Squaramide-based supramolecular polymers: from self-assembly to in vivo application
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CHAPTER 2

Aromatic gain in a supramolecular polymer

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Chapter 2

2.1 Abstract
The synergy of aromatic gain and hydrogen bonding in a supramolecular polymer is explored. Partially aromatic bis-squaramide bolaamphiphiles were designed to self-assemble through a combination of hydrophobic, hydrogen-bonding, and aromatic effects into stiff, high-aspect-ratio fibers. UV and IR spectroscopy show electron delocalization and geometric changes within the squaramide ring indicative of strong hydrogen bonding and aromatic gain of the monomer units. The aromatic contribution to the interaction energy was further supported computationally by nucleus-independent chemical shift (NICS) and harmonic oscillator model of aromaticity (HOMA) indices, demonstrating greater aromatic character upon polymerization: at least 30% in a pentamer. The aromatic gain–hydrogen bonding synergy results in a significant increase in thermodynamic stability and a striking difference in aggregate morphology of the bis(squaramide) bolaamphiphile compared to isosteres that cannot engage in this effect.
2.2 Introduction
Aromatic gain is considered to be a thermodynamic driving force in several organic reactions. In aromatic substitutions, Bergman cyclizations, aromatic Cope rearrangements, [1,5]H sigmatropic shifts and [4+2] cyclizations, among others, the restoration of aromaticity helps to explain their exergonic character and increased efficiency.1–6 In supramolecular polymers,7–17 where monomers are held together by non-covalent interactions resulting in higher-order aggregates with various topologies, the concept of aromatic gain in the construction of such systems is unexplored.

Aromaticity has captivated chemists since its introduction as a concept 150 years ago by Kekulé.18 In contrast to other chemical concepts, such as electronegativity or van der Waals radii, aromaticity is not a direct physical observable and its exact definition is the subject of much debate.19–22 Classically, cyclic π-conjugated compounds are aromatic when they show differences in geometric, energetic, and magnetic criteria relative to their acyclic analogues.23–25 In recent years, computational methods such as nucleus-independent chemical shift (NICS),24,26 harmonic oscillator model of aromaticity (HOMA),27 and aromatic stabilization energies (ASE),25 have grown in use to describe aromaticity. In the case of NICS, excellent correlation has been reported with experimental nuclear magnetic resonance data as well as other descriptors of aromaticity,19 thus opening the door to predict the aromaticity of new compounds and structures a priori. Very recently, NICS calculations demonstrated reciprocal hydrogen-bonding–aromaticity relationships that can have important consequences on the strength of hydrogen-bonding interactions.28

Squaramides,29,30 which are composed of two NH hydrogen-bond donors opposite two carbonyl hydrogen-bond acceptors on a conformationally rigid cyclobutene ring, are predicted to show partial aromatic character.29,30 This character arises from the delocalization of the nitrogen lone pair into the cyclobutenedione ring system (Hückel's rule: $(4n + 2)$ π electrons, $n = 0$).31 In the solid state, catemers of dissecondary squaramides arranged in a head-to-tail motif have been reported32 and they may benefit from strong resonance-assisted hydrogen bonding (RAHB) interactions similar to squaric acids.33 Applications of the squaramide unit have been found in medicinal chemistry, catalysis, and anion recognition.34–36 The capacity of squaramides to form strong hydrogen bonds that simultaneously
Figure 2.1. (a) Structure of the squaramide-based bolaamphiphile 1. (b) Self-assembly of 1 into fibrillar structures, and depolymerization by hexafluoroisopropanol (HFIP). Within the fibrillar structure, hydrogen bonds are proposed to occur parallel to the fiber axis while π-interactions between squaramide bolaamphiphiles occur in the lateral direction, as depicted. (Bottom) Proposed hydrogen-bonding interactions between squaramide monomers.
influence their aromatic character is highly appealing to guide the formation of increasingly stable supramolecular polymers. Herein, I incorporate the squaramide synthon into a bolaamphiphilic construct that self-assembles into stiff fibers in water, and I explore the coupling of hydrogen-bonding and aromatic gain using experiment and computation.

2.3 Results and discussion

Compound 1 consists of two oligo(ethylene glycol) methyl ether chains opposite a central hydrophobic core with two embedded squaramide units (Figure 2.1). \(^1\)H NMR spectra of 1 in D\(_2\)O were suggestive of strong aggregation that is resistant to thermal denaturation up to 65 °C (Figure S2.1). Only \(^1\)H NMR spectra recorded in CDCl\(_3\) or HFIP-d\(_2\) were well-resolved and suggestive of various degrees of depolymerization.

![Cryo-TEM images of 1 in aqueous solution (1 wt%) after sonication: (a) t=0, and (b) t=2 weeks. Inset: Histograms of length (a) and width (b).](image)

The effect of the squaramide synthon on the self-assembly of 1 in water was evaluated by cryo-transmission electron microscopy (cryo-TEM) and atomic force microscopy (AFM). Cryo-TEM images of 1 (1 wt%) displayed stiff, micrometer-long fibrils with a uniform diameter. Short, rod-like structures on the order of 12.6 ± 2.4 nm in length were found upon sonication (Figure 2.2a) and slowly progressed into micrometer-long fibers. Fibers of 1 were 6.4 ± 1.2 nm in diameter, on par with the length of the hydrophobic region of the bolaamphiphile (Figure 2.2b). By small-angle X-ray scattering measurements (SAXS, Figures 2.3, S2.3 and S2.4), a
cross-sectional radius \( (r_{cs}) \) of 3.5 nm and a cross-sectional mass per unit length \( (M_L) \) of \( 2.5 \times 10^{20} - 6.0 \times 10^{20} \) g nm\(^{-1} \) was determined for fibers of 1, indicating that approximately 10–30 squaramide bolaamphiphiles per nm can be found along the fiber axis. These results suggest that hydrogen bonds parallel to the fiber axis drive the formation of highly anisotropic fibers, meanwhile the combination of hydrophobic and π-interactions between squaramide moieties facilitate the assembly of several bolaamphiphiles in the lateral direction (Figure 2.1). To better understand the consequence of self-assembly on the squaramide synthon, spectroscopy at the molecular level was pursued. UV-Vis spectroscopy of 1 in water showed maxima at 255 and 329 nm, and a shoulder around 310 nm at the various concentrations tested (Figure S2.5). Disruption of the polymerized state was achieved using both temperature (Figure 2.4a) and various solvents (Figure 2.4b). More specifically, hexafluoroisopropanol (HFIP), a potent hydrogen bond disruptor, promoted depolymerization resulting in the gradual loss of the red-shifted hydrogen-bonded squaramide N-H proton-donor π–π* bands (329 nm) and the blue-shifted C=O proton-acceptor n–π* bands (255 nm), concomitant with the growth of the non-hydrogen bonded monomer band (310 nm) (Figure 2.5).\(^{37} \) These experimental trends are in agreement with TD-DFT calculations (Table S2.2), where two superimposed absorption bands of similar intensity corresponding to the HOMO–LUMO and HOMO–(LUMO+1) transitions are predicted for the monomer; in oligomers, the high wavelength band is

**Figure 2.3.** Small-angle X-ray scattering profiles of squaramide fibers collected at concentrations of 4 and 5 mg mL\(^{-1} \).
progressively red-shifted while the other appears blue-shifted. Self-assembly of 1 through strong hydrogen bonding interactions results in increased orbital overlap between squaramide units and further electron delocalization within the individual squaramide rings, enabling aromatic gain to occur (see supporting information chapter 2).

Figure 2.4. (a) Temperature-dependent UV-Vis spectra of 1 (0.029 mM) from 20 to 75 °C. (b) UV-Vis spectra of 1 (0.029 mM) in various solvents at room temperature.
Geometric changes to the squaramides upon self-assembly were examined by IR spectroscopy. Solutions of 1 (2 wt%) in D$_2$O were measured at room temperature. Above the amide I region, a small broad band at 1796 cm$^{-1}$, consistent with squaramide ring breathing, was found experimentally (Figure 2.6) and confirmed by modeling (Table S2.3). In the amide I region, asymmetric and symmetric C=O stretches (1687, 1676, and 1642 cm$^{-1}$) of the squaramide and carbamate moieties were recorded. Strong hydrogen bonding of the squaramide units was observed through the N-H stretch at 3162 cm$^{-1}$ (inset in Figure 2.6). In HFIP-d$_2$, the blue-shifting of several bands such as the ring breathing (13 cm$^{-1}$) and symmetric C=O stretch (14 cm$^{-1}$) modes were observed and suggestive of depolymerization. Owing to lack of transparency of HFIP-d$_2$ in the N-H region, an approximation for free N-H stretch (3452 cm$^{-1}$) was made for 1 in CDCl$_3$ (Figure 2.7) The experimental data correlated well with ab initio calculations. These results revealed that bond lengths in the squaramide are systematically altered as a function of oligomer length (Figure 2.8a): double bonds become longer, whereas single bonds shorten, resulting in a ring with less bond length alternation. With these bond lengths, it was computed HOMA values of -0.015 and 0.516 for the isolated monomer and the central monomer in a pentamer, respectively, while a value of one is defined for aromatic compounds. Experiments point to strong

**Figure 2.5.** UV-Vis spectrum of 1 in water (0.029 mM) as a function of HFIP concentration (red line before HFIP titration, blue line after HFIP titration with 5 v/v%).
Figure 2.6. IR spectrum of 1 recorded in the amide I region and amide II in D$_2$O and [D$_2$]HFIP. Inset: N-H and C-H stretch region.

Figure 2.7. Infrared spectrum of 1 (2 wt%) in CHCl$_3$. 
hydrogen bonding and computed geometric considerations demonstrate an increase in aromatic character within the squaramide unit due to supramolecular polymerization.

NICS-scan profiles, a measurement of the magnetic shielding above and at the center of the ring, were computed on an axis passing though the center of the squaramide ring for monomers to pentamers to quantify the aromatic character upon oligomerization (NICS and NICS-scan profiles were computed by positioning

\[ \text{Figure 2.8.} \text{ (a) Geometric changes (computed at the M06-2X/6-311+ +G(d,p) level of theory) in N-methyl squaramide between an isolated monomer (normal text) and the central monomer in a pentamer (in bold italics). (b) NICS values at a point 0.6 Å from the ring plane for an isolated monomer and each monomer in oligomers of length 2–5 (GIAO-M06-2X/6-311+ +G(d,p)).} \]
a ghost atom at the center of the squaramide ring ranging from between 0 and 5 Å above it, see figure S2.10 with the NICS-scans for dimers, trimers, tetramers and pentamers). The profiles for the individual squaramide units were negative overall and exhibited a minimum around 0.6 Å, consistent with an aromatic ring. Upon increasing oligomer length, the NICS values became more negative without a change in the shape of the curves, suggestive of increased aromaticity. In particular, the change in NICS at 0.6 Å from the ring plane (Figure 2.8b) when going from a monomer (-6.8) to the central monomer of a pentamer (-8.4), is in line with previous reports. Additionally, the aromatic stabilization energy accounts for at least 30% of the total interaction energy in a squaramide pentamer (-85.6 kJ mol⁻¹ out of -271.7 kJmol⁻¹ including BSSE correction) using a heterodimer of vinylogous amides that cannot exhibit aromaticity as a reference (Table S2.8 and S2.9).
I further investigated the thermodynamic consequence of aromatic gain experimentally by measuring the critical aggregation concentration (CAC) using static light scattering (SLS). An order of magnitude lower CAC, corresponding to a free energy difference $\Delta \Delta G_{\text{agg}} = -5.25 \text{ kJ mol}^{-1}$, was obtained for molecule 1 ($7.94 \times 10^{-6} \text{ M}$) in comparison to urea-based analogue 5 ($7.41 \times 10^{-5} \text{ M}$; see the supporting information section 2.6.15) (Figure 2.9a). These results are further supported by DFT calculations, where the interaction energy computed per hydrogen bond of
urea oligomers was found to be smaller (-23.9 vs. -35.6 kJ mol$^{-1}$ for pentamers) and does not increase as steeply with oligomer length (+18% vs. +30%). Intriguingly, a striking difference in the fiber morphology was found above the CAC of both molecules. Whereas 1 consistently formed long and stiff micron-sized fibers (Figure 2.9b), short worm-like or spherical aggregates were obtained for 5 (Figure 2.9c). Given the similarity of the hydrophilic and hydrophobic blocks, these results suggest that the coupling of aromatic gain and hydrogen-bonding in addition to the structural rigidity of the squaramide units act collectively to lower the critical aggregation concentration and propagate the formation of high-aspect-ratio fibers in water.

2.4 Conclusions
I found that the capacity of squaramides to couple hydrogen bonding and aromaticity facilitates the formation of robust supramolecular polymers. The gain in aromatic character upon assembly is demonstrated through bond length equalization, decreased NICS values, high aromatic stabilization (ASE) values, and increased thermodynamic stability of the resultant aggregates. These changes are in accordance with the geometric, magnetic, and energetic criteria used to describe aromaticity. Moreover, the aromatic gain is a significant component of the total interaction energy of squaramide-based supramolecular polymers, explaining the observed increase in thermodynamic stability relative to the monomers and to their urea counterparts. In summary, this self-tuning behavior between hydrogen bonding and aromaticity within the squaramide ring system cannot be achieved by other simple ditopic synthons, such as ureas or amides, commonly used to construct supramolecular polymers. Therefore, I anticipate that the information gained here can enrich the palette of hydrogen-bonding monomers used for supramolecular polymer assembly by implementing aromaticity as a design consideration.

2.5 References


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3513–3560.


2.6 Supporting Information

2.6.1 Materials
All solvents and reagents were obtained from commercial suppliers and were used without further purification. O-methyl-undecaethylene glycol was obtained from Polypure. Deuterated dimethylsulfoxide and chloroform were purchased from Euriso-top. Palladium on matrix activated carbon, triethylsilane and all other commercial grade reagents and chemicals were bought from Sigma Aldrich and used as received. Water was deionized before use.

2.6.2 General methods
The bis-squaramide and bisurea bolaamphiphiles were purified using a Grace Reveleris X1 flash chromatography system equipped with a C18 column. $^1$H NMR and $^{13}$C NMR spectra were acquired on a Bruker DMX-400 (400 MHz) operating at 400 MHz for $^1$H NMR and 100 MHz for $^{13}$C NMR at 298K. LC-MS analysis was performed on a Finnigan Surveyor HPLC system equipped with a Gemini C18 50 x 4.60 mm column (UV detection at 200-600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI. The mobile phase was a gradient of 10-90% of H$_2$O-CH$_3$CN with 0.1% trifluoroacetic acid over 13.5 minutes. MALDI-TOF-MS (Matrix-assisted laser desorption ionization–time-of-flight) spectra were recorded on a Bruker microflex LRF mass spectrometer in reflection positive-ion mode using α-cyano-4-hydroxycinnamic acid as a matrix on a ground steel target plate. AFM images were collected on a Veeco Multimode AFM with a Nanoscope IIIa controller device at ambient temperature in tapping mode. Silicon cantilever tips were used for image acquisition (OMCL-AC240TS-R3, 50-90 kHz, 0.6-3.5 N/m from Olympus). The scanning speed was at a line frequency of 1.0 Hz, and the original images were made at a resolution of 512 x 512 pixels. Cryogenic TEM samples were applied to a Quantifoil R2/2 holey carbon film and freeze-plunged into liquid ethane in a FEI Vitrobot Mark IV. The grids were imaged in a FEI Titan Krios at 300 KV using a Falcon Direct Detector (Netherlands Centre for Nanoscopy (NeCN)) or an FEI F20 at 200 kEV using a Gatan Ultrascan camera (Leids Universitair Medisch Centrum (LUMC)). Small angle X-ray scattering measurements were performed on a SAXSLAB GANESHA 300 XL SAXS system equipped with a GeniX 3D Cu Ultra Low Divergence micro focus sealed tube source producing X-rays with a wavelength $\lambda = 1.54$ Å at a flux of 1x10$^8$ ph/s and a Pilatus 300K silicon pixel detector with 487 x 619 pixels of 172 μm$^2$ in size placed at two sample-to-detector distances of 713 and 1513 mm respectively to access a
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$q$-range of $0.006 \leq q \leq 0.44 \ \text{Å}^{-1}$ with $q = 4 \pi / \lambda (\sin \theta / 2)$. Silver behenate was used for calibration of the beam centre and the $q$ range. Samples were contained at room temperature in 2 mm quartz capillaries (Hilgenberg GmbH, Germany). The two-dimensional SAXS patterns were brought to an absolute intensity scale using the calibrated detector response function, known sample-to-detector distance, measured incident and transmitted beam intensities, and azimuthally averaged to obtain one dimensional SAXS profiles. The scattering curves of the self-assembled fibers were obtained by subtraction of the scattering contribution of the solvent and quartz cell. The small angle X-ray scattering profiles were analyzed using the software package SasView (http://www.sasview.org/). Absorption spectra were obtained on a Cary 300 UV-Vis spectrophotometer. All measurements were carried out using a quartz cuvette with a 1 cm path length. Transmission FTIR spectra were measured using a Bio-Rad Excalibur spectrometer equipped with a nitrogen cooled MCT detector. Static light scattering (SLS) measurements were performed using a Malvern Zetasizer Nano ZS ZEN3500. The laser wavelength was 633 nm and the scattering angle was 173°.
2.6.3 Synthetic routes

Synthesis of 1

Scheme S2.1. Synthetic route to obtain 1.

Synthesis of compound 2:

N-(benzyloxy carbonyloxy)succinimide (2.89 g, 11.60 mmol) was dissolved in 150 mL chloroform and added dropwise over 1 hour to a cooled (0°C), stirring solution of 1,10-diaminodecane (9.98 g, 58.00 mmol) dissolved in 150 mL chloroform. The reaction was allowed to reach room temperature and continued to stir for 18 hours. The solution was then concentrated by evaporation, ethyl acetate was added and the mixture washed 3x with water. The aqueous layers were discarded and the ethyl acetate layer was washed 3x with 1 M HCl. A white precipitate
formed in the organic layer, which was collected by filtration and washed with ethyl acetate to yield the product as a white crystalline solid.

Yield: 2.38 g, 67.1 % ¹H-NMR (δH[ppm], DMSO-d₆, 400 MHz): 7.90 (br s, 3H), 7.41-7.25 (m, 5H), 5.02 (s, 2H), 3.02-2.95 (m, 2H), 2.79-2.72 (m, 2H), 1.57-1.50 (m, 2H), 1.42-1.26 (m, 14H). ¹³C-NMR (δC[ppm], DMSO-d₆, 100 MHz): 156.03, 137.29, 128.29, 127.68, 127.64, 65.01, 40.13, 38.73, 29.35, 28.82, 28.72, 28.64, 28.46, 26.92, 26.18, 25.76.

**Synthesis of compound 3:**

O-methyl-undecahydropolyglycol (0.60 g, 1.16 mmol) was activated with 1,1'-carbonyldiimidazole (0.75 g, 4.64 mmol) in 25 mL chloroform for 2 hours at room temperature (LC-MS: t=4.14 min, m/z: 611.27 [M+H]+). Subsequently, 2 (0.43 g, 1.40 mmol) and DIPEA (0.404 mL, 2.32 mmol) were added to the reaction mixture and refluxed overnight. The product was purified by flash column chromatography using a 10-90% CH₃CN/H₂O gradient over 25 minutes on a C18 silica column. The product was concentrated by evaporation and lyophilized overnight to obtain a white solid.

Yield: 0.7 g, 68.7 % ¹H-NMR (δH[ppm], CDCl₃, 400 MHz): 7.38-7.34 (m, 5H), 5.12 (s, 2H), 4.24-4.22 (m, 2H), 3.71-3.56 (m, 42H), 3.40 (s, 3H), 3.21-3.15 (m, 4H), 1.52-1.48 (m, 4H), 1.32-1.27 (m, 12H). ¹³C-NMR (δC[ppm], CDCl₃, 100 MHz): 156.41, 136.71, 128.52, 128.12, 128.08, 71.93, 70.59, 70.51, 69.73, 66.60, 63.90, 59.04, 41.10, 29.94, 29.41, 29.20, 26.71, 26.69. LC-MS: t = 7.64 min, m/z: 849.20 [M+H]+. MALDI-TOF-MS: m/z calc: 849.07; found: 871.426 [M+Na]+, 887.384 [M+K]+.

**Synthesis of compound 4:**

Compound 3 (0.20 g, 0.24 mmol) was dissolved in 5 mL methanol and Pd/C (2.5 mg, 0.024 mmol) was added. The solution was briefly degassed with argon, prior to the dropwise addition of triethylsilane (0.38 mL, 2.40 mmol). The addition of triethylsilane resulted in an effervescent solution and once complete, the solution was filtered over Celite to remove the remaining Pd/C. The filtrate was concentrated by evaporation and a gentle stream of air. The dried product was redissolved in 25 mL chloroform and 3,4-dibutoxy-3-cyclobutene-1,2-dione (67 µL, 0.31 mmol) and DIPEA (31 µL, 0.24 mmol) were added to the reaction mixture. The reaction mixture was stirred and refluxed overnight and purified by flash
column chromatography using a gradient of 10-90% CH$_3$CN/H$_2$O over 25 minutes on a C18 silica column. The product was concentrated by evaporation and lyophilized overnight to obtain compound 4 as white solid.

Yield: 168.6 mg, 81.0 % $^1$H-NMR (δ$_H$[ppm], CDCl$_3$, 400 MHz): 6.34 (br s, 1H), 4.96 (br s, 1H), 4.79-4.74 (m, 2H), 4.25-4.22 (m, 2H), 3.72-3.41 (m, 47H), 3.20-3.15 (m, 2H), 1.84-1.79 (m, 2H), 1.66-1.30 (m, 18H), 1.00 (s, 3H). $^{13}$C-NMR (δ$_C$[ppm], CDCl$_3$, 100 MHz): 177.16, 172.53, 156.70, 73.32, 71.90, 70.53, 70.46, 69.64, 63.78, 58.99, 44.85, 41.01, 32.03, 30.69, 29.92, 29.37, 29.18, 29.08, 26.68, 26.35, 18.66, 13.70. LC-MS: $t$=7.28 min, $m/z$: 867.40 [M+H]$^+$.

MALDI-TOF-MS: $m/z$ calc: 867.08; found: 889.481 [M+Na]$^+$, 905.479 [M+K]$^+$.

**Synthesis of compound 1:**

Compound 4 (160.0 mg, 0.19 mmol) was dissolved in 25 mL chloroform with DIPEA (50 µL, 0.38 mmol) and 1,7-heptanediame (14 µL, 0.10 mmol) were added and refluxed overnight. The product was purified by flash column chromatography using a gradient of 10-90% CH$_3$CN/H$_2$O over 25 minutes on a C18 silica column. The product was concentrated down by evaporation and lyophilized overnight to obtain a white solid.

Yield: 100.3 mg, 62.8 % $^1$H-NMR (δ$_H$[ppm], CDCl$_3$, 400 MHz): 4.24-4.22 (m, 4H), 3.71-3.56 (m, 84H), 3.40 (s, 6H), 3.18-3.15 (m, 4H), 3.01-2.82 (m, 8H), 1.68-1.64 (m, 8H), 1.51-1.29 (m, 34H). $^{13}$C-NMR (δ$_C$[ppm], CDCl$_3$, 100 MHz): 184.30, 168.40, 136.24, 71.99, 70.63, 70.59, 69.74, 64.12, 59.12, 45.09, 43.99, 41.09, 30.90, 30.43, 29.96, 29.85, 29.47, 29.26, 29.00, 28.74, 28.33, 26.76, 26.44, 25.19. LC-MS: $t$ = 6.97 min, $m/z$: 1715.73 [M+H]$^+$. MALDI-TOF-MS: $m/z$ calc: 1716.16; found: 1716.306 [M+H]$^+$, 1738.167 [M+Na]$^+$, 1754.129 [M+K]$^+$. 
**Synthesis of 5**

Scheme S2.2 Synthetic route of 5.

**Synthesis of compound 5:**

Compound 3 (0.41 g, 0.48 mmol) was dissolved in 10 mL methanol and Pd/C (5.00 mg, 0.048 mmol) was added. The solution was degassed with argon and triethylsilane was added dropwise (0.70 mL, 4.8 mmol). The addition of triethylsilane resulted in an effervescent solution and once finished, the solution was filtered over Celite. The filtrate was concentrated by evaporation and dried by a gentle stream of air. The dried product was redissolved in 10 mL of water and stirred at 0 °C. Initially, 1,1'-carbonyldiimidazole (86 mg, 0.53 mmol) was added to the reaction mixture at 0 °C. After 30 min, another identical aliquot of 1,1'-carbonyldiimidazole was added. The reaction mixture was stirred at 0 °C for another 30 minutes and progress of the reaction was followed by the appearance of the product peak by LC-MS (t=5.50 min, m/z: 809.40 [M+H]+). Once CDI activation of the free amine was achieved, the solution was lyophilized and redissolved in CHCl₃. Aliquots of 1,7-heptanediame (7.27 µL, 0.048 mmol at a time) were added to the solution gradually until the reaction mixture showed full conversion to 5 by LC-MS. The product was purified by flash column chromatography on a C18 silica column using a gradient of 10-90% CH₃CN/H₂O.
over 40 minutes. The product was concentrated by evaporation and lyophilized overnight to obtain compound 5 as a white solid.

Yield: 183 mg, 23.7 %. \(^1\)H-NMR (\(\delta_H [\text{ppm}]\), CDCl\(_3\), 400 MHz): 4.25-4.22 (m, 4H), 3.73-3.57 (m, 84H), 3.41 (s, 6H), 3.21-3.16 (m, 12H), 1.58-1.45 (m, 12H), 1.41-1.26 (m, 30H). \(^{13}\)C-NMR (\(\delta_C [\text{ppm}]\), CDCl\(_3\), 100 MHz): 158.90, 156.59, 72.01, 70.64, 69.79, 63.88, 59.14, 41.10, 40.47, 39.91, 30.41, 29.97, 29.78, 29.43, 29.39, 29.32, 29.21, 28.02, 26.94, 26.71, 26.12. LC-MS: t = 7.34 min, \(m/z\): 1611.87 [M+H]\(^+\). MALDI-TOF-MS: \(m/z\) calc: 1612.08; found: 1612.338 [M+H]\(^+\), 1634.286 [M+Na]\(^+\), 1650.253 [M+K]\(^+\).

### 2.6.4 Lower critical solution temperature

![Graph showing LCST determination of 1 in water (2.33 mM) by UV-vis spectroscopy. The optical density was recorded as a function of temperature at 450 nm, where the molecules do not absorb light. The large increase in optical density above 68 °C represents the LCST for 1.](image)

**Figure S2.1.** LCST determination of 1 in water (2.33 mM) by UV-vis spectroscopy. The optical density was recorded as a function of temperature at 450 nm, where the molecules do not absorb light. The large increase in optical density above 68 °C represents the LCST for 1.
2.6.5 Cryogenic-Transmission Electron Microscopy (cryo-TEM)

Cryogenic TEM samples were prepared by applying 3 μL sample of 1 (5.82 mM) and 5 (6.21 mM) in water to a glow-discharged Quantifoil R2/2 holey carbon film, blotting excess liquid off for 1 second and plunge-freezing it into liquid ethane using a FEI Vitrobot Mark IV. The grids were imaged in a FEI Titan Krios at 300 KV using a Falcon Direct Electron Detector (Netherlands Centre for Electron Nanoscopy, NeCEN) (sample 1) or a FEI Tecnai F20 at 200 kEV using a Gatan UltraScan camera (Leids Universitair Medisch Centrum (LUMC)) (sample 5) at between -2 and -5 micron under focus.

2.6.6 Atomic force microscopy (AFM)

A stock solution of 1 (6 mM) was diluted to 12 μM in water, and drop-casted (25 μL) on freshly cleaved mica. The samples were dried overnight at room temperature. The obtained data were processed using the WSxM software (Nanotec Electronica).40

Figure S2.2. AFM height (a), amplitude (b) and phase (c) images of 1, deposited on mica 1 hour after dissolution (3 x 3 μm scale).
2.6.7 Small-angle X-ray scattering (SAXS)

![Graph showing SAXS profiles](image)

**Figure S2.3**: Small angle X-ray profiles of squaramide fibers collected at a concentration of 4 and 5 mg mL\(^{-1}\) normalized by weight concentration (i.e., the symbols correspond to experimental data (I/c vs. q)).

Small angle X-ray scattering (SAXS) experiments were performed to probe the structure of the self-assembled squaramide fibers in water at room temperature. The SAXS profiles of the 4 and 5 mg mL\(^{-1}\) sample are given in Figure S2.3. In the low-q regime the scattering profiles decay with a powerlaw slope of unity, which is characteristic for scattering profiles of 1D objects with a length beyond the resolution of the experiment (\(\sim \pi/q_{\text{min}} = 48\) nm). Upon normalization to 1 mg mL\(^{-1}\) the SAXS profiles taken at 4 and 5 mg mL\(^{-1}\) nearly superpose, which means that interfiber interactions are insignificant at the length scales probed. A Casassa–Holtzer plot of the same data is given in Figure S2.4.

The experimental data can be readily modeled using a form factor developed for homogeneous cylinders (lines represent fit to experimental data in Figure S2.3). Several other, more elaborate form factors were also tested, including those of core-shell, and flexible homogeneous cylinders. Since these more complex models do not significantly improve the goodness of fit, only the results of the simplest form factor are presented. Since the length L of the fibers is beyond the experimental resolution we fix L at an arbitrary value of 200 nm. We also fix \(\rho_{\text{solvent}} = 9.37 \times 10^6\) Å\(^{-2}\). From the modeling of the SAXS data, we obtain values for the
radius of the fibers, \( r_{cs} \), and their electron length density, \( \rho_{cyl} \). For the 4 and 5 mg/mL samples, we obtain \( <r_{cs}> = 3.5 \text{ nm} \), and \( \rho_{cyl} = 10.4 \times 10^6 \text{ Å}^{-2} \), respectively.

To extract the cross-sectional mass per unit length, \( M_L \), from the scattering profiles we use:

\[
\frac{d\Sigma(q)}{d\Omega} = I(q) = \frac{\pi I_{cs}(q)}{q}
\]

\[
M_L = \frac{I_{cs}(0)}{c \Delta \rho_M^2}
\]

with the electron length density difference per mass, \( \Delta \rho_M \), and the height of the Holtzer plateau, \( I_{cs} \) as indicated by the dash-dotted lines in Figure S2.4. Computation of \( \Delta \rho_M \) from the squaramide composition \( C_{53}H_{93}N_{5}O_{30} \) and the measured specific volume, \( \nu = 0.83 \text{ cm}^3 \text{ g}^{-1} \) gives \( \Delta \rho_M = 1.29 \times 10^{10} \text{ cm} \text{ g}^{-1} \), which is comparable to the value obtained from the form factor fit, which gives \( \Delta \rho_M = 0.87 \times 10^{10} \text{ cm} \text{ g}^{-1} \). Using these values we obtain \( M_L = 2.5 - 6.0 \times 10^{20} \text{ g nm}^{-1} \), which corresponds to 10 – 30 squaramide bolaamphiphiles per nm.

Table S2.1. Structural parameters extracted from the SAXS profiles of the squaramide bolaamphiphiles

<table>
<thead>
<tr>
<th>( \nu ) [wt%]</th>
<th>( \nu ) [cm$^3$ g$^{-1}$]</th>
<th>( \frac{q(q)/c}{\text{cm}^2 \text{g}^{-1}} )</th>
<th>( I_{cs} = \frac{q(q)}{\pi} ) [cm$^2$]</th>
<th>( \Delta \rho_M^2 ) [10$^{10}$ cm$^{-1}$ g$^{-1}$]</th>
<th>( M_L ) [10$^{20}$ g nm$^{-1}$]</th>
<th>SQ/L nm$^{-1}$</th>
<th>( \Delta \rho_M^2 ) [10$^{10}$ cm$^{-1}$ g$^{-1}$]</th>
<th>( M_L ) [10$^{20}$ g nm$^{-1}$]</th>
<th>SQ/L nm$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4%</td>
<td>0.83</td>
<td>1.29x10$^5$</td>
<td>1.64x10$^5$</td>
<td>1.68</td>
<td>2.45</td>
<td>11.5</td>
<td>0.74</td>
<td>5.51</td>
<td>25.9</td>
</tr>
<tr>
<td>0.5%</td>
<td>0.83</td>
<td>1.39x10$^5$</td>
<td>2.22x10$^5$</td>
<td>1.68</td>
<td>2.65</td>
<td>12.5</td>
<td>0.74</td>
<td>5.96</td>
<td>28.0</td>
</tr>
</tbody>
</table>

$^a$Taken from Holtzer plateau at \( 0.0065 \leq q \leq 0.0164 \text{ Å}^{-1} \)
Figure S2.4: Casassa–Holtzer plot of the scattering profiles in Figure S2.3. The Holtzer plateaus (0.0065 ≤ q ≤ 0.0164 Å⁻¹) are indicated by dash-dotted red and black lines.

2.6.8 UV-Vis spectroscopy
Serial dilutions of a stock solution of 1 (5.82 mM) were performed in water. A UV-Vis spectrum for each sample was recorded from 400-200 nm. Two peaks were observed for 1 at 255 and 329 cm⁻¹ and a shoulder at 310 cm⁻¹.

Figure S2.5.1. Concentration-dependent UV-Vis spectra of 1 (absorbance vs wavelength).
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Figure S2.5.2. Concentration-dependent UV-Vis spectra of 1 (ε vs wavelength).
2.6.9 TD-DFT calculations
The time-dependent DFT (TD-DFT) method\textsuperscript{41} was used to compute UV-Vis absorption spectra for a squaramide monomer up to a pentamer. The M06-2X/6-31+G(d,p) level of theory was used, which significantly reduces the computational cost, but still yields a spectrum comparable with M06-2X/6-311+G(d,p) (a test was performed with the dimer structure). We employed a continuum model of water of type SMD\textsuperscript{42} to describe the effect of solvation.

**Table S2.2.** Major absorption bands for squaramide monomers (n=1) and oligomers (n=2-5).

<table>
<thead>
<tr>
<th>n</th>
<th>λ</th>
<th>Strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>266.75</td>
<td>0.3555</td>
</tr>
<tr>
<td></td>
<td>263.98</td>
<td>0.4299</td>
</tr>
<tr>
<td>2</td>
<td>271.85</td>
<td>0.8728</td>
</tr>
<tr>
<td></td>
<td>261.16</td>
<td>0.7281</td>
</tr>
<tr>
<td>3</td>
<td>274.43</td>
<td>1.4391</td>
</tr>
<tr>
<td></td>
<td>258.25</td>
<td>0.5650</td>
</tr>
<tr>
<td>4</td>
<td>276.07</td>
<td>2.0098</td>
</tr>
<tr>
<td></td>
<td>257.79</td>
<td>1.3904</td>
</tr>
<tr>
<td>5</td>
<td>277.18</td>
<td>2.5755</td>
</tr>
<tr>
<td></td>
<td>257.57</td>
<td>1.3952</td>
</tr>
</tbody>
</table>

**Figure S2.6.** Frontier molecular orbitals of the squaramide monomer computed at the M06-2X/6-31+G(d,p) level of theory. Left: HOMO; right: LUMO.
**Figure S2.7.** Frontier molecular orbitals of the squaramide dimer computed at the M06-2X/6-31+G(d,p) level of theory. Top left: HOMO-1; top right: HOMO; bottom left: LUMO; bottom right: LUMO+1.
2.6.10 Fourier transform infrared (FTIR)

Samples of 1 were dissolved in D$_2$O, HFIP-d$_2$ or CHCl$_3$ to provide a final concentration of 11.7 mM. Measurements in liquid phase were performed in a CaF$_2$ cell for liquid samples at room temperature. Liquid samples were measured after the addition of the solvent to the solid.

**Figure S2.8.** Infrared spectrum of 1 in the solid state. Solid FTIR was recorded in ATR mode.

**Figure S2.9.** Infrared spectrum of 1 (5.82 mM, 11.7 mM, 29.1 mM) in water.
2.6.11 Infrared spectra predictions
A frequency calculation was performed to verify that structures were at a minimum of energy and to obtain vibrational spectroscopy predictions at the same theory level of geometry optimization. The zero point energy correction was also included in our calculations.

Table S2.3. Infrared spectra assignment (wavenumbers in cm\(^{-1}\)) of vibrational modes in the 1500-2000 cm\(^{-1}\) region for squaramide monomer (1) and oligomers (2-5), computed from structures optimized at the M06-2X/6-311+G(d,p) level of theory.

<table>
<thead>
<tr>
<th>Mode</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C=O sym</td>
<td>1719</td>
<td>1696</td>
<td>1678</td>
<td>1666</td>
<td>1661</td>
</tr>
<tr>
<td>C=O antisym</td>
<td>1849</td>
<td>1817</td>
<td>1783</td>
<td>1774</td>
<td>1762</td>
</tr>
<tr>
<td>Ring breathing</td>
<td>1919</td>
<td>1916</td>
<td>1909</td>
<td>1909</td>
<td>1908</td>
</tr>
</tbody>
</table>

2.6.12 DFT optimization and geometries
DFT calculations were performed with the Gaussian 09 suite of programs.\(^{43}\) The M06-2X hybrid functional was selected for its good performance in taking into account non-covalent interactions, such as hydrogen bonds and π-interactions.\(^{44}\) A monomer of N,N'-dimethylsquaramide was built and optimized at the M06-2X/6-311+G(d,p) level of theory, and subsequently oligomers from dimers to pentamers were optimized at the same level. Bond lengths were measured for each of the squaramide units in monomer and oligomers, and the HOMA index was computed as:

\[
HOMA = 1 - \frac{98.89}{4} \sum (r_i - 1.397)^2
\]
Table S2.4. Bond lengths for squaramide monomer (n=1) and oligomers (n=2-5). # is the monomer number in the oligomer (#1 always has non-hydrogen bonded NH, #n has non-hydrogen bonded C=O). HOMA was computed with equation shown above.

<table>
<thead>
<tr>
<th>n</th>
<th>#</th>
<th>C(O)-C(N)</th>
<th>C(O)-C(O)</th>
<th>C(N)-C(N)</th>
<th>C=O</th>
<th>C-N</th>
<th>HOMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1.492</td>
<td>1.549</td>
<td>1.402</td>
<td>1.206</td>
<td>1.353</td>
<td>-0.015</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.472</td>
<td>1.512</td>
<td>1.400</td>
<td>1.210</td>
<td>1.336</td>
<td>0.393</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1.477</td>
<td>1.525</td>
<td>1.396</td>
<td>1.207</td>
<td>1.339</td>
<td>0.277</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1.471</td>
<td>1.508</td>
<td>1.405</td>
<td>1.218</td>
<td>1.335</td>
<td>0.424</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>1.469</td>
<td>1.504</td>
<td>1.411</td>
<td>1.223</td>
<td>1.330</td>
<td>0.459</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1.477</td>
<td>1.522</td>
<td>1.401</td>
<td>1.216</td>
<td>1.338</td>
<td>0.301</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1.470</td>
<td>1.505</td>
<td>1.407</td>
<td>1.220</td>
<td>1.334</td>
<td>0.445</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>1.466</td>
<td>1.497</td>
<td>1.416</td>
<td>1.226</td>
<td>1.327</td>
<td>0.508</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>1.466</td>
<td>1.495</td>
<td>1.417</td>
<td>1.227</td>
<td>1.326</td>
<td>0.516</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>1.467</td>
<td>1.499</td>
<td>1.415</td>
<td>1.225</td>
<td>1.328</td>
<td>0.489</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>1.476</td>
<td>1.520</td>
<td>1.402</td>
<td>1.217</td>
<td>1.337</td>
<td>0.314</td>
</tr>
</tbody>
</table>
2.6.13 Nucleus-independent chemical shift (NICS) calculations

Figure S2.10. NICS-scan profiles for squaramide monomer and oligomers (n=2-5). For the monomer, the isotropic shielding as well as the individual out-of- and in-plane contributions are displayed. For the oligomers, only the isotropic shielding is shown, and the markers are hidden for clarity; the lines are meant as a guide for the eye.
Table S2.5. NICS(0), computed on the plane of the squaramide ring. n is the number of monomers in the oligomer, and # is the monomer number in the oligomer (#1 always has non-hydrogen bonded NHs, #n has non-hydrogen bonded C=Os).

<table>
<thead>
<tr>
<th>n</th>
<th>Monomer #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Table S2.6. NICS(0.6), computed 0.6 Å from the plane of the squaramide ring on an axis perpendicular to it. Idem Table S2.5.

<table>
<thead>
<tr>
<th>n</th>
<th>Monomer #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Table S2.7. NICS(1), computed 1.0Å from the plane of the squaramide ring on an axis perpendicular to it. Idem Table S2.5.

<table>
<thead>
<tr>
<th>n</th>
<th>Monomer #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2

2.6.14 Interaction energies

The total interaction energy for each oligomer \((n=2-5)\) was computed as

\[
\Delta E(n-\text{mer}) = E(n-\text{mer}) - nE(\text{monomer})
\]

| \(n\) | \(n_{\text{Hbond}}\) | Squaramide | | | Urea |
|---|---|---|---|---|
| | | \(\Delta E\) | \(\Delta \text{BSSE}\) | \(\Delta E/n_{\text{Hbond}}\) | \(\Delta E\) | \(\Delta \text{BSSE}\) | \(\Delta E/n_{\text{Hbond}}\) |
| 1 | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 2 | 2 | -51.7 | 3.2 | -25.8 | -38.4 | 2.0 | -20.2 |
| 3 | 4 | -120.3 | 6.0 | -30.1 | -84.4 | 3.9 | -22.1 |
| 4 | 6 | -194.4 | 9.5 | -32.4 | -134.5 | 6.2 | -23.4 |
| 5 | 8 | -271.7 | 12.8 | -34.0 | -182.6 | 8.4 | -23.9 |

To calculate the contribution of the aromatic stabilization to the interaction energy in squaramide oligomers, we computed the interaction energy of systems analogous to squaramide that can form the same hydrogen bond pattern but cannot exhibit aromatic stabilization, heterodimers 5 and 6 (see Figure S2.11). The former is a minimal system that generates the same arrangement of hydrogen bonds as present in a squaramide dimer, while the latter exhibits a di(vinyligous amide) pattern. The structure of the complex was optimized at the M06-2X/6-311+G(d,p) level of theory, and the interaction energy was calculated by subtracting the energy of the individual isolated molecules. The interaction energy was corrected for Basis Set Superposition Error by single point counterpoise method,\(^{45}\) the BSSE value represented 4-6% of the total interaction energy. The contribution of the aromatic stabilization was calculated as the difference between the interaction energy of the oligomer and the interaction energy of the non-aromatic heterodimer times the number of bonds between monomers in the oligomer (i.e., \(n-1\)) (see Table S2.9):

\[
E^{aro}(n-\text{mer}) = \Delta E(n-\text{mer}) - (n - 1)\Delta E(\text{nonaromatic})
\]
Figure S2.11. Reference non-aromatic systems for the calculation of the aromatic stabilization energy in a squaramide dimer. \( \Delta E \) is the interaction energy corrected for BSSE (\( \Delta \text{BSSE} \)).

Table S2.9. Interaction energy (\( \Delta E \)), aromatic stabilization energy (\( E_{\text{aro}} \)) and percentage of the total interaction energy of the oligomers \( E_{\text{aro}} \) accounts for compared to heterodimers 5 and 6.

<table>
<thead>
<tr>
<th>n</th>
<th>( \Delta E )</th>
<th>5 ( E_{\text{aro}} )</th>
<th>5 ( E_{\text{aro}}/\Delta E )</th>
<th>6 ( E_{\text{aro}} )</th>
<th>6 ( E_{\text{aro}}/\Delta E )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-51.7</td>
<td>-26.5</td>
<td>51%</td>
<td>-5.2</td>
<td>10%</td>
</tr>
<tr>
<td>3</td>
<td>-120.3</td>
<td>-69.8</td>
<td>58%</td>
<td>-27.2</td>
<td>23%</td>
</tr>
<tr>
<td>4</td>
<td>-194.4</td>
<td>-118.8</td>
<td>61%</td>
<td>-54.9</td>
<td>28%</td>
</tr>
<tr>
<td>5</td>
<td>-271.7</td>
<td>-170.8</td>
<td>63%</td>
<td>-85.6</td>
<td>32%</td>
</tr>
</tbody>
</table>

2.6.15 Critical aggregation concentration

The CAC was determined by static light scattering through serial dilutions of the self-assemblies of 1 and 5. Individual stock solutions of 1 and 5 of the same concentration (2.91 mM), were prepared by dissolving each of the compounds in Milli-Q water and leaving to stand overnight. A dilution series was prepared in a range from 0.29 mM to 2.91 mM and their respective scattered light intensities measured in a polystyrene cuvette at room temperature. The measurements were recorded in triplicate. The CAC value was obtained from the intersection between the lines drawn to the points representing the aggregated and non-aggregated species. Subsequently, Gibbs free energy for molecules 1 and 5 were calculated from the experimentally determined CAC values using the equation \( \Delta G = RT \ln(CAC) \).
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