Trifluoromethyldiazirine: an effective photo-induced cross-linking probe for exploring amyloid formation†

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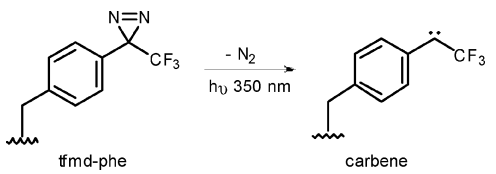
The separative and analytical power of ion mobility spectrometry-mass spectrometry combined with photo-induced cross-linking of site-specifically incorporated trifluoromethyldiazirine provides a powerful approach towards structural characterisation of amyloid fibrils.

Amyloid fibrils, with a characteristic cross-β structure, are associated with multiple diseases, including Alzheimer's, Parkinson's and prion diseases.[1–4] Despite intensive research, the molecular mechanism of fibril formation[5] and the molecular basis of toxicity are poorly understood. In addition to fibrils, small soluble oligomers, which may or may not represent on-pathway intermediates, have been implicated in amyloid-related diseases.[1–4,6,7] Photo-induced cross-linking (PIC) offers an attractive approach to structural studies of amyloid fibrils[8–10] and their mechanisms of formation. The technique makes it possible to trap transient non-covalent associations, enabling 'snapshots' of assembly reactions to be taken and otherwise metastable intermediates to be isolated for structural studies.[11,12] Identifying cross-links also provides important spatial restraints for modelling the structure of amyloid fibrils. Herein we describe the development of a toolkit that is highly promising for such a study, utilising a fragment of amyloid beta peptide (Aβ) as a model system. Amyloid fibrils were created from this peptide, which incorporated a single trifluoromethyldiazirine (tfmd) phenylalanine. We demonstrate that tfmd can be incorporated into an amyloidogenic fragment without inhibiting fibril formation, and show cross-linking permits identification of interacting residues within the amyloid fibril using electrospray ionisation-ion mobility spectrometry-mass spectrometry (ESI-IMS-MS).[13,14]



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The aryl-tfmd group is non-invasive, non-toxic, and photolyses rapidly (nanosecond timescale) at non-damaging wavelengths (350 nm) to generate singlet carbene. This species is highly reactive, even towards aliphatic CH bonds,[15–17] generating products that are sufficiently stable to permit isolation, purification and analysis.[11,12] As importantly, ESI-IMS-MS[13,14] has been used to separate, mass analyse and sequence the resulting cross-linked species in a rapid, sensitive and accurate single experiment, avoiding lengthy purification procedures and ensuring simultaneous characterisation of the entire range of products.

We synthesised tfmd–phenylalanine (tfmd–Phe) in high yield, following a previously published route (Fig. S1, ESI†).[18] To test its capacity to probe intermolecular interactions in amyloid fibrils we incorporated tfmd–Phe at the fourth position in a seven-residue fragment of Aβ ($A\beta_{16–22}$) using standard Fmoc-based chemistry ($A\beta^*_{16–22}$; *N*-acetyl-KLV[tfmd–F]FAE–NH_2). Residues 16–22 constitute the central hydrophobic cluster of Aβ and the unmodified peptide ($A\beta_{16–22}$; *N*-acetyl-KLVFFAE–NH_2) has been shown to form cross-β amyloid fibrils, with an anti-parallel organisation of β-strands aligned perpendicular to the fibril axis.[19] Fibrils of $A\beta_{16–22}$ and $A\beta^*_{16–22}$ were grown under identical conditions. Both peptides formed morphologically identical fibrils on the same timescale (~1 week), as assessed by negative-stain electron microscopy (Fig. 1), and by addition of the amyloid specific dye Thioflavin T (Fig. S2, ESI†). The introduction of tfmd–Phe therefore does not appear to perturb fibril formation significantly despite the high degree of structural order observed in cross-β amyloid fibrils[20] ($A\beta_{16–22}$ in particular).[19]

To demonstrate cross-linking, the $A\beta^*_{16–22}$ fibrils were irradiated with light of wavelength ~350 nm for 1 hour using a TLC UV lamp. The insoluble cross-linked fibrils were then

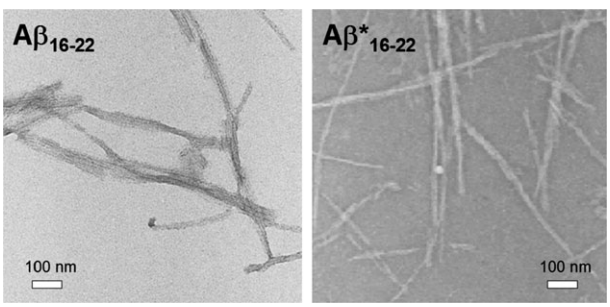

Fig. 1 Negative-stain electron microscopy images of amyloid fibrils formed by $A\beta_{16–22}$ and $A\beta^*_{16–22}$ after incubation for 7 days.

harvested and fully dissociated in hexafluoroisopropanol prior to analysis by ESI-IMS-MS (Fig. 2). To interpret these data it should be noted that activation of tfmd–Phe at 350 nm gives rise to an unreactive linear diazo isomer at 20–30%.[11,12,15,16] This isomer can be photolysed to the reactive carbene by irradiation at ~300 nm,[11,12,15,16] although we did not attempt to optimise this in the experiments presented here.

The ESI-IMS-MS data of the cross-linked products are consistent with the formation of monomers and dimers (Fig. 2, S3, S4 and S5, ESI†). The advantage of the IMS facility coupled to ESI-MS is apparent by the clear separation of species A and B, which have the same m/z ratio despite A being a monomer and B a dimer. In contrast, no changes in the m/z spectrum of the unmodified peptide were observed after irradiation (Fig. S6, ESI†). To locate the position of the inter-peptide cross-link, the two principal dimers (species B & C) were fragmented *via* collision-induced dissociation after IMS separation (ESI-IMS-MS/MS). The resulting tandem MS m/z spectra of peptide fragments (Fig. S4 & S5, ESI†) demonstrate conclusively that the majority of inter-peptide cross-links in species B and C occur between the two (tfmd)–Phe residues, *i.e.* pairs of tfmd–Phe side chains are in close contact in the fibril. It should be noted that a minor fragment results from an inter- and intra-chain cross-linking event, which confirms further that cross-linking arises due to structural features present within the fibril (Fig. S4(C), ESI†).

As cross-linking occurs between two tfmd–Phe residues in the self-assembled fibril they must be in mutual proximity. Assuming the modified peptide adopts an anti-parallel β-sheet structure as observed previously for the unmodified peptide[19] there are two possibilities *via* which the tfmd–Phe residues can be organised within a sheet (Fig. 3). Tfmd–Phe can pair in an anti-parallel β-sheet with H-bonds (HB) directly flanking the paired residues (Fig. 3b), or with no H-bonds (NHB) directly flanking the paired residues (Fig. 3a). The HB and NHB sites alternate down the length of the sheet (Fig. 3a and b). Bioinformatic analyses[21] show that most aromatic–aromatic pairs at HB sites adopt conformations where the face or edge of one aromatic ring interacts with the Cβ atom of its partner (Fig. 3b). Most aromatic–aromatic pairs at NHB sites, by contrast, adopt conformations where archetypical off-set stacking of the aromatic rings is achieved (Fig. 3a). Our cross-linking studies are consistent with the latter case, where inter-peptide cross-links would be expected to form exclusively between pairs of tfmd–Phe residues at NHB sites. The former case would be expected to give rise to larger species (trimers, tetramers, *etc.*) *via* cross-strand and cross-sheet cross-links (*i.e.*, the aromatic ring in this conformation at the HB site would point tfmd towards groups in the adjacent sheet).

In summary, we have demonstrated that tfmd is a suitable non-invasive probe for site-specific PIC studies of amyloid, and shown how combining this methodology with the separative and analytical power of ESI-IMS-MS allows identification of cross-linked pairs within amyloid fibrils and provides detailed information relating to their structural organisation. The toolkit we have developed should also be useful for mechanistic studies of self-assembly reactions including

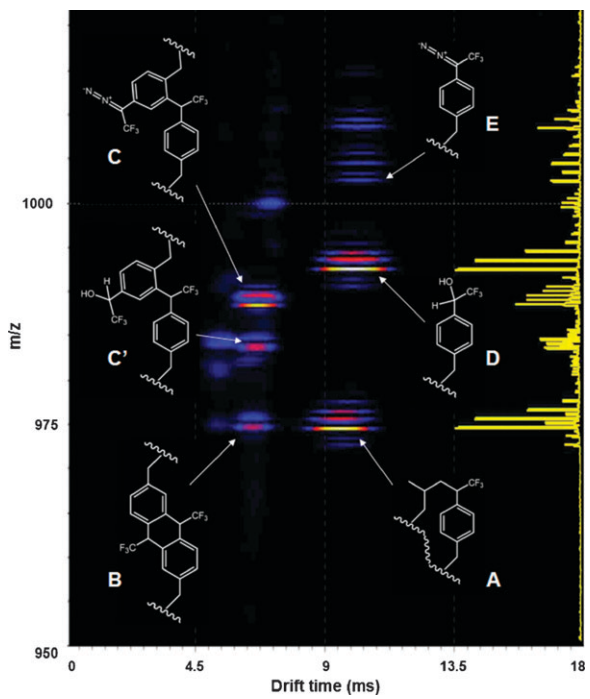

Fig. 2 ESI-IMS-MS data (m/z 950–1025): A) (m/z 974.4(1+)) monomer resulting from intra-peptide cross-link; B) (m/z 974.4(2+)) dimer resulting from two inter-peptide cross-links, or one intra- and one inter-peptide cross-link; C) (m/z 988.2(2+)) dimer resulting from one inter-peptide cross-link (other tfmd isomerised to unreactive diazo); C′) (m/z 983.2(2+)) as C but other carbene has formed a water adduct; D) (m/z 992.4(1+)) monomer, where carbene has formed a water adduct; E) (m/z 1002.4(1+)) monomer, where tfmd has isomerised to unreactive diazo. Hypothetical cross-links are shown. The corresponding ESI-MS spectrum is shown in yellow.

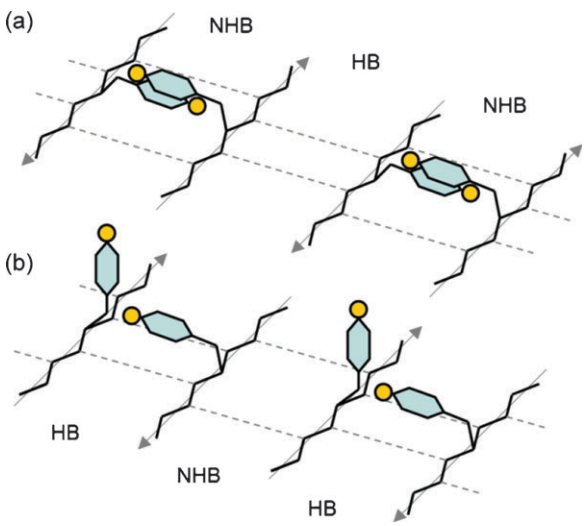

Fig. 3 Possible organisation of tfmd–Phe pairs within an anti-parallel β-sheet: (a) tfmd–Phe adopts an off-set stacking arrangement with no flanking H-bonds (NHB); (b) tfmd–Phe adopts an edge-to-face stacking arrangement with flanking H-bonds (HB). The peptide backbone is shown as a dark line; peptide backbone H-bonds are shown as dashed lines and are parallel to the fibril axis; arrows indicate backbone N–Cα–C direction; the gold sphere is tfmd and all other α-substituent's have been omitted except Phe in blue.

investigations of transient species populated during amyloid fibril formation that complement existing MS methods for characterising co-populated, non-covalent complexes.[22,23] These non-covalent assembly intermediates are dynamic, metastable and structurally heterogeneous, making it challenging to isolate individual species for detailed structural and biochemical analyses. Such studies are vital for rationalising toxicity and for elaborating therapeutic strategies that target amyloid formation.[24,25] For this particular self-assembly reaction, the tfmd probe may be advantageous over benzophenone[8] in time-resolved PIC studies due to the irreversible nature of excitation and rapid reactivity of the resultant carbene. Our approach may similarly complement the studies[9,10] using the method of Fancy and Kodadek[26] where activation is diffusion controlled. With only one probe molecule, our experiments provide evidence for the structural integrity of $A\beta_{16-22}$ fibrils incorporating tfmd–Phe. More detailed studies of fibrils of unknown structure and their assembly intermediates will require the incorporation of tfmd in different and multiple positions to facilitate covalent fixation of the entire assembly at any given time point. Our future studies will focus on extending these preliminary studies in these directions.

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