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Halogen bonding modulates hydrogel formation from Fmoc amino acids

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The single crystal X-ray structures of a series of *p*-halogenated Fmoc phenylalanines highlight the role of halogen bonding in their supramolecular organization in the solid state. The amino acids showing the occurrence of halogen bonding resulted in gels with the strongest mechanical properties. All gelation parameters could be ranked according to halogen atom polarizability, *i.e.*, halogen bond strength.

Amino acids modified with the 9-fluorenylmethyloxycarbonyl (Fmoc) moiety[1] possess an ideal balance of hydrophilic/hydrophobic/aromatic interactions[2-6] that are responsible for their efficient self-assembly in water.[7] In fact, Fmoc-protected amino acids are widely used as low-molecular-weight hydrogelators,[8-10] where an entangled network of fibres, which are typically a few tens of nanometres in diameter and can be microns in length, is formed.[11] In particular, Fmoc-phenylalanine (Fmoc-Phe) is a well-known hydrogelator due to its excellent ability to form self-assembled fibres.[12] Introducing chemical modifications at the *para* position of the Phe aromatic ring is a fruitful strategy to further tune the gel formation efficiency of this class of amino acids, as well as the mechanical properties of the formed hydrogels.[13]

Among the many possible modifications[14-17], in 2010 Nilsson *et al.* demonstrated that halogenation is particularly effective.[18] He found that among the four possible *p*-halogen-substituted Fmoc-Phe derivatives, the fluorine one was the most efficient gelator. Incorporation of a phenyl/perfluorophenyl pair in the structure of a Phe-based peptide hydrogelator was also proven to be effective.[19-20] On the other hand, we recently reported that introduction of iodine on Phe may result in additional supramolecular interactions stemming from the ability of iodine to behave as halogen-bond donor as well as hydrogen and halogen-bond acceptor. In particular, we demonstrated that iodination promoted the formation of fibrils from short amyloidogenic peptide sequences[21] (*i.e.*, DFNKF[22-23]). However, a clear understanding of the role of halogen bonding,[24] *i.e.*, the noncovalent interaction involving halogen atoms as electrophilic species,[25-26] in promoting interlacing of the nanofibrils to induce the formation of the hydrogel by entrapping the solvent molecules, is still missing. Here we report a series of Fmoc-Phe derivatives halogenated at the *para* position of the benzene ring and hydrogels formed thereof.

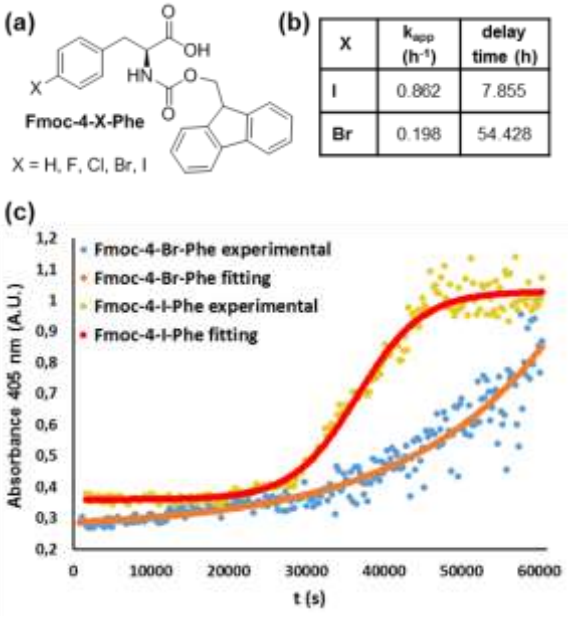

Fig. 1. a) Molecular formula of the amino acid gelators used in this study, namely *N*-Fmoc-4-substituted-phenylalanines (Fmoc-4-X-Phe); b) Table with the kinetic parameters extrapolated from the UV-controlled kinetic measurements on Fmoc-4-Br-Phe and Fmoc-4-I-Phe fibrillation processes; c) Fibrillation kinetic measurements for 0.1 mM solutions of Fmoc-4-Br-Phe (blue) and Fmoc-4-I-Phe samples (yellow). The fitting of the data to the Equation 1 are reported as solid line (Equation 1 in ESI).

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We demonstrate that various gelation properties, such as fibrillation efficiency, minimum gelation concentration, temperature stability, and rheological properties follow the halogen atom polarizability order, with the iodine derivative being the most efficient. Single crystal X-ray structures of the gelator molecules show the occurrence of halogen bonds in the I and Br derivatives, but not in the Cl and F ones. This suggests that halogen bonding plays a role in the improved gelation properties of halogenated Fmoc-Phe derivatives.

Halogenated amino acid derivatives **Fmoc-4-X-Phe**, where X = H, F, Cl, Br, and I (Fig. 1a), were obtained from commercial providers and used without further purification. We first measured the fibrillation kinetics of each amino acid by monitoring over time UV-*vis* absorbance of diluted solutions (0.1 mM, 5% DMSO in PBS) under controlled temperature (25 °C) and shaking conditions.[27] This experimental procedure was profitably used to investigate Fmoc-4-X-Phe self-assembly kinetics since the formation of bundle of fibrils in solution cause significant light scattering.[28] Usually, the occurring of the fibrillation process results in a sigmoidal curve, showing the three peculiar stages of fibril formation: a lag phase, a rapid growth representing the elongation process, and a final equilibrium stage. By fitting experimental data to canonical rate law already described for similar self-assembling systems (see ESI),[27] it was possible to extrapolate two different kinetic parameters: the delay time (T) and the apparent rate constant of fibril growth (k_{app}). Both of these parameters indicated that the fastest self-assembling derivative is the iodinated one, followed by the brominated, which has a delay time of one order of magnitude greater than **Fmoc-4-I-Phe** (Fig. 1b). The faster kinetics of the iodinated amino acid over the brominated one is well represented by the shape of the curve. In fact, while for **Fmoc-4-I-Phe** kinetics was complete, the corresponding bromo-derivative was still in its growth phase (Fig. 1c) in the observed time range. Similarly, for the fluorinated and chlorinated Fmoc-amino acids, the UV-*vis* absorbance evolution was so slow that no kinetic parameters could be evaluated (Fig. S1). An opposite ranking following halogen atom polarizability[29], *i.e.* F, C l > Br > I, was also found for the minimum gelation concentration values (MGC). The lowest MGC was shown by the iodinated amino acid (MGC = 0.25 mM, 5% DMSO in PBS), followed in the order by bromo- (0.50 mM), chloro- (0.75 mM), and fluoro-amino acids (3.0 mM) (see Tables S2 and S3). Interestingly, an opposite MGC ranking was obtained when performing the experiments in deuterated solvents, which confirms that hydrophobicity here is not playing a major role.[30]

Rheology properties were instead measured on 5 mM solutions, where every **Fmoc-4-X-Phe** derivative, except the fluorinated one, formed a stable gel. Furthermore, the non-halogenated Fmoc-Phe was also studied, for comparison. In general, the storage modulus value (G') increases as a function of halogen atom polarizability, confirming the same trends observed with both fibrillation kinetics and opposite to MGC values (Fig. 2a, bottom, Fig.S2, Tables S2 and S3). This finding demonstrates that fibrillation efficiency and hydrogel rigidity are intimately related in this kind of systems. However, it was not possible to obtain reliable and reproducible measurements on the gel of the fluorinated derivative, as it formed only a very weak gel.

The obtained hydrogels were all thermoreversible with gel–sol transition temperatures ranging from 58°C (Fmoc-Phe) to 105°C (**Fmoc-4-I-Phe**) (Fig. 2a, top). In particular, the observed transition temperatures followed the same trend of the above discussed gelation properties. These results clearly show that, in general, halogenation of Fmoc-Phe promotes hydrogel formation, with the iodinated amino acid being the most efficient. This efficiency clearly decreases with the polarizability of the halogen atom.

Transmission electron microscopy (TEM) allowed assessing the morphology of the fibrillary networks constituting the hydrogels. All the samples showed fibrillary networks, with apparent increasing interlacing and fibril dimensions (Fig. S3) on moving from F to I (Fig. 2b-e).

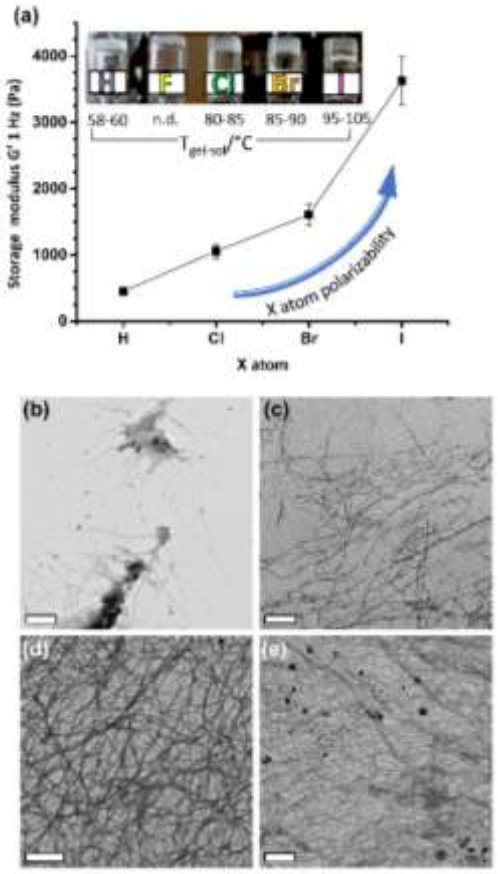

Fig. 2. a) Storage modulus G' (at ω = 1Hz) as a function of the X atom in 5 mM gel samples of Fmoc-4-X-Phe derivatives. Error bars correspond to the standard deviations calculated over 5 different measurements. The solid line is a guide for the eyes. The inset shows the appearance of the gels and their $T_{gel-sol}$. b-e) TEM images of 1 mM samples of **Fmoc-4-X-Phe** amino acids showing an increasing interlacing of the fibrillary network on going from F (b) to I (e). Scale bar is 0.5 μm.

According to Adams *et al.*,[11] extrapolating from single-crystal X–ray structures how the gelator molecules pack in gel phase, may lead to erroneous conclusions.[31] On the other hand there are only two structures of Fmoc-Phe amino acids in the Cambridge Structural Database (CSD).[11] Therefore, with the two-fold objective of increasing the number of available structures of Fmoc-Phe derivatives, as well as to assess the role of the supramolecular interactions involving the halogen

atoms in the self-assembly of **Fmoc-4-X-Phe** derivatives, we undertook their detailed single crystal X-ray diffraction (XRD) study.

All of the **Fmoc-4-X-Phe** derivatives formed small, weakly diffracting, and poorly ordered crystals directly from solutions, by using various crystallization conditions (see ESI). However, accurate structure solution was possible by using synchrotron radiation. **Fmoc-4-I-Phe** and **Fmoc-4-Br-Phe** were isostructural and crystallized in the *P* 6_3 space group with one molecule of amino acid and a disordered water molecule in the asymmetric unit. Water is presumably taken either from the solvent mixture or from air. **Fmoc-4-Cl-Phe** and **Fmoc-4-F-Phe** crystallized, instead, in the *P* 2_1 space group with no solvent molecule and in the *P* $2_12_12_1$ space group with one disordered DMSO molecule, respectively. All amino acid molecules stack by forming infinite 1D chains connected by N–H···O=C intermolecular hydrogen bonds and overlapping their aromatic surfaces (Fig. 3, left). These hydrogen-bonded stacks perfectly resemble the molecule stacking observed in the two Fmoc-Phe structures reported in the CSD (OGIXOT and OGIXUZ). Interestingly, identical to OGIXOT, in the **Fmoc-4-Cl-Phe** structure, the carboxylic acid groups of adjacent 1D stacks of molecules interact *via* O–H···O hydrogen bonds forming a wavy chain that extend along a twofold screw axis parallel to the crystallographic *b* axis (Fig. 3, right). This crystal packing mode of adjacent 1D stacks was, instead, prevented in the structures of the I, Br, and F derivatives by the interaction of the carboxylic group with the crystallized solvent molecules, similar to what observed in the case of OGIXUZ.

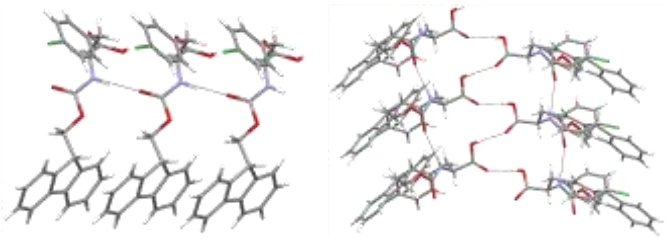

Fig. 3. Subpackings of the single crystal X-ray structure of **Fmoc-4-Cl-Phe**, showing the molecules stacking thanks to hydrogen bonding and aromatic interactions (left), and interactions between two stacks by hydrogen bonds involving the exposed carboxylic groups (right).

As far as the contacts involving the halogen atoms in the structures of **Fmoc-4-X-Phe** are concerned, I and Br atoms show a different pattern of interactions compared to Cl, while F was not involved in any relevant interaction. In particular, the I atom of **Fmoc-4-I-Phe** points towards the C15-C16 bond of the fluorenyl ring giving rise to a classical over-the-bond halogen···pi interaction, where I···C16 and I···C15 distances are 3.34(2) Å and 3.59(2) Å, and C-I···C16 and I···C15 angles are 166.5(7)° and 169.8(7)°, respectively. The distance of the I atom from the delocalized bond centroid is 3.39(2) Å, with a C-I···centroid angle of 176.4(7)° (Fig. 4). The high directionality of this interaction, as well as its distance well below the sum of vdW radii for C and I, *i.e.*, 3.68 Å, highlights the halogen-bond (XB) nature of this interaction, and demonstrates how **Fmoc-4-I-Phe** may behave as an XB-donor. Furthermore, three bent **Fmoc-4-I-Phe** molecules interact through these iodine···pi interactions, resulting in the formation of triangular hydrophobic pockets where the fluorenyl rings are confined. This halogen-bond driven assembly of hydrophobic pockets may be relevant for the understanding of the enhanced

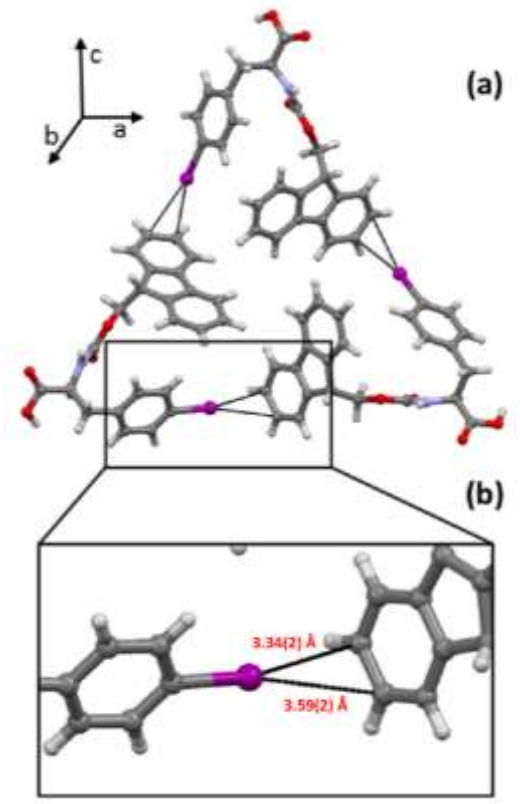

Fig.4. Single crystal X-ray structure of the **Fmoc-4-I-Phe** derivative. a) Iodine···pi interactions lead to the self-assembly of three bent amino acid molecules into a triangular hydrophobic pocket where the fluorenyl rings are confined. b) Magnification of the iodine···pi interactions involving iodine and one of the bonds of the fluorenyl moiety.

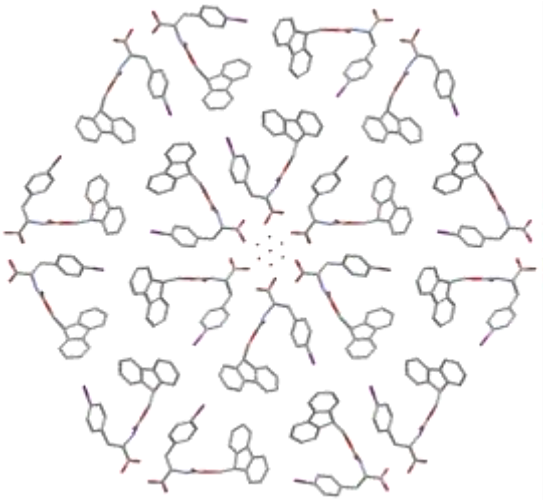

Fig. 5. View down crystallographic *c* axis of the single crystal X-ray structure of **Fmoc-4-I-Phe**, showing the triangular stacks of molecules packing in a hexagonal manner and surrounding the water channel running along *c* and lined with the amino acid carboxylic groups.

gelation properties demonstrated by **Fmoc-4-I-Phe** compared to Fmoc-Phe. The triangular stacks then pack in a hexagonal manner around a water channel lined with the amino acid carboxylic groups running along the crystallographic *c* axis (Fig. 5).

Importantly, the same triangular hydrophobic pocket is also present in the structure of **Fmoc-4-Br-Phe**. In particular, Br···C16 distance of 3.26(1) Å and C-Br···C16 angle of 167.9(4)° were found (Fig. S6). On the other hand, no such kind of halogen bonding pattern was found in the structures of the Cl and F derivatives, which yielded the weakest gels. While the Cl atom in **Fmoc-4-Cl-Phe** is only involved in weak hydrogen bonds with the H11B and H12 of the fluorenyl moiety, the F atom in **Fmoc-4-F-Phe** is not involved in any relevant interaction (Figs. S4 and S5).

In conclusion, we have successfully reported the single crystal X-ray structures of four *p*-halogen substituted Fmoc-phenylalanine derivatives. This work contributes to increase by twofold the number of structures reported in the CSD related to this important class of hydrogelators. Halogen for hydrogen substitution on the *p* position of phenylalanine benzene ring is a minimal structural modification, however, it deeply influences its self-assembly features as demonstrated by the reported crystal structures. In particular, I and Br derivatives show the emergence of halogen bonding, which dictated the organization of hydrophobic pockets alternating to water channels. On the other hand, the Cl and the F derivatives do no present such halogen bonding and show structures very similar to the two previously published Fmoc-Phe crystals, which is understandable due to the fact that halogen bond strength decreases with the halogen atom polarizability. Gelation properties of the halogenated amino acids were also studied and all measured parameters similarly follow the polarizability scale. Although the reported crystal structures were not obtained from the same solvent mixture used for gelation studies, the influence exerted on the Fmoc-Phe self-assembly by the heavier halogen atoms is evident. The reported study try to correlate, for the first time, the various halogen-bond donor abilities of halogenated amino acids to their gelation efficiency. This understanding may pave the way to the routine incorporation of halogen bonding as a strategy to modulate gelator molecule structures and properties.[32-34]

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