sizes and morphologies were obtained; one type being benign to cells and the other population avidly perforating lipid bilayers. Under different temperatures, the huntington protein with extended polyglutamine misfolds into conformational distinct fibrils, through the formation of β-strands and its side chain projects outward of Ca2+.

Results

Shifting the toxicity of amyloid fibrils by an N-to-Q substitution. IAPP is known for its propensity to self-assemble into cross-β amyloid structures, which are associated with the pathogenesis of type II diabetes. As reported by solid-state NMR, each monomeric unit of the amyloid fibrils are composed of two β-strands connected by a disordered loop involving positions H18 to L27. Recent cryo-EM studies have revealed conformational polymorphism of IAPP fibrils, with monomers composed of three β-sheets of variable lengths connected by disordered loops. Notwithstanding the models of IAPP fibrils, residue N21, which is critical for self-assembly and toxicity, is located in a disordered loop joining β-strands and its side chain projects outward into protofilament interface, likely participating in protofilament packing (Fig. 1a). As reported for other amyloidogenic proteins, fibrils assembled from IAPP are poorly cytotoxic, whereas oligomers and prefibrillar species damage cells. Strikingly, the substitution of Asn at position 21 by a Gln (N21Q), which corresponds to displacement of the amide group away from the peptide backbone by approximately 1.5 Å, led to the formation of fibrils that are highly toxic to β-pancreatic cells (Fig. 1). Fibrils were prepared by incubating the monomerized peptide in 20 mM Tris-HCl, pH 7.4 for 48 h at 150 μM under quiescent conditions at room temperature. Under these conditions, N21Q derivative self-assembled into protofilotypical twisted filaments (Fig. 1b), showing a concentration-dependent cytotoxicity. Viability measured by the metabolic activity of pancreatic INS-1E cells, a cell line commonly used to study IAPP, decreased below 20% of control at 25 μM N21Q, whereas IAPP fibrils showed no toxicity (Fig. 1c). IAPP-mediated toxicity is associated with numerous cellular events, including oxidative stress, mitochondrial dysfunction, plasma membrane disruption and apoptosis. Considering that caspase-3 activation is an upstream event to viability measured by the metabolic activity, cells were incubated for 1 to 6 h with fibrils before measuring caspase-3 activity. N21Q fibrils induced a significant increase of caspase-3 activation, with roughly a 3-fold increase compared to IAPP fibrils after 150 min (Fig. 1d). Cytotoxicity of N21Q fibrils was further evaluated using the Live/Dead assay. INS-1E β-cells treated with IAPP fibrils showed a similar green/red ratio to the cells treated with the control (Fig. 1e, Supplementary Fig. 1). The red fluorescence correlates with loss of plasma membrane integrity and the green fluorescence is associated with intracellular esterase activity. In sharp contrast, cells treated with N21Q fibrils were all red-positive, indicative of perturbation of the plasma membrane.

We investigated if the unexpected toxicity of N21Q fibrils could result from the presence of soluble oligomers associated with an incomplete fibrillization and/or dissociation of oligomers from the fibrils. First, peptides were assembled for up to 5 days and cytotoxicity of the aggregation mixtures were periodically evaluated. Prolonging self-assembly did not modify the divergent toxicity observed between WT and N21Q fibrils (Supplementary Fig. 2). Secondly, fibrils were isolated by centrifugation to remove...
INS-1E cells were incubated for 24 h with pre-assembled data represent mean ± S.D. of at least three independent experiments performed in triplicate. the distribution of live (green) and dead (red) INS-1E cells after treatment with 50 freshly dissolved monomerized peptides incubated under quiescent conditions for 48 h at a concentration of 150 β-spectroscopy. According to the thermal unfolding of the fiber morphology of the N21Q toxic fibrils is observed by transmission electron microscopy (TEM) and cytotoxicity was evaluated. N21Q fibrils isolated in the pellet, with or without sonication, remained toxic to pancreatic cells (Supplementary Fig. 3). Thirdly, by amyloid fibrils can disassemble and release oligomers, we evaluated the thermal unfolding of the fibrils by circular dichroism (CD) spectroscopy. According to the β-sheet signal at 218 nm, IAPP and N21Q fibrils in presence of 4 M urea showed a similar thermal unfolding midpoint ($T_m$); 58.1 ± 0.2 °C for IAPP and 61.3 ± 0.5 °C for N21Q (Supplementary Fig. 4). Fourthly, stability against proteolysis was assessed by subjecting the assemblies to 120 U/mL of proteinase K (PK) for 1 h and digestion was evaluated by MALDI-TOF mass spectrometry. N21Q and WT fibrils showed an equivalent stability against PK proteolysis, suggesting a similar degree of fibril compactness and undetectable dissociation of soluble species (Supplementary Fig. 5). These observations indicate that the cytotoxicity of N21Q assemblies is induced by defined fibrils and is not associated with presence of soluble oligomers or fibril dissociation.

Supramolecular characterization of N21Q toxic fibrils. To obtain insights into the molecular basis of the discrepancy in toxicity between IAPP and N21Q fibrils, their (supra)molecular characteristics were compared. As observed by TEM and atomic force microscopy (AFM), both peptides assembled at 150 μM for 48 h formed two major distinctive fibril morphologies: flat ribbons and twisted filaments with varied pitches (Fig. 2a, b). Morphological heterogeneity of IAPP fibrils prepared in vitro is well-known. Quantification of over 3000 fibrils using AFM images revealed that the prevalence of twisted fibrils in the N21Q samples was significantly higher (Fig. 2c). N21Q fibrils were somewhat shorter, with an average length of 1.08 ± 0.17 μm, compared to 1.42 ± 0.36 μm (Fig. 2c, Supplementary Fig. 6). The average height of N21Q fibrils was approximatively half of the diameter measured for IAPP fibrils, i.e. 3.12 ± 1.24 nm vs 5.98 ± 2.62 nm (3.07 vs 6.11 nm, considering the median), suggesting that the number of protofilaments composing the fibrils and/or their compactness diverge. Measurement of Young’s modulus revealed that N21Q fibrils displayed lower stiffness. Structural organization was probed by attenuated total reflection Fourier transform infrared (ATR-FTIR) and far-UV CD spectroscopy. The amide I region of IAPP and N21Q spectra (1700−1600 cm−1) were practically identical with three characteristic amide peaks (Fig. 2d, Supplementary Fig. 7). The amide I region of IAPP and N21Q spectra (1700−1600 cm−1) were practically identical with three characteristic amide peaks. The amide I region of IAPP and N21Q spectra (1700−1600 cm−1) were practically identical with three characteristic amide peaks. The amide I region of IAPP and N21Q spectra (1700−1600 cm−1) were practically identical with three characteristic amide peaks.
The amyloid architecture was probed by measuring the fluorescence of thioflavin T (ThT), whose emission increases sharply upon binding to cross-β quaternary structure. Whereas fibrils assembled from IAPP presented a sharp ThT signal, N21Q fibrils led to a modest increase of fluorescence, suggestive of less defined ThT binding sites. Although low-ThT binding is generally ascribed to prefibrillar assemblies, this characteristic has been reported for fibrils assembled from different peptides, including pufferfish IAPP and the Japanese mutant of Aβ (ΔE22). Accessibility of hydrophobic clusters was evaluated using the 8-anilino-1-naphtalenesulfonic acid (ANS) that binds to solvent-exposed hydrophobic domains, leading to an increase and blue shift of fluorescence emission. In comparison to WT fibrils, the N21Q fibrils led to a strong increase of ANS fluorescence, indicative of higher surface hydrophobicity. Powder X-ray diffraction (XRD) revealed a diffraction pattern characterized by two sharp peaks and Bragg reflections corresponding to 4.7 Å and 8.9 Å periodic spacing were measured.
spawning. While the 8.9 Å inter-sheet distance is rather short for amyloids, which is typically around 10 Å, the inter-sheet spacing is shorter in dry interface 3-5, as for powder XRD. Overall, these results indicate that IAPP and N21Q fibrils exhibit a similar structural arrangement, although dissimilarities were observed in the mesoscopic morphology and in the ability to interact with dyes.

SS-NMR was used to evaluate changes in the microenvironment of specific residues induced by the N-to-Q substitution that could reveal the molecular fingerprints of toxicity. Peptides, with uniformly labelled 13C and 15N residues at positions A13, F23, and V32, were assembled at 375 µM for 48 h and fibrils were recovered by centrifugation and lyophilized. Fibrils conserved their distinctive biophysical properties and cytotoxicity (Supplementary Fig. 7). Labelled residues were selected to differentiate the segments according to SS-NMR and cryo-EM models of fibrils (Fig. 3a). Cross-polarization (CP) dipolar assisted rotational resonance (DARR) spectra of IAPP (black) and N21Q (green) fibrils were overlaid (Fig. 3b). Assignments of residue chemical shifts were established based on the connectivity pattern and the typical ranges for each carbon. Secondary 13C chemical shifts (Δδ) for Ca, Cβ, and CO of labelled residues were calculated as Δδ = δRes – δQC (Supplementary Fig. 8). Chemical shifts indicated that the cross-β-sheet structure is retained in both fibril preparations, as indicated by Δδ of Cβ being positive, and CO and Ca being negative. Spectra obtained from both fibrils were similar in the aliphatic and aromatic regions, with two minor changes regarding A13 Cβ and V32 Cτ. Pronounced changes were observed in the carbonyl region of the spectra. N21Q fibrils carbonyl resonances were deshielded compared to IAPP, indicative of either a change in structure or local environment (Fig. 3c, Supplementary Fig. 8). F23 exhibited the largest change in CO shift, from 16.9 ppm in IAPP to 173.4 ppm in N21Q fibrils, leading to alteration of F23 CO secondary shift, from strongly negative (−3.3 ppm) in IAPP to 0.3 ppm in N21Q. Considering the lack of changes in the Ca and Cβ secondary shifts of F23, this is not likely related to a major alteration in secondary structure, but may instead be a result of a local increase in hydrophobicity 3-5 and/or from altered hydrogen-bond interactions 44. Change in V32 CO secondary shifts, from negative value towards zero, suggest the possibility that a similar effect was occurring around the C-terminal region.

Kinetics of self-assembly and time-resolved cytotoxicity. The observed distinct biological properties, i.e. cyto compatible vs toxic, from fibrils assembled from closely related peptides, i.e. IAPP vs N21Q, could arise from divergent aggregation pathways. Accordingly, we evaluated the kinetics of self-assembly by ThT and ANS fluorescence, as well as using an assay based on fluor escein arsenical hairpin (FIAsH). Although a weak ThT signal was measured for N21Q, typical sigmoidal traces characterized with three distinct phases (lag, elongation, saturation) were observed, suggestive of a nucleation-dependent polymerization (Fig. 4a, Supplementary Fig. 9). Lag-times of 9.9 ± 2.0 h and 7.0 ± 1.5 h were extracted respectively from IAPP and N21Q ThT kinetics, indicating that the N21Q substitution hastens nucleation. Considering the low ThT-signal of N21Q, the fluorogenic probe FIAsH was used. FIAsH, whose fluorescence quantum yield dramatically increases upon its binding to a tetracycstic tag 45, has been recently used to detect IAPP self-assembly through the formation of a non-contiguous tetra-Cys binding motif involving the N-terminal C2 and C7 46. This method is well-suited to detect ThT-negative fibrils, as those assembled from N21Q. Self-assembly monitored by FIAsH and performed under reducing conditions, revealed a typical sigmoidal growth with lag-time of 7.3 ± 1.4 h and 2.6 ± 1.9 h for IAPP and N21Q, respectively (Fig. 4b, Supplementary Fig. 9). Kinetics of aggregation monitored by ANS fluorescence confirmed that the N21Q substitution accelerates nucleation (Fig. 4c). Gradual augmentation of the molar ratio of N21Q into IAPP self-assembly (from 1 to 10%) progressively hastened nucleation and led to reduced final ThT fluorescence and increased final ANS fluorescence, while the opposite effect was observed for the reverse experiment, i.e. IAPP into N21Q assembly reaction (Supplementary Fig. 10). These observations suggest that IAPP and N21Q monomers co-assemble, leading to fibrils that progressively acquire the characteristics of their co-assembling counterpart.

Next, the toxicity of the proteospecies assembled along the aggregation pathway was evaluated. When freshly dissolved monomerized peptides (0 h) were immediately applied to INS-1E cells, a concentration-dependant toxicity was observed for both peptides, albeit N21Q was significantly more toxic (Supplementary Fig. 11). The higher toxicity of monomeric N21Q correlated with a hastened capacity to induce caspase-3 activation and to perturb synaptic large unilamellar vesicles (LUVs) composed of phosphocholine/phosphoglycerol (DOPC/DOPG) (Supplementary Fig. 11). For time-resolved toxicity, peptides were incubated at 150 µM and after different incubation periods, the aggregation mixture was characterized by CD spectroscopy, ThT and ANS fluorescence, and TEM, and the toxicity of the proteospecies was evaluated by monitoring the viability of INS-1E cells after 5 h incubation with 50 µM pre-assembled proteospecies. As previously reported 37, WT IAPP cytotoxicity correlated with self-assembly time; the proteospecies populating the lag phase decreasing cell viability and the fibrils, i.e. over 12 h self- assembly time, being non-cytotoxic (Fig. 4d). In sharp contrast, N21Q species of the elongation and saturation phases remained cytotoxic, although their toxicity was less compared to the one induced by the assemblies of the lag phase. In agreement with the self-assembly kinetics, the random coil-to-β-sheet secondary structural conversion occurred after 2 h for N21Q and after 6 h for IAPP (Fig. 4e). ThT and ANS fluorescence reached the plateau after 6 h incubation for N21Q and well-defined filaments were observed by TEM imaging after only 2 h incubation (Fig. 4f), i.e. when cytotoxicity remains very high. In sharp contrast for IAPP, ThT and ANS signal reached the plateau after 12–24 h incubation and defined fibrils could be observed by TEM after 6–12 h. These data suggest that the N21Q substitution hastens IAPP self-assembly into fibrillar assemblies, and these N21Q fibrils conserve high toxicity upon elongation.

N21Q fibrils share conformational characteristics with pre-fibrillar oligomers. Time-resolved analysis suggested that the toxic oligomer-like characteristics of N21Q are maintained upon elongation. This hypothesis was evaluated using conformational antibodies that specifically recognize distinct supramolecular structures 47. As revealed by dot blot analysis, isolated IAPP and N21Q fibrils were recognized by the anti-amylloid LOC and 4G8 antibodies (Fig. 5a). The oligomer-specific A11 antibody recognized oligomers, which were prepared by incubating IAPP at 150 µM for 15 min (Supplementary Fig. 12). No binding of the A11 antibody to freshly dissolved monomerized peptides was observed (Supplementary Fig. 13). Strikingly, the A11 antibody recognized N21Q fibrils (Fig. 5a). ELISA confirmed these results (Supplementary Fig. 14). In contrast to IAPP fibrils, immunogold TEM images revealed that N21Q fibrils were simultaneously recognized by the anti-amylloid 4G8 (10 nm gold-particles, white arrows) and the anti-oligomer A11 (20 nm gold-particles, yellow arrows) antibodies (Fig. 5b). IAPP and N21Q fibrils separately incubated with A11, LOC and 4G8 primary antibodies
confirmed the orthogonality of the double labelling approach (Supplementary Fig. 15). Thus, N21Q peptide preserves the A11-specific epitope upon elongation into fibrils. Several conformational models have been proposed for IAPP oligomers, including α-helical coiled-coil48, β-hairpin assemblies49 and parallel β-sheets involving the 20–29 segment50. The latter model proposes that the 20–29 segment of IAPP mediates initial self-recognition and that the oligomers contain stacks of solvent-exposed β-sheets involving the 23FGAIL27 region. Considering that N21Q fibrils exhibit oligomeric characteristics, exposure of the 20–29 region during self-assembly was evaluated by replacing F23 by a p-cyano-phenylalanine (FCN) whose fluorescence is modulated by solvent exposure. As previously reported51, fluorescence of F23FCN IAPP decreased over self-assembly time, indicating that position 23 is being buried with fibril growth (Fig. 5c). In sharp contrast, high fluorescence persisted during F23FCN N21Q self-assembly. This observation suggests that the 23FGAIL27 region remains solvent-exposed in the fibrils, in agreement with the SS-NMR data showing alterations of F23 chemical shifts in N21Q fibrils (Fig. 3).

**N21Q fibrils disrupt plasma membrane.** Several mechanisms mediating the toxicity of oligomers have been proposed, and plasma membrane disruption remains one of the most abundantly described52. The capacity of N21Q fibrils to disrupt lipid membranes was compared to the one of IAPP oligomers. Disruption of lipid bilayer was first evaluated using DOPC:DOPG (7:3) LUVs loaded with self-quenched calcein. WT oligomers (Supplementary Fig. 12) and N21Q fibrils induced high permeabilization of LUVs with leakage of 40–60% after 3 h incubation, whereas IAPP fibrils induced below 10% of leakage after 5 h incubation (Fig. 6a). Plasma membrane disruption of pancreatic β-cells was evaluated by measuring the release of the cytoplasmic enzyme lactate dehydrogenase (LDH). IAPP fibrils did not lead to a significant release of LDH, whereas N21Q fibrils and WT oligomers induced high LDH activity in the media (Fig. 6b). IAPP oligomer mixture and N21Q fibrils induced the equivalent time-dependent activation of caspase-3, suggestive of a similar mechanism of toxicity (Fig. 6c). Interaction of N21Q fibrils with the plasma membrane was evaluated by confocal microscopy and compared with toxic oligomers and cytocompatible fibrils. Fluorescent quaternary species were prepared by co-assembling Alexa Fluor 488-labelled monomers with unlabelled monomerized peptides at a molar ratio of 0.5:99.5, allowing the formation of fluorescent fibrils and oligomers with equivalent (supra)structural and biological properties to unlabelled peptides. CHO cells were used, since INS-1E cells detached promptly in presence of N21Q fibrils, precluding the washing steps necessary.
Fig. 4 Kinetics of self-assembly and time-resolved analysis of cytotoxicity. a–c Kinetics of self-assembly monitored by a ThT, b FlAsH, and c ANS fluorescence. Monomerized peptides were incubated at 12.5 μM under quiescent conditions in 20 mM Tris-HCl buffer, pH 7.4 in the presence of ThT (40 μM), FlAsH (0.5 μM) or ANS (50 μM). Fluorescence of ThT (Ex 440 nm, Em 485 nm), FlAsH (Ex 508 nm, Em 533 nm) and ANS (Ex 355 nm, Em 480 nm) was measured every 10 min. Data from triplicates were averaged and fitted with a Boltzmann sigmoidal curve. d–h Time-resolved cytotoxicity of proteospecies evaluated by measuring the metabolic activity of INS-1E upon 5 h incubation with 50 μM pre-assembled peptides. e–h Time-resolved self-assembly of IAPP and N21Q monitored by e CD spectroscopy, f ThT fluorescence, g ANS fluorescence and h TEM. d–h Freshly dissolved monomerized peptides were incubated under quiescent conditions at 150 μM in 20 mM Tris-HCl buffer, pH 7.4 and after the indicated time of self-assembly, the aggregation mixture was characterized and evaluated for cell toxicity.
The oligomer-like conformation of N21Q fibrils propagates to IAPP. It is known that the conformation of an aggregate can propagate to a naive polypeptide building block, whose assemblies will ultimately acquire the properties of the pre-formed aggregates. This could occurred through secondary nucleation, which corresponds to the catalyzed nucleation of monomers or aggregates. This could occur through secondary nucleation, or via secondary nucleation, depending on the properties of the pre-formed aggregates.

Discussion

Inconsistency among the reported toxicity of the proteospecies of the amyloid cascade has been associated with their high polymorphism and/or dynamic transient nature. Site-specific mutagenesis can lead to the formation of assemblies with discrete morphologies and each of these structural ensembles can exert distinct biological functionalities. Herein, we took advantage of a trivial N-to-Q substitution to identify a fibrillar nanostructure with oligomer-like characteristics and high cytotoxicity, underlining the high susceptibility of amyloid self-assembly to point mutation. Toxicity of defined and mature amyloid assemblies has been ascribed to fibril breakage and leakage of soluble oligomers. This is likely not the case for N21Q fibrils, as these assemblies for microscopy analyses. CHO cells showed similar susceptibility towards concentration-dependent membrane disruption and toxicity induced by the proteospecies (Supplementary Fig. 16). Z-stack reconstruction images revealed that IAPP fibrils gather at the cell surface and do not incorporate within the plasma membrane (Fig. 6d). In sharp contrast, IAPP oligomers colocalized with lipids and extracted them from the membrane. Strikingly, N21Q fibrils damaged plasma membrane by pulling-out lipids, as observed with the formation of large yellow puncta containing the fluorescent fibrils in green and lipid vesicles in red (Fig. 6d). These data reveal that the N21Q fibrils mediate plasma membrane disassembly and permabilization, perhaps an upstream event to cell death, as observed for toxic oligomers.

Fig. 5 N21Q fibrils exhibit oligomer-like conformation and expose residue F23. a Dot-blot analysis of IAPP and N21Q oligomers and fibrils. b Immunogold labelling electron microscopy images of IAPP and N21Q fibrils, scale bar: 100 nm. c× zoom image of the region indicated by the rectangle. a, b Peptides were incubated under quiescent conditions for 15 min (oligomers) and 48 h (fibrils) at 150 µM in 20 mM Tris-HCl buffer, pH 7.4. c FcN fluorescence emission spectra over self-assembly time. Peptides were incubated under quiescent conditions for 48 h at 150 µM in 20 mM Tris-HCl buffer, pH 7.4.

(a) LOC 4G8 A11
IAPP oligomers
N21Q oligomers
IAPP fibrils
N21Q fibrils
(b) 4G8 (10-nm gold particles) + A11 (20-nm gold particles)
IAPP fibrils
N21Q fibrils
(c) FcN15 IAPP
FcN15 N21Q
FcN23 IAPP
FCN23 N21Q
Wavelength (nm)
Wavelength (nm)
Wavelength (nm)
Wavelength (nm)
Fluorescence (a.u.)
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showed comparable thermal and enzymatic stability with cytocompatible IAPP fibrils. The oligomer properties of N21Q assemblies are preserved after fibril elongation and are transmissible. On the one hand, N21Q fibrils share prototypical amyloid characteristics with IAPP fibrils, including the 4.7 Å distance between stacked β-strands, a conformation rich in β-sheet, low solvent-exposition of F15, recognition by anti-amyloid antibodies, and long and linear morphology. On the other hand, cytotoxic N21Q fibrils diverge from IAPP fibrils by low ThT-binding, high surface hydrophobicity, solvent exposure of F23, recognition by A11 antibody, short thickness and higher proportion of twisted filaments. These divergent properties, which arise from the incorporation of a methylene group within N21 side chain, are associated with high ability to disrupt lipid membranes and to cause cellular dysfunction.

Previous studies have proposed cytotoxic determinants of prefibrillar species, including size, hydrophobicity, compactness, rigidity and morphology. For the Aβ peptide, it has been shown that toxicity correlates inversely to the size of aggregates. It has been proposed that low molecular weight oligomers, with a high diffusion coefficient, diffuse rapidly within biological environment, favoring aberrant interactions with cellular components. Herein, we showed that cytotoxicity is mediated by defined fibrils with microscale length, indicating that toxicity does not necessarily correlate with oligomerization state, suggestive of conformational-dependent toxicity. Oligomers assembled from Sup35, HypF-N62, Aβ60,63, and α-synuclein with similar size and morphology can have divergent toxicity, which correlates with exposure of hydrophobic clusters and capacity to disrupt plasma membranes. Poorly toxic oligomers have compact structures with the hydrophobic region buried inside, as for mature amyloid fibrils. Conversely, loose oligomers with high degree of hydrophobic exposure tend to be more cytotoxic. In the present study, N21Q fibrils exhibited high hydrophobicity and these hydrophobic clusters likely involve organized stacks of the 23FGAIL27 segment. Surface hydrophobicity of N21Q assemblies promoted its insertion within plasma membrane, resulting in disassembly of lipid bilayers and membrane permeabilization. Although both fibril preparations showed polymorphism, the prevalence of twisted fibrils was significantly more important for N21Q. Moreover, as observed by AFM imaging, the height of N21Q filaments was approximatively half of the height of IAPP fibrils. Thus, the N21Q substitution likely blocks the self-assembly process into twisted protofibrils by precluding their packaging in fibrils, as previously observed for the amyloid self-assembly of the immunoglobulin light chain. Aβ twisted fibrils have been shown to be more toxic to their striated ribbon counterpart, and twisted morphology has been observed for fibrils extracted from...
Alzheimer patients. The capacity of N21Q fibrils to perturb lipid membranes could be associated with twisted supramolecular organization, which increases surface hydrophobicity. Twisted morphology, either from twisting of protofilament(s) around a central axis or wrapping of protofilaments around an internal axis, emerges early during elongation. While stacking of monomers, or oligomers, onto growing fibrils is driven by hydrophobic contacts, protofilament twisting and wrapping are modulated by a fine balance of specific interactions, including steric repulsions and hydrogen bonding, both being dependent of residue side chains. The N-to-Q substitution affects twisting and/or wrapping of protofilaments as well as the height and stiffness of the resulting fibrils, which could be related to changes in the strength and/or the positioning of hydrogen bonds involving residue-21.

IAPP self-assembly can be ascribed to a nucleated conformational conversion, in which hydrophobic collapse of monomers prompts oligomerization into ensemble of aggregates followed by the slow conversion of competent oligomers into protofilaments. The 20–29 segment has been proposed to drive self-recognition into on-pathway oligomers, which could be composed of stacks of β-sheets. Accordingly, nucleation is kinetically controlled by an energy barrier involving the conversion of this segment from a β-sheet into a loop, which allows formation of the hairpin or S-shaped structure within monomers of the protofilament. Not only N21 acts as a molecular hinge modulating primary nucleation, it forms extensive interlayer hydrogen-bonding interactions within protofilaments, known as a polar ladder. As observed by time-resolved ThT, ANS and FlAsH fluorescence, CD spectroscopy, and solvent exposition of FCN, N21Q substitution reduced the lag phase, indicating that the amide group at position 21 is a key player in nucleation and, perhaps, elongation. Particularly, the conformation recognized by the A11 antibody is preserved during elongation. As observed by the solvent-exposure of FCN and SS-NMR analysis, this conformational epitope is likely composed of repeating arrays of 23FGAIL that remain exposed in N21Q mature fibrils. Seeding experiments revealed that the oligomer-like conformation of N21Q fibrils is organized into a repeating display of monomers, wherein all monomers share identical conformation. The transmissible and exposed lattice of monomers recognized by the A11 antibody and involving the 20–29 domain likely represents the toxic quaternary epitope. Considering that N21Q fibrils

![Fig. 7 The oligomer-like conformation of N21Q fibrils propagates to IAPP. a–d] AThT fluorescence and ANS fluorescence, b dot-blot analysis with the anti-oligomer A11 antibody, c SS-NMR (CP-DARR) of F23 labelled IAPP, and d FCN fluorescence of the F23FCN IAPP. e Cell viability of INS-1E treated with 50 μM IAPP fibrils seeded with N21Q pre-assembled fibrils. N21Q fibrillar seeds were grown for 48 h (quiescent conditions, 150 μM in 20 mM Tris-HCl buffer, pH 7.4) before being isolated and used to seed the amyloid formation of IAPP under quiescent conditions for 48 h.
share conformational and biological characteristics with toxic soluble oligomers, including the ability to pull out lipids from plasma membrane and disassemble lipid bilayers, these stable fibrils offer a unique opportunity to define the structural determinants of oligomer-induced cell death. The present study showed that the conformational A11 epitope can be preserved upon fibril elongation and can propagate, demonstrating that cytotoxicity of amyloid is associated with specific structural features and not uniquely to the presence of soluble oligomers. The identification of these defined filaments expands the diversity of amyloid assemblies and increases the complexity of quaternary structures associated with amyloid-related disorders.

**Methods**

**Peptide synthesis, purification and sample preparation.** Peptides were synthesized on solid support using Fmoc-chemistry and 2-(6-chloro-1H-benzo-triazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU) coupling strategy with the incorporation of oxazolidine pseudoproline derivatives. After cleavage, crude peptides were purified by RP-HPLC and collected fractions were analysed by LC/MS-TOF. Disulfide bond formation between Cys-2 and Cys-7 was achieved by dimethyl sulfoxide oxidation under mild agitation. Cycлизed peptides were purified by RP-HPLC and fractions corresponding to the desired product with purity higher than 95% were pooled and lyophilized. Peptides labelled with Alexa488 NHS ester were prepared as previously described. Aliquots of monomerized peptides were prepared by dissolving the lyophilized peptide in hexafluoro-2-propanol (HFIP). The solution was sonicated for 30 min, filtered through a 0.22 µm hydrophilic filter and lyophilized. The resulting peptide powder was weighted using an ultra-microbalance, solubilized for a second time in HFIP to 1 mg/ml, sonicated for 30 min and lyophilized again. Peptide concentrations were validated by measuring absorbance at 280 nm using a theoretical molar extinction coefficient of 1940 M⁻¹ cm⁻¹ and by measuring the area under curve at 229 nm by RP-HPLC. Monomerized dried samples were kept dried at −80 °C until used, but not for longer than 4 weeks.

**Preparation of oligomers and fibrils.** Prefibrillar species and fibrils were prepared by incubating freshly dissolved monomerized peptides under quiescent conditions at 150 µM in 20 mM Tris-HCl buffer, pH 7.4 for 15 min and 48 h, respectively, unless otherwise stipulated. Fluorescently labelled peptide oligomers and fibrils were prepared by incubating non-labelled peptides with Alexa Fluor 488-labelled peptides (99.5:0.5, molar ratio) under quiescent conditions for 48 h at a final concentration of 150 µM.

**Fluorescence microscopy**. Peptide samples were diluted to a concentration of 50 µM in presence of 40 µM of ThT or 100 µM ANS. Fluorescence was measured in a quartz ultra-micro plates 10 mm length. For ThT, excitation was set at 440 nm and emission from 450 nm to 550 nm was recorded. For ANS, the excitation was set at 355 nm and emission was recorded from 385 nm to 585 nm. Data are the average of at least four independent experiments.

**Preparation of oligomers and fibrils.** Prefibrillar species and fibrils were prepared by incubating freshly dissolved monomerized peptides under quiescent conditions at 150 µM in 20 mM Tris-HCl buffer, pH 7.4 for 15 min and 48 h, respectively, unless otherwise stipulated. Fluorescently labelled peptide oligomers and fibrils were prepared by incubating non-labelled peptides with Alexa Fluor 488-labelled peptides (99.5:0.5, molar ratio) under quiescent conditions for 48 h at a final concentration of 150 µM.

**Kinetics of amyloid formation.** Solutions were prepared by dissolving the monomerized peptide in 20 mM Tris-HCl, pH 7.4. Assays were performed at 25 °C without agitation in sealed black-wall clear-bottom 96-well non-binding surface plates with a total volume of 100 µl per well. Final peptide concentrations were 12.5 or 25 µM in presence of ThT (40 µM), ANS (50 µM) or FlAsH (0.5 µM). Fluorescence of ThT (Ex. 440 nm, Em. 485 nm), ANS (Ex. 355 nm, Em. 480 nm) or FlAsH (Ex. 508 nm, Em. 533 nm) were measured from the bottom of the well every 10 min. over the course of 20 h. Data obtained from triplicate wells were averaged, corrected by subtracting the corresponding control reaction and plotted as fluorescence vs time. Data of time-dependence of ThT fluorescence were fitted to a single exponential function, i.e. the apparent first-order constant k, where Y_max and Y_0 are, respectively, the maximum and initial fluorescence values (Eq. 1).

\[
Y = Y_0 + (Y_{max} - Y_0)e^{-kt}
\]

The lag time is described as \(t_{lag} = \frac{\ln(2)}{k}\). Data (lag time and final fluorescence) of at least four different lots of peptides were averaged and were expressed as the mean ± S.D.

**Circular dichroism spectroscopy.** Peptide samples were diluted to a final concentration of 50 µM and incorporated into a 1 mm path length quartz cell. Far-UV CD spectra were recorded from 190 to 260 nm using a Jasco J-815 CD spectro-polarimeter at 25 °C. The wavelength step was set at 0.5 nm with an average time of 10 s per scan at each wavelength step. Spectrum was background subtracted with peptide-free buffer. Raw data were converted to mean residue ellipticity (MRE). Thermal unfolding transitions were monitored by the variation of CD signal at 222 nm between 25 °C and 104 °C with a heating rate of 1.0 °C min⁻¹. Transitions were evaluated using a nonlinear least square fit assuming a two-state model, i.e. assembled and unassembled.

**Attenuated total reflectance-Fourier transform infrared spectroscopy.** ATR-FTIR spectra were recorded using a Nicolet Magna 560 spectrometer equipped with a nitrogen-cooled MCT detector. Each spectrum was an average of 128 scans recorded at a resolution of 2 cm⁻¹ using a Happ–Genzel apodization. Data analysis were performed using Grams/Al 8.0 software.

**Powder X-ray diffraction.** Fibrils were deposited on an X-ray diffraction lamella and dried overnight. Powder XRD was performed using a Bruker D8 Advance X-ray diffractometer. The current and the voltage were 40 mA and 40 mV, respectively, with a step size of 0.012° s⁻¹ in the 20 range of 5°–50°. Interplanar distances were determined from powder raw pattern (20), satisfying Bragg’s condition.

**Transmission electron microscopy.** Prefibrillar species and fibrils were diluted to 10 µM in 1% acetic acid, and immediately applied to freshly cleaved mica. The mica was washed twice with deionized water and air-dried. Images were acquired on a Veeco/Bruker Multimode AFM using tapping mode with a silicon tip (2–12 nm tip radius, 0.4 N/m force constant) on a nitride lever. Images were taken at 0.2 Hz and 1024 scan/minute.

**Solid-state nuclear magnetic resonance.** Fibrils for SS-NMR were prepared using uniformly labelled 13C, 15N amino acids at positions Ala13, Phe23 and Val22. Fibrils assembled at 375 µM for 48 h were concentrated by ultracentrifugation at 100,000 × g for 45 min at 4 °C. The supernatant was discarded and the pellet was resuspended in water and lyophilized. The lyophilized fibrils were then packed dry into NMR rotors, with a final recovery ranging between 50 and 80% of the mass of the initial peptide. For IAPP fibrils, approximately 50% of the starting mass was recovered, whereas for N2IQ fibrils, almost 80% of the starting mass was recovered. NMR spectra were recorded on a 400 MHz wide-bore Bruker Avance III-HD using a triple-resonance 1.9 mm magic-angle spinning (MAS) probe operating in double resonance mode. MAS speed was set to 26 kHz for 2D DARR experiments with a 200 ms mixing time and the 1H and 13C radiofrequency fields were 83 kHz and 100 kHz, respectively. Cross-polarization was used (1.3 ms) during acquisition with a 70–100% intensity ramp on the 1H radiofrequency channel, and protons were decoupled using the spiral-64 sequence. All experiments were recorded at 277 K and data were externally referenced using adamantane, setting the trimethylsilyl (CH3) signal to 38.48 ppm, relative to tetramethylsilane (TMS)74. Data were acquired and processed using Topspin and Mestrenova.

**Dot blot.** Ten µl of peptide sample (150 µM) was applied to a nitrocellulose membrane. The membranes were allowed to air dry and were blocked in 5% non-fat dried milk in TBS-T for 30 min at RT. Membranes were incubated with the primary antibodies (A11, LOC, 4G8) for 2 h. After three washes in TBS-T, membranes were incubated with HRP-conjugated secondary antibody (mouse anti-lgG for 4G8, goat anti-rabbit IgG for LOC and A11 antibodies) for 1 h. The secondary antibodies were diluted 1:10,000 in 5% non-fat dried milk in TBS-T. Blots were washed three times with TBS-T, and secondary antibody activity was revealed by enhanced chemiluminescence.

**Cell viability.** INS-1E cells (Millipore Sigma) were plated in black wall clear-bottom 96-well plates at a density of 20,000 cells/well (100 µl/well) in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, 50 mM β-mercaptoethanol. After 48 h incubation at 37°C in a 5% CO2, cells were treated by the addition of 50 µl of peptides (monomers, prefibrillar or fibrils) at 3 x 10⁻¹⁰ M final concentrations (50 µM to 1 µM) solubilized in 20 µl Tris, pH 7.4. Cells were incubated for 5 h (for time-resolved assay) or 24 h and cellular viability was measured by the resazurin reduction assay. For CHO-K1 (ATCC), cells were plated at a density of 20,000 cells/well (100 µl/well) in Ham F-12 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin. Cell viability (in %) was calculated from the ratio of the fluorescence of the treated sample to the control cells (non-treated). Data (in %) of at least four lots of
peptides were averaged and expressed as the mean ± S.D. For Live/Dead assays, cells were plated in 12-well plates at a density of 150,000 cells/well for 48 h before treatment. Analyses were performed by the addition of the reagent solution (4 μM of ethidium homodimer-1; 2 μM of calcine-AM) and after 45 min incubation, cells were imaged by fluorescent microscopy.

**Caspase-3 activity assay.** Cells were cultured in 12-well plates at a density of 400,000 cells/well. After 48 h incubation, cells were treated with peptide solutions for different time (0–24 h), at final peptide concentration of 50 μM. Cells were lysed on ice for 30 min followed by a centrifugation at 16,000 × g for 20 min. Caspase-3 activity in the supernatant was measured by a colorimetric assay and the caspase-3 activity was expressed in μmol pNA released per min per ml of cell lysate. Data of at least three individual experiments performed in triplicate were averaged and expressed as the mean ± S.D.

**Lactate dehydrogenase (LDH) release assay.** INS-1E and CHO-K1 cells were seeded in 96-well plates at 30,000 cells/well. After 48 h incubation, cells were treated with peptide solutions. Media (50 μl) was collected at different times (0–12 h) and incubated with the reaction mixture from the Pierce LDH Cytotoxicity Detection Kit for 30 min at room temperature. The reaction was stopped and absorbance was measured from 490 to 680 nm. To determine LDH activity, the 680 nm absorbance value (background) was subtracted from the 490 nm absorbance before calculation. Percentage of LDH leakage was calculated (Eq. 2):

\[
\% \text{LDH leakage} = \frac{\text{LDH activity}_{\text{peptide-treated}} - \text{LDH activity}_{0}}{\text{LDH activity}_{\text{max}} - \text{LDH activity}_{0}} \times 100
\]

where LDH activity, is the absorbance measured in absence of peptide and LDH activity \(_{\text{max}}\) is the absorbance measured for cells treated with lysis buffer. Data of at least three individual experiments performed with different lot of peptides were averaged and expressed as the mean ± S.E.M.

**Leakage of large unilamellar vesicles.** DOPE/DOPG (7:3, molar ratio) lipids were solubilized in chlorella in a glass tube and solvent was evaporated with a nitrogen stream. Lipid film was rehydrated in a 20 mM Tris-HCl pH 7.4 buffer containing 70 mM calcine for 30 min. Solution was freeze-thawed five times before being extruded with a 100 nm polycarbonate membrane for 20 cycles. Free calcine was separated from the LUVs using by size-exclusion chromatography (Sephadex G25-fine). Lipid concentration was determined by a colorimetric assay and size distribution of LUVs was evaluated by dynamic light scattering. For leakage, peptide assemblies (monomers, prefibrillar and fibrils) were prepared as described above and used at a final concentration of 50 μM. Calcine-LUVs were used at a final concentration of 500 mM. Fluorescence was measured in sealed black-well, clear-bottom 96 well non-binding surface plates with a total volume of 100 μl per well. Measurements were performed every 15 min using an excitation wavelength of 495 nm and emission at 517 nm. The control used to determine 100% leakage (F\(_{\text{max}}\)) was calcine-LUVs with 0.1% of Triton X-100. Dye leakage was reported using the following equation (Eq. 3):

\[
\% \text{membrane leakage} = \frac{F - F_0}{F_{\text{max}} - F_0} \times 100
\]

where F0 is the fluorescence of the LUVs in absence of peptide. Data of at least four individual experiments performed with different lot of peptides were averaged and expressed as the mean ± S.E.M.

**Confocal microscopy.** CHO-K1 cells were cultured in 8-well cell culture chamber at a density of 15,000 cells/well for 48 h before treatment. Alexa488-labelled oligomers or fibrils were added to the cell media and cells were incubated at 37 °C for 4 h. After incubation, cells were washed three times with PBS. Membrane was stained with 0.2 μg/ml of Cell mask and 0.1 μg/ml of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Cells were then washed three times with PBS, fixed with 4% paraformaldehyde. Fluorescence was analysed with a confocal Nikon microscope equipped with a 60x oil immersion objective. Images were analysed using ImageJ software.

**Statistical analysis.** Statistical analyses were performed using the Student’s t-test and statistically significant statistical difference (between IAPP and 21Q1, otherwise stated) was established at p < 0.001 (**), p < 0.0001 (***), p < 0.00001 (****). Statistical analyses were carried out using GraphPad Prism 8.0 software.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The data supporting the findings of this manuscript are available from the corresponding authors upon reasonable request.
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Author contributions P.T.N. and S.B. conceived and designed the overall study. P.T.N. performed the cell-based assays, fluorescence and CD spectroscopy, dot blot, membrane leakage and AFM. X.Z. performed the PXRD, FTIR, TEM analysis and the immunogold labelling, M.S. and L.M. performed, designed and analysed the NMR experiment. P.T.N. and S.B. analysed data and prepared the figures. P.T.N. and S.B. wrote the manuscript and all authors contributed to its revision.

Competing interests The authors declare no competing interests.
