Synthesis and characterization of squaramide-based supramolecular polymers

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Introduction

1.1 Supramolecular biomaterials

Biomaterials are a class of materials that are in contact with biological systems.[1] The growing demand for smart materials that are designed to interact with the biological environment to improve the patient's condition and experience in injury and disease is rapidly increasing.[2] Although this is a field that has been classically associated with covalent polymers, that are largely irreversible in character, there is a second class of polymers that have been applied more recently in this area and are based on dynamic or non-covalent interactions, such as electrostatic, hydrogen-bonding, π-π, and hydrophobic effects; these are supramolecular polymers.[3-6] This class of dynamic or supramolecular materials are extremely attractive in areas such as tissue engineering, regenerative medicine and drug delivery due to their modular, stimuli-responsive, tunable, and biomimetic character.[7-12]

Supramolecular polymers can be further subdivided into two main classes based on their mode of assembly. In the first class the monomers stack together through non-covalent interactions into fibrillar aggregates, whereas in the second one macromers with molecular recognition units interact to form networked structures. This thesis will focus on the former and explores the use of hydrogen bonding interactions in combination with hydrophobicity to drive monomer assembly in water.

1.1.2 Supramolecular polymerization mechanisms of stacked monomers

Supramolecular monomers can self-assemble into polymer structures through one of three mechanisms: ring-chain, isodesmic or cooperative (**Fig 1.1**).[13-15] In this section, I will only focus on stacked aggregates that self-assemble through isodesmic and cooperative mechanisms.

The isodesmic mechanism involves the successive addition of monomers with the same binding constant to a growing polymer chain.[16] The addition of each monomer results in an equivalent decrease in free energy with monomer affinity being independent of the length of the polymer. The polymers formed according to this mechanism are polydisperse and there is a lack of critical concentration and temperature.

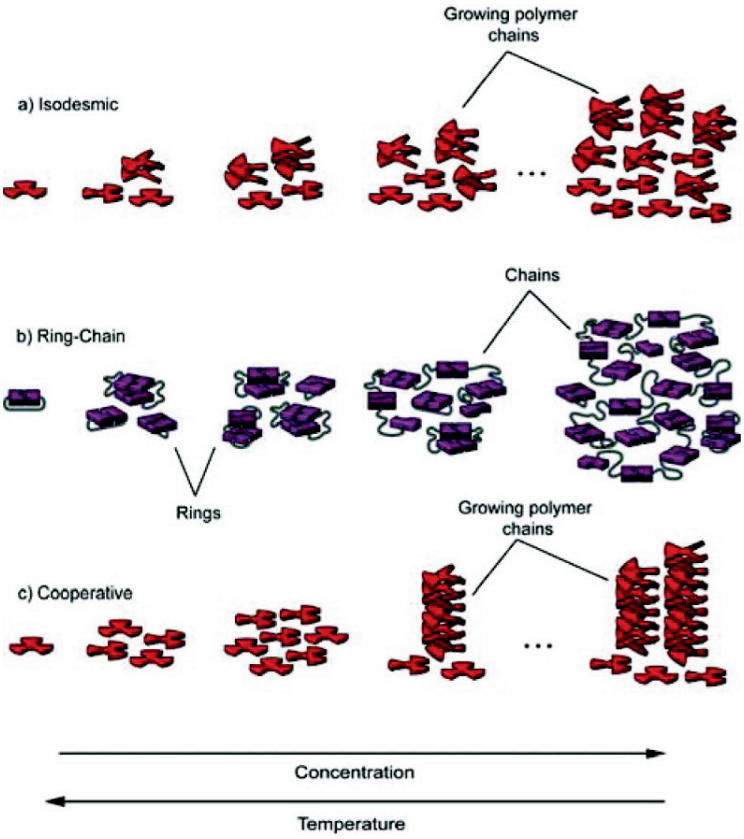

Figure 1.1 Mechanism of supramolecular polymerization: isodesmic (a), ring-chain (b), and cooperative (c). Figure adapted from reference 13

In the cooperative mechanism, supramolecular polymerization occurs in two steps, one involving formation of the nucleus of length s, that is often an unfavourable process and then an energetically favourable second step where elongation of the polymer occurs. Each of these steps are represented by their own binding constant; K_n for the nucleus formation and K_e for the elongation step. Additionally, the result of such polymerizations are nanostructures with a high degree of internal order. The degree of cooperativity of such polymerizations can be determined from the ratio of $\sigma=K_n/K_e$, when $\sigma < 1$ the polymerization is cooperative whereas polymerization with $\sigma > 1$ are anticooperative.

To determine the mechanism and to gain insight into the thermodynamic properties of the polymerization, several experiments can be performed and

then fit with the various models that rely on input parameters involving: i) concentration ii) temperature or iii) solvent-based changes.[13, 15] Changes in polymerization are followed by spectroscopic methods such as circular dichroism (CD), ultraviolet (UV), nuclear magnetic resonance (NMR) or infrared (IR) spectroscopy.[15, 17] From these experiments, the fraction of polymerized material, (φ) as a function of the changing parameter, monomer concentration, temperature, or good solvent, can be determined yielding insight into the polymerization mechanism.

Whereas the sigmoidal curves are obtained for isodesmic polymerizations, sharper and asymmetric curves are obtained for cooperative polymerizations. For cooperative growth, the thermodynamic models consider the formation of an initial nucleus with an unfavorable binding constant, followed by an elongation step with a very high binding constant.[17] For example, the mean-field chemical equilibrium model developed by Goldstein and Stryer[18] describes the two stages of a cooperative polymerization process ($\sigma=K_n/K_e < 1$) by their equilibrium constants, K_n and K_e. The equilibrium constant of elongation can be defined as $K_e = \exp(-\Delta G_0'/RT)$ where ΔG_0 is the Gibbs energy gain for each successive monomer, R is the gas constant and T is temperature. Mejier and coworkers[19] expanded this model to consider the effect of a 'good' solvent on the supramolecular polymerization process and where a linear relationship between the Gibbs free energy and solvent volume fraction is observed:

$$\Delta G_0' = \Delta G_0 + m \cdot f$$

where f is volume fraction of the good solvent and ΔG^0 is the Gibbs free energy gain upon monomer addition in the pure solvent.

In this study, both simulations and predicted models demonstrate the presence of a critical solvent composition for a cooperative polymerization ($\sigma < 1$) and the lack of it for an isodesmic polymerization ($\sigma = 1$). Additionally, the obtained values for ΔG^0 and σ agreed with temperature-dependent analysis enabling extrapolation of the thermodynamic parameters such as the nucleation enthalpy, elongation enthalpy (ΔH_{nucl}, ΔH_{el}) and the entropy (ΔS) that can be used to calculate the two binding constants and the cooperativity factor.[20, 21]

Electronic, structural and hydrophobic effects are known to be involved in cooperative growth of supramolecular polymers, often in combination with one another. C_3-symmetric monomers based on 1,3,5-benzenetricarboxamides have been demonstrated to self-assemble through a cooperative mechanism. The molecular origin of cooperativity in this system arises due to the three amide units that engage in hydrogen bonding and π-π stacking of the aromatic rings.[22, 23] In the case of OPV (oligo(p-phenylenevinylene)), the formation of supramolecular polymers in chloroform was achieved through π-π interactions of the aromatic core and the hydrogen bonding of the attached ureidotriazines driven by the structure.[24] Conversely in aqueous solution, cooperativity is driven by the hydrophobic effect. Fernandez and coworkers explored this effect investigating the self-assembly of oligophenylenethynylenes (OPE)-based bolaamphiphiles functionalized with poly(2-ethyl-2-oxazoline) (PEtOx) moieties of different lengths.[25] While the more water soluble derivatives self-assembled in spherical aggregates by an isodesmic mechanism, the formation of 2D anisotropic platelets according to cooperative mechanism were determined by the self-assembly of the molecules with shorter PEtOx chains.

Although most mechanistic investigations are performed in organic solvents, the growing interest in the self-assembly in supramolecular biomaterials that are self-assembled in water calls for more investigation into understanding their aggregation processes. In the next section, the effect of the monomer designs on their self-assembly behavior and application is discussed.

1.1.3 Characterization of supramolecular polymers

Spectroscopic methods can assist in revealing the supramolecular polymerization mechanism of the monomers in their response to a particular stimulus (e.g. heat, solvent), but they can also provide the much needed insight into arrangement of the monomers and non-covalent interactions between them. For monomers containing a chromophore, UV-Vis spectroscopy can be used to pinpoint their arrangement (e.g. stacking face-to-face or slipped) in the polymers by comparing blue and/or red-shifting and increase or decreased intensity of the monomer bands relative to the absorbance spectrum of the polymer.[15] Wurthner and coworkers investigated the intramolecular aggregation of a series of bis(merocyanine) dyes by UV-Vis spectroscopy revealing a marked hypsochromic shift as a consequence of the π–π-stacking disposition of the dyes on aggregation.[26] Circular dichroism can be used to understand the influence of chiral substituents in directing supramolecular polymer (e.g. sergeants and soldiers[27]) assembly by examining increases in signal intensity. Infrared spectroscopy can be used to understand if hydrogen-bonding interactions are present between the monomers following changes in absorbance of the N-H and C=O stretch regions. Bouteiller and coworkers exploited the infrared spectroscopy to investigate the cooperativity in the self-assembly of bis-urea based supramolecular polymers[28] Nuclear magnetic resonance experiments can confirm such interactions in the solution[29] and solid state[30] depending on solvent, but also monomer stacking looking at aromatic ring protons in response to changes in concentration or solvent composition.[31] Often such experiments are used collectively to understand how monomers interact through non-covalent interactions at the molecular level.

To further characterize supramolecular polymer morphology at the nano- and microscales, imaging techniques such as scanning (SEM) and transmission electron (TEM), atomic force (AFM) and stochastic optical reconstruction microscopy (STORM) can be used. SEM and conventional TEM require the use of electrons either being reflected or removed, or transmitted through the sample, respectively to image dried supramolecular polymer samples. AFM involves scanning of the supramolecular polymer surface by a sharp tip connected to a cantilever interfaced with a laser light that is reflected onto a photodiode yielding an image that reveals the morphology of the aggregates. These imaging techniques have been used within the field to analyze the

morphology of concentrated[32] and dilute solutions[33] of supramolecular polymers from a range of solvents, however use of dried samples and staining artifacts (e.g. through use of heavy metals) can play a role in the observed structures.[15] Alternatively, this issue can be mitigated in water using the cryo-EM technique, where the rapid cooling (vitrification) of the sample is executed to enable its visualization.[34] More recently, STORM has been used to understand the structure and dynamics of supramolecular polymers in water. The technique relies on the use of fluorescently labelled supramolecular monomers that are switched on and off in multiple cycles and their localization enables the reconstruction of a high resolution image with sub-diffraction resolution on the nanometer scale.[35, 36] As an example, Albertazzi and coworkers use this technique to study the self-assembled peptide nanostructure[37] unmasking the dynamic nature of supramolecular polymers.

Light scattering techniques are also routinely used to probe the morphology and internal order of supramolecular polymers. Small angle X-ray or neutron scattering (SAXS/SANS) are frequently performed on solution phase samples. This technique measures the scattering cross section of a sample subtracting the scattering of the solvent.[38] With the mathematical equations, information on the size, shape, and internal diameters of self-assembled objects are obtained.[39] Previously, we determined the cross sectional mass (M_L) of the squaramide-based tripodal and bolaamphiphile assemblies by SAXS.[40, 41] Specifically, the transition from bolaamphiphile to tripodal monomer geometry resulted in a reduction M_L and thus, the number of monomers in the cross-section from approximately 10-30 molecules to a single monomer, a reduced lateral aggregation and an increased fiber length. Often labeling and the staining of the supramolecular polymers can be circumvented due to sufficient contrast. Additionally, through static light scattering (SLS) information regarding morphology and the critical aggregation concentration (cac) of the monomers to form polymers can be determined.[42] For certain monomers above a critical concentration gel phase materials can be formed through entanglement or physical interactions between supramolecular polymers.[43] Oscillatory rheology can used to characterize the bulk mechanical properties of hydrogel materials, such as stiffness, viscoelasticity or strain stiffening.[44, 45] These properties are highly relevant to understand for their applications in the biomedical field, such as in 3D cell culture.[46] The group of Dankers and coworkers designed Upy-based hydrogels where changing the

ratio between molecules with a bivalent (B) and monovalent (M) presentation modulating the viscoelastic properties for its application as a cell scaffold.[47]

1.2 Supramolecular monomers

In aqueous media, the self-assembly process of amphiphilic monomers is driven mainly by the hydrophobic effect, in combination with other forces such as hydrogen bonding and π-π interactions. Commonly applied monomer geometries are tripodal, C_3-symmetric and linear. These are described in the following paragraphs.

1.2.1 Bolaamphiphile monomers

Bolaamphiphiles are a class of amphiphilic molecules characterized by two hydrophilic head groups connected by a hydrophobic spacer that can self-assemble into several stable nanostructures such as micelles, fibers, tubes, cylindrical aggregates.[48] In the literature, a wide variety of bolaamphiphile scaffolds based on molecular building blocks such as peptides, carbohydrates and lipids can be found.[49] Although a major application focus is based on pharmaceutical formulations, there is a growing interest for their abilities to self-assemble in stable nanostructures. The properties and structures of these assemblies are tuned by length of the central hydrophobic core or the type of hydrophilic head groups.

In biomedical field since many drugs suffer of very low solubility in water, bolaamphiphile architectures are considered promising candidates for drug delivery applications. For example, Sharma and coworkers reported the self-assembly of a series of symmetric bolaamphiphiles with distinct hydrophilic domains (polyethylene glycol (PEG) or dendritic polyglycerol (dPG)) and examined their potential as nanocarriers (**Fig. 1.2a**).[50] Moreover, Jianxi and coworkers explored the use of a bolaamphiphile scaffold consisting of peptides to prepare collagen mimetic materials.[51] A family of peptides consisting of the triple helical collagen sequence ($(GPO)_m$ or $(PPG)_m$) with aspartic acid groups at its periphery was synthesized forming nanospheres in a broad pH range.

In contrast to conventional bolaamphiphiles, asymmetric bolaamphiphiles are characterized by two hydrophilic head groups of different size. They can contain a functional moiety with a cell-targeting[52] or pH-sensitive function that can be used for specific gene and drug delivery applications.[52-54] For example, Cui and coworkers designed a reverse asymmetric bolaamphiphile (RAB) tagged with an metalloproteinases (MMP-2) cleavable peptide to engineer enzyme-responsive supramolecular hydrogels.[55] The structure was characterized by two different peripheral hydrophobic units that were MMP-2 sensitive (**Fig. 1.2b)**. Since most supramolecular filaments lack accessibility to enzymes, this work demonstrates successful fiber degradation by MMP-2 with additional anticancer drug release.[55]

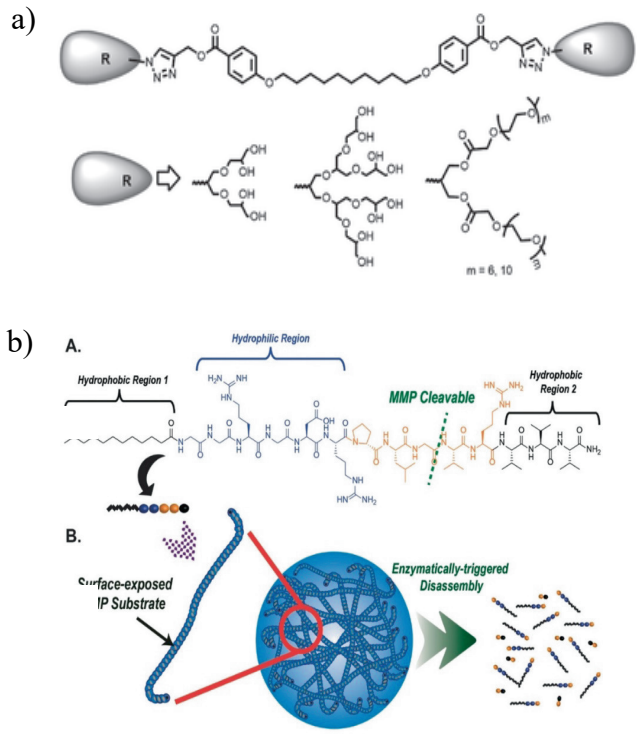

Figure 1.2 a) Pegylated (PEG) and dendronized (dPGs) symmetrical bolaamphiphiles for drug delivery applications. b) Molecular design of asymmetric reverse bolaamphiphile (RAB). The monomers self-assemble in water forming hydrogels and are degraded in presence of MPP-2 (metalloproteinases). Figure adapted from reference 28 (a), 29 (b).

1.2.2 C_3-symmetric and tripodal monomers

Monomers bearing C_3 –symmetric cores are one of the most often used and exploited geometries in the formation of self-assembled supramolecular polymer materials. The main reasons for their use are the easy and established synthetic protocols that enable the modification of C_3-symmetric synthons, the formation of helical supramolecular aggregates and cooperativity in comparison to their C_2-symmetric counterparts.[56,57] More specifically, their rigidity compared to their non-helical counterparts, results in their entanglements forming gel phase materials in a range of solvents. When in water, the hydrophobic environment shields the hydrogen-bonding units from the surrounding solvent.[58] Concentration also plays an important role, with its increase resulting in gel phase materials.

An often-used core is 1,3,5-benzenetricarboxamide (BTA) whose self-assembly is predominantly driven by π-π stacking of the aromatic core and the formation of the intramolecular hydrogen bonds between the amide units. Initially, the investigation of the BTAs self-assembly, and their supramolecular polymerization, was performed in organic solvents.[22, 23] More recently, the self-assembly of pegylated BTAs in aqueous solution has opened up numerous potential biomedical applications. Besides fundamental studies on supramolecular polymerization,[59-61] BTA-based assemblies were used for intracellular drug delivery (**Fig. 1.3a**).[62] Moreover Webber and coworkers explored the effect of pH on the self-assembly of isopeptide-modified BTAs.[63] Connecting the BTA core to glutamic acids by benzoic acid linkers, the effect a pH stimulus on the morphology of supramolecular polymers was examined (**Fig. 1.3b**). Rapid acidification with HCl resulted in the formation of highly branched entanglements, whereas the slow acidification with D-glucono-delta-lactone (GdL) enabled a more thermodynamic self-assembly process with the formation of a less interconnected structure.

van Esch and coworkers explored the effect of catalysis on the reaction coupled self-assembly of cyclohexane-based low molecular hydrogelators (**Fig. 1.3c).** Hydrazone bond formation between a cyclohexane trishydrazide building block and three aldehydes was executed in presence and in absence of a catalyst (acid and base) to obtain in situ fiber self-assembly and gelation and their mechanical properties were assessed. Scanning electronic microscopy confirmed the formation of a dense homogeneous network in the

catalyzed systems, while a less dense and more open network was formed in the absence thereof.[64]

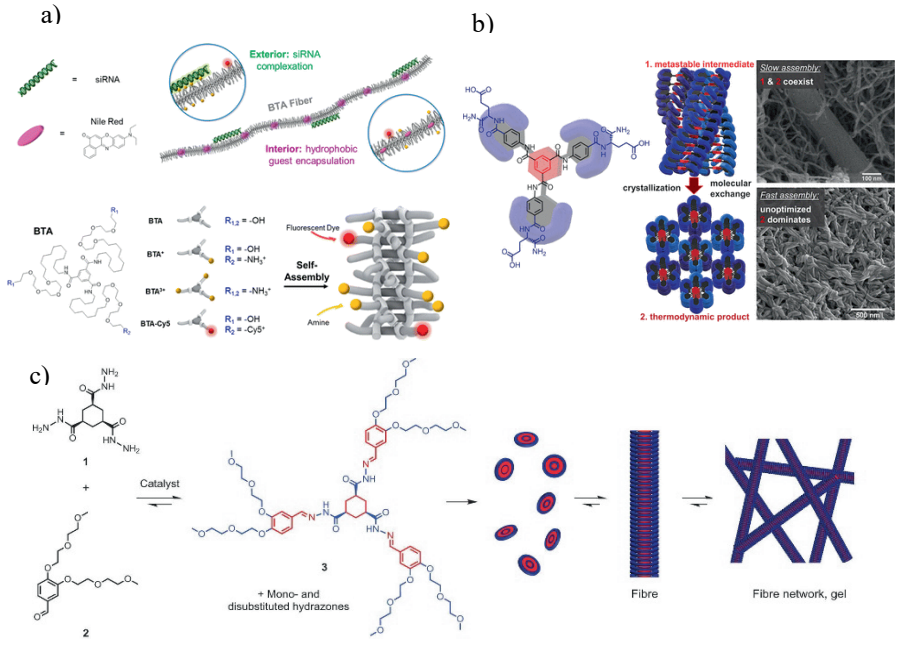

Figure 1.3 a) BTA supramolecular polymers for applications in intracellular delivery. The system was prepared through co-assembly of nonfunctional, positively charged, and fluorescently-labeled BTA monomers; b) Self-assembly of BTA-(bE)$_3$ monomer in water varying the rates of pH stimulus; c) Reaction-coupled self-assembly between cyclohexane trishydrazide building block (1) and three molecules of aldehydes (2). The formation of hydrazone bond gives the formation of fibers. Figures adapted from references 40 (a), 41 (b) and 42 (c).

Next, the effect of crosslinking by DNA and PEG on the properties of a cyclohexyl trishydrazone core-based hydrogel was investigated.[65] The addition of 1 mol% of DNA-crosslinkers showed a 4.5-fold increase of the stiffness of the native hydrogel while at higher percentage (2 and 3 mol%) a decrease in stiffness was reported. Oscillatory rheology and their structures imaged by cryoTEM and CSLM revealed the presence of spherical aggregates or a collapsed network at higher concentrations of crosslinkers. In contrast, the presence of 1 mol% PEG crosslinkers resulted in the formation of weaker hydrogel in comparison to the native material. These findings reveal that the physical properties of the polymer and the concentration of crosslinkers are

critical in the hydrogelation process and on the final mechanical properties of the materials.[65]

1.2.3 Peptide-based monomers

A monomer class that has gained the attention of chemists for the development of supramolecular biomaterials is based on peptides. Their ability to be biodegradable, biocompatible and to adopt a range of nanostructures makes them attractive for numerous applications in the biomedical domain.[66-68]

Peptide-based self-assembly is dictated by a combination of several non-covalent interactions such as hydrogen bonds, π-π, van der Waals, hydrophobic and electrostatic interactions. Functional groups such as amides, amines and carboxylic acids in the peptides facilitate hydrogen bond interactions which facilitate the formation of higher order peptide structures.[69] Moreover, the π-π stacking contribution of aromatic side groups is relevant not only in water, but also in organic solvents such as toluene where this effect plays an important role.

Peptide-based scaffolds have been actively employed as mimics of the natural extracellular matrix.[70, 71] The main classes of peptide-based hydrogels are: short oligopeptides, peptides with secondary structure and peptide amphiphiles. Individual amino acids or dipeptides protected with Fmoc (fluorenylmethyloxycarbonyl) group are already able to self-assemble in well-defined structures.[72] The self-assembly is determined by the combination of π-π interactions from the Fmoc group and hydrogen bonds from the amides.[72-74] Tyrosine dipeptides also belong to this class and self-assemble into nanofibers.[75]

In the second class, the formation of specific secondary structures such as beta-sheet, alpha-helix, and beta-hairpin gives rise to self-assembled nanofibers that can form a 3D network in water.[72] Alternating hydrophobic and hydrophilic residues in combination with electrostatic interactions can facilitate this process.[76] This family of peptides has been employed for cell culture, namely cell adhesion and osteogenic differentiation.[77, 78]

Peptide amphiphiles are the third class, and are characterized by a charged, beta-sheet and bioactive domain peptide sequence coupled to an aliphatic or aromatic spacer (**Fig 1.4).** In the last decade it was extensively studied not

only how to modulate their self-assembly behaviour changing the aliphatic spacer, the aminoacids, the solvent, the co-assembly with other molecules, but also their potential in tissue engineering.[79, 80]

The co-assembly of two or more peptides is gaining a particular attention to tune the mechanical properties of peptide-based supramolecular systems.[81-83] According to the type of monomers that are combined and the type of interactions between them (aromatic, electrostatic, enantiomeric and enzymatic), either cooperative, disruptive co-assembly and self-sorting can take place.[84] In cooperative co-assembly, the final properties of the materials are generally improved in comparison to the single component systems. When the two components interfere with one another in the self-assembly process, weaker mechanical properties of the materials are observed and is called disruptive co-assembly. When the components self-assemble independently of one another, this phenomenon is called self-sorting.

Adler-Abramovich and coworkers explored the cooperative co-assembly of the gels formed by Fmoc-FF (Fmoc-diphenylalanine) and Fmoc−pentafluorophenylalanine. The mixing of these two components in a 1:1 molar ratio gave rise to a hybrid hydrogel having a stiffness with an order of magnitude greater than the single component systems.[81, 85]

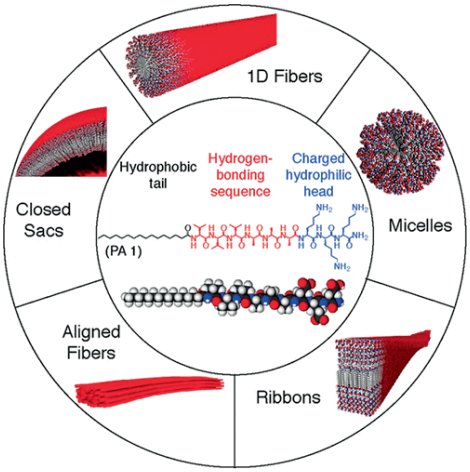

Figure 1.4 Chemical structure of the peptide amphiphile (PA) (center): charged, hydrogen-bonding sequences are coupled to an aliphatic spacer. Possible supramolecular nanostructures are presented surrounding the PA**.** Figure adapted from reference 57.

1.2.4 Hydrogen bonding interactions

Although an isolated hydrogen bonds is typically too weak to control self-assembly, the combination of several hydrogen bonds results in a selective, directional non-covalent interaction to control self-assembly in solution.[86] Consequently, triple hydrogen bonding arrays have been investigated extensively for the formation of stable aggregates.[87-90]

Despite a linear correlation between the free energy and the number of hydrogen bonds in a monomer,[91] the type of hydrogen bond, secondary interactions, and the solvent also need to be considered for their effect on strength of such systems. Moreover, the acidity of hydrogen bond acceptor and basicity of the hydrogen bond donor play a key role on the interaction.[92] Generally, the more basic the hydrogen bond acceptor (A) is and the more acidic the hydrogen bond donor (D) is, the stronger will be their interaction.[93]

Another important factor to consider are the secondary interactions between adjacent hydrogen bond donor and acceptor groups; this feature has an effect on the association constants of the monomers and on the stability of their interactions.[94] The order of donors and acceptors DDAA, results in an association constant ($K_a = 10^5$ L mol^{-1}) that is three orders of magnitude greater than when the order is DADA ($K_a = 10^2$ L mol^{-1})). This difference in association constants can significantly impact the stability of the formed self-assembled polymers (**Fig 1.5a**).

The solvent further plays a critical role in affecting the stability of the self-assembly monomers formed. Although in organic solvent the formation of well-defined architectures can be controlled by the combination of several non-covalent interactions,[58] in water hydrogen bonds are often weakened because the solvent molecules compete with the monomers. Therefore, hydrogen bonds have been combined with the hydrophobic effect in monomer design to drive monomer assembly into well-defined structures.[95, 96] In BTA assembly, the hydrogen-bonded amides of adjacent aromatic cores are shielded by an aliphatic or aromatic spacer to drive self-assembly in water.[97] A similar same design strategy has been applied to drive the self-assembly of bisurea derivatives in water. Boué and coworkers have demonstrated the importance of hydrophobic spacer for the formation of hydrogen bonds between the ureas units.[98]

More recently, the effect of the aromatic character on hydrogen bonding in supramolecular systems has been examined. The effect of aromaticity on multiarray hydrogen bonding scaffolds was examined by the Wu group.[99] They showed through DFT calculations that heterocycles that participate in 4n+2 π-delocalization show increased hydrogen bonding strength than those that do not (**Fig 1.5b**). The limitation of SEI (secondary electrostatic interaction) model introduced by Zimmermann, based on the hydrogen bond donor/acceptor pattern was demonstrated through BLW (block-localized wavefunction) calculations showing a linear correlation between the aromatic gain and association constant of multiarray hydrogen bond scaffolds further highlighting its importance.[99] Moreover, in the Kieltyka group, the contribution of aromaticity to squaramides in their self-assembly in aqueous media was uncovered.[40] Hydrogen-bonded squaramide monomers showed an increase in the aromatic character of the synthons pointing out the interplay of these two variables on supramolecular polymerization of such monomers.

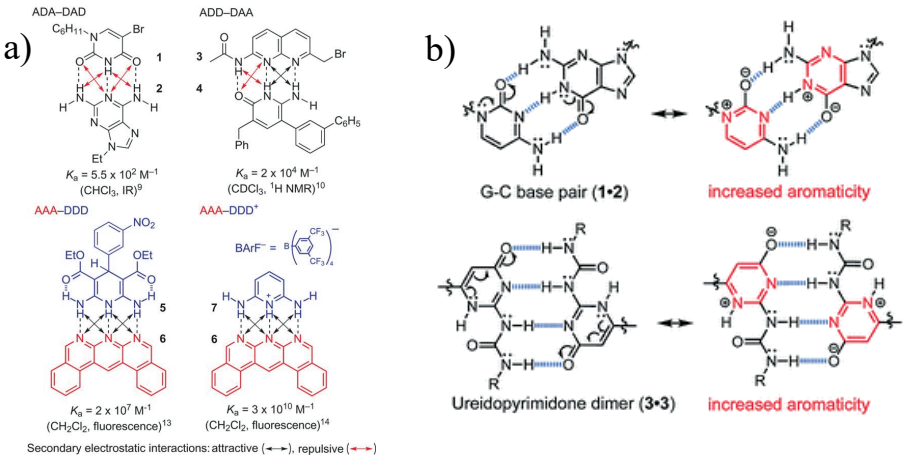

Figure 1.5 a) Examples of triple hydrogen-bond arrays and their association constants in various solvents. b) Aromaticity-modulated hydrogen bonding (AMHB) in the guanine–cytosine (G–C) base pair, 1·2, and ureidopyrimidone (UPy) dimer, 3·3. Figures adapted from references 72 and 77.

1.3 Squaramides

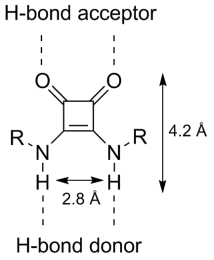

Figure 1.6 Chemical structure of squaramide moiety

Squaramides consist of a cyclobutenedione ring with two NH hydrogen bond donors opposite two CO hydrogen bond acceptors.[100] They can be easily synthesized from combining the desired amine with their commercially available squarate esters. Moreover, Liu *et al.* reported that their squarate ester precursors can be synthesized in high yield (77-97%) starting from squaric acid in the presence of a specific alcohol.[101] Because of their high synthetic accessibility, squaramides have been applied in areas such as catalysis, medicinal chemistry, bioconjugation, anion recognition and also in the field of supramolecular materials.[102, 103]

In organocatalysis, squaramides have been applied using the hydrogen bond formation between the catalyst and the substrate to drive chemical transformation. The increased donor character of squaramide in comparison to thioureas results in the formation of stronger hydrogen bonds with various substrates.[104]

Moreover, Bae and Song explored the addition of beta-carbonyl compounds to nitroolefins 'on water' using a cinchona squaramide as a catalyst.[105] The success of the reaction 'on water' compared to the reaction in DCM was due to the hydrophobic hydration effect of the catalyst. Notably, the isolated products showed very high stereoselectivity (90-93%) and yields (86-99%) at low catalyst loading (0.01 mol%).

Another area where the squaramide moiety has been used is in the bioconjugation chemistry. Recently diethyl squarate (DES) has been used as a novel chemical crosslinker for gelatin-based hydrogels. In particular Cipolla and coworkers examined the ability of 5% gelatin – DES as 3D cell culture scaffolds for chondrocyte growth.[106]

Additionally, squaramides have been applied as drug precursors in the field of medicinal chemistry because of their isosterism with ureas.[102] Kitov and coworkers, for example, demonstrated the ability of di- and trisaccharides conjugated with squaramides to inhibit verotoxins produced by *E. coli* that cause gastrointestinal and urinary disorders.[107] Moreover, the chemoselectivity of squaric esters for the amino acid groups in the peptides were exploited in the synthesis of potential antitumor vaccines based on glycopeptides.[108]

One of the peculiar properties of squaramide moiety is its partial aromatic character (Hückel's rule $(4n + 2)\ \pi$ electrons, $n = 0$, as a consequence of the delocalization of the nitrogen lone pair attached to the squaramide inside the cyclobutenedione ring.[109] In contrast to other H-bonding motifs such as urea, [110-112] thiourea or pyrimidones, the aromatic character of squaramide is enhanced in a synergistic manner with the formation of stronger and directional hydrogen bonding.[109] This remarkable property renders squaramide an attractive and minimalistic module for the design of supramolecular materials.[100] Kieltyka group for the first time demonstrated that the squaramide synthon can be used to drive the self-assembly of a squaramide-based bolaamphiphile into nanostructured fibers in water. More specifically, the combination of spectroscopic and computational studies (NICS, HOMA, ASE) confirmed how the synergy between the increased aromaticity of squaramide and stronger hydrogen bonds is determining in the self-assembly process of these polymers **(Fig.1.6a)**.[40] In a follow up work, Kieltyka group reported a family of squaramide-based bolaamphiphiles where the length of the PEG chains and the aliphatic chains were independently modulated.[113] It was shown that at least 8 carbons between undecaethylene glycol chains and the squaramides were critical to enable monomer self-assembly into fibrillar aggregates. In contrast, maintaining the length of the hydrophobic core and changing the length of the oligoethylene glycol chains resulted in a morphological transition from fibrillar to spherical aggregates opening the door to their exploration in the field of nanomedicine.[113]

Hydrogel materials could not be formed from the bolaamphiphilic monomers, therefore in a follow up work, squaramides were introduced into a tripodal monomer geometry to achieve such materials.[41] Specifically, self-assembly of these tripods resulted in a decreased number of monomers within nanofiber cross-section, increased fiber length and hydrogelation. Moreover, the effect

of the hydrophobic spacer and introduction of a urea moiety in the place of the squaramide were examined, with spacer lengths of C8 and C10 providing hydrogel materials whereas the use of urea did not yield the same result. These hydrogels were also examined for their capacity to easily seed and release human induced pluripotent stem cell (hiPSCs) aggregates using the supramolecular nature of the material. High cell viability and retention of the pluripotent character of the hiPSCs were observed (**Fig.1.6b**).

Intermolecular hydrogen bonding between squaramides is not always involved in the self-assembly process. Soberats and coworkers demonstrated the self-assembly of squaramide-based monomers by the combination of hydrophobic effect and π-π dipolar interaction to form hydrogel materials.[114] A small library of aryl-squaramide amphiphiles hydrogelators were prepared with a benzoic acid moiety making the molecule easily ionizable.[114] The aromatic ring had different substituents, but only the electron-deficient ones can give rise to self-assembly. The absence of hydrogen bonding was confirmed by FT-IR and NMR measurements due to the lack of an N-H signal at 3160 cm^{-1}. In NMR spectra, the aromatic protons were shielded and no N-H signal was present in water (**Fig. 1.6c**).[114]

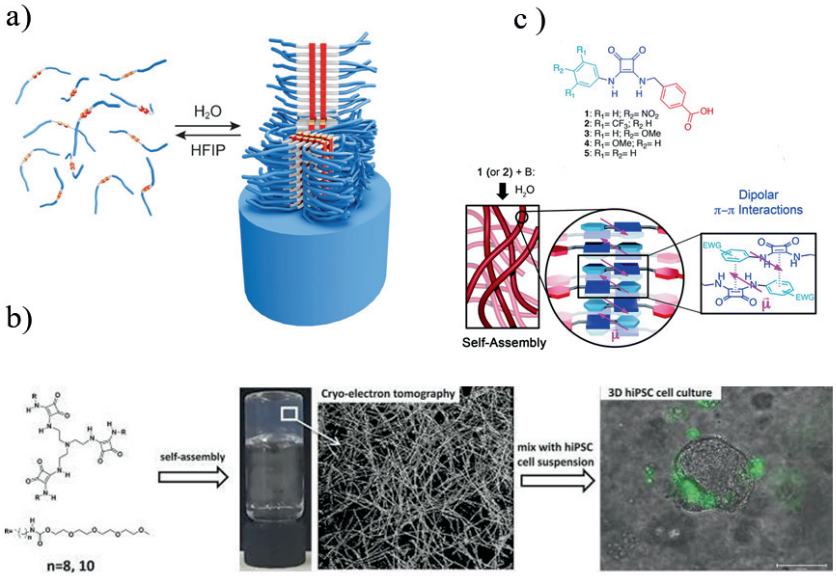

Figure 1.6 (a) Self-assembly of squaramide-based supramolecular polymers in water; (b) Self-assembly of tripodal squaramide-based supramolecular hydrogels for hiPSCs encapsulation; c) Chemical structure of aryl squaramides synthesized by the Soberats group. The self-assembly in water of this class of molecule is determined by π-π dipolar interactions (pink arrows). Figures adapted from the reference 77 (a), 90 (b) and 91 (c).

1.4 Thiosquaramides

Thiosquaramides are prepared from oxosquaramides by a thionation reaction that involves the exchange of oxygen for sulfur on the cyclobutenedione ring. The thiosquaramide was synthesized for the first time in 1966 when N,N'-dicyclohexylsquaramide was thionated using diphosphorus pentasulfide in dichloromethane. More recent synthetic methods involve the use of pyridine complex (pentathiodiphosphorus(V) acid-P,P′-bis(pyridinium betaine))).[115]

From supramolecular point of view, thiosquaramides form less directional hydrogen bonds in comparison to oxosquaramides due to the more polarizable nature of thiocarbonyl groups. According to Cambridge structural database (CSD), C=S···H interactions show an angle of almost 90° from the lone-pair

plane, in contrast with the C=O·····H interactions where small deviations from the lone pairs plan were observed (0-20°).[116]

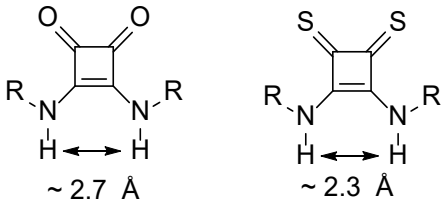

Figure 1.7 Chemical structure of squaramide and thiosquaramide

Several works have shown oxosquaramides to be excellent catalysts in enantioselective reactions because of their ability to form very strong hydrogen bonds.[117] However, oxosquaramides are characterized by low solubility in non-polar solvents due to the self-aggregation.[117] This issue is solved by substitution of carbonyl by the thiocarbonyl group resulting in its increased solubility in apolar solvents opening the door for its use in organocatalysis.[117]

Pedrosa and coworkers reported the synthesis of chiral, bifunctional thiosquaramide catalysts that were applied in nitro-Michael addition of 3,3-disubstitued oxindole to beta-aryl substituted nitroalkenes.[118] In comparison to thiourea and oxosquaramide catalysts, high reaction yields (82%) and enantioselectvity (94:6) using a catalyst loading of 5 mol% was demonstrated. The enantioselectivity of thiosquaramide was also demonstrated by Kupai and coworkers in an asymmetric Michael reaction between 2,4-pentanedione and trans-nitrostyrene, and a Diels-Alder reaction between siloxydiene and benzylideneacetone.[119] Althougth oxo and thio derivative cinchona catalyst catalysts provided the same yield (90 %) in both reactions, the thiosquaramide showed improved enatioselectivity over the oxo (99% ee compared to 93% ee).

Earlier publications demonstrated the ability of oxosquaramide to act as an ion transporter as an alternative to ureas and thioureas.[120] More recent works show that thioureas have an improved capacity to transport ions in comparison to ureas because of the increased acidic character of NH and greater lipophilicity of sulfur atom.[121] Bussacaert showed that thiosquaramide-based transporters demonstrate a pH-dependent ion transport behavior.[121] The ion transporter was synthesized by a thionation reaction using the pyridine complex

(pentathiodiphosphorus(V) acid-P,P′-bis(pyridinium betaine). While anion transport is favored in acidic conditions (pH < 7), at high pH (pH >7) deprotonation of thiosquaramide occurs promoting anion release.

Although thiosquaramides have been examined for use in organocatalysis and anion transport, they have not been explored for the self-assembly of supramolecular polymers.

1.5 Multicomponent reaction

Most of the synthetic pathways for the preparation of supramolecular monomers are characterized by several synthetic steps. In the case of peptide-based materials with various functionalities, the synthesis of peptides on solid phase is low yielding and costly on a large scale. Thus, it is necessary to find a methodology that improves the synthetic efficiency of such monomers.

Multicomponent reactions (MR) involve the reaction of 3 or 4 components under mild conditions with high yield and selectivity in one pot with high atom efficiency. This strategy is in contrast to a multistep synthetic approach, where the product of each reaction step is the reagent for the next reaction. In a multicomponent reaction, all the synthetic steps are condensed in one reaction. This approach avoids the challenges related to the purification and, isolation of the formed chemical compounds, which are typical of other synthetic strategies.[122] Commonly used reagents in these reactions are amines, carboxylic acids, isonitriles, aldehyde or ketones. The most widely used multicomponent reactions are the Ugi, Passerini and Biginelli reactions that have been known for more than 100 years.[123] With these methodologies it is possible to make large libraries of compounds with minimal synthetic effort. Multicomponent reactions are considered to be an important approach to design and discover biologically active scaffolds.[124] Moreover, the structural complexity on MCR compound can be increased by post-modification with bifunctional or multifunctional scaffolds. When this approach is used, MCR compounds can then be involved in another MCR.[125]

One of the main applications of MCRs is related to the synthesis of stapling peptides and their macrocyclization. This compounds class can be applied as antimicrobials, anticancer agents and enzyme inhibitors[126] and are usually

synthesized by click chemistry or lactamization. By an MCR approach, it is possible to obtain compounds with high structure diversity.[127]

An important class of multicomponent reactions involves the reactive isocyanide group that is often found in peptide chemistry. The first isocyanide-based MCR, discovered in 1921 was the Passerini reaction that has been extensively used for the synthesis of cyclic peptides.[128] This reaction is performed in presence of isocyanide, aldehyde and carboxylic acid, resulting in the formation of an ester bond. Another important reaction that belongs to this class is the Ugi reaction that will be discussed in the following section.

1.5.1 Ugi reaction

The Ugi reaction is an isocyanide based-multicomponent reaction that involves the use of an amine, carboxylic acid, isocyanide, and aldehyde or ketone. The formation of the final compound that has a peptide-like structure is due to the reactivity of isocyanide that reacts with both electrophiles or nucleophiles to form alpha-adducts. The mechanism starts with the condensation of the aldehyde or ketone with an amine, forming an imine that is protonated by a carboxylic acid. Imine formation is then followed by nucleophilic attack of the isocyanide and addition of the carboxylic acid to give rise to the formation of a nitrilium intermediate. In a final step, an irreversible Mumm rearrangement takes place resulting in the alpha-amide product (**Fig. 1.8a**).[129]

The high yield, selectivity and mild reactions conditions render the Ugi reaction a useful synthetic strategy for a wide variety of applications. This reaction has been applied in post polymerization reactions that are difficult to perform by conventional strategies due to the steric hindrance of the polymeric chains.[130] Moreover, polymers formed by the Ugi reaction have also been reported and exploited in the synthesis of α,ω-diene monomers by Meier et coworkers[131] and acrylate monomers.[132]

The generation of peptide-like structures, or peptidomimetics, by the Ugi reaction is gaining attention. Classical solid-phase peptide synthesis is often low yielding and can be costly depending on the peptide sequence. The Ugi reaction provides an alternative to this approach in the synthesis of polypetoids and hybrid copolymers such as peptide-peptoids for applications in biomedicine.[133-136]

In supramolecular chemistry, the Ugi reaction was exploited in the synthesis of peptoid-based low molecular hydrogelators. Mild conditions were used and very high yields were obtained for a small family of low molecular gelators, and their capacity to gelate in aqueous solutions such as water/DMSO and in water/EtOH mixtures (20 % of organic solvent) was examined.[137] The Ugi reaction has found several application in the covalent functionalization of supramolecular materials (**Fig.1.8b**).[138] Mironov and coworkers exploited the Ugi reaction to crosslink microgels of cellulose. With this synthetic method particles with a diameter in a range of 170-230 nm were obtained and used as stabilizers for oil/water emulsions.[139]

These preliminary results have opened new possibilities in the design of new supramolecular biomaterials where their synthesis is challenged by low yields and efficiency.[137]

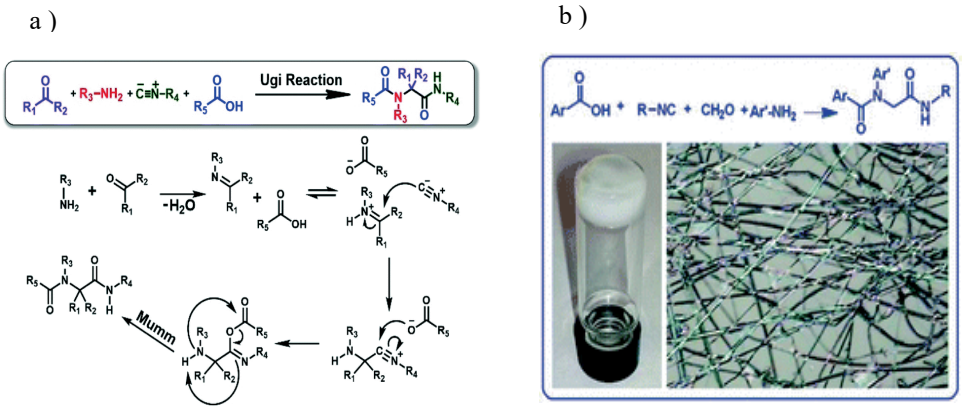

Figure 1.8 (a) Mechanism of Ugi reaction; (b) A small family of supramolecular hydrogelators synthesized by Ugi reaction. Figure adapted from reference 109 (a) and 117 (b).

1.6 Aim and outline

The field of squaramide-based supramolecular polymers has been growing steadily over the past few years. The remarkable ability of squaramide moiety to form directional hydrogen bonds that can synergistically benefit from an increase in their aromatic character, can be exploited in the design of supramolecular biomaterials. In water, the self-assembly of stable nanostructures is largely driven by the hydrophobic effect in combination with hydrogen bonding determining the final properties of the nanostructures. The formation of a particular nanostructure depends on several variables such as the chemical structure of the building block, if any other building blocks are used and the environmental conditions. Moreover, as the synthesis of the building blocks can be challenging and costly, and thus novel strategies to synthesize tripodal squaramide supramolecular polymers such as using multicomponent strategies will be examined.

In **Chapter 2**, the substitution of O → S in squaramide monomers for supramolecular polymerization results in the formation of rod-like nanostructures with a decreased persistence length. This morphological difference results from the less directional hydrogen bonds afforded by the thiocarbonyl group, resulting in a distinct self-assembly mode. From computational and UV-vis studies the oxosquaramide can self-assemble in a head-to-tail manner, while thiosquaramide prefers a stacked configuration. In this chapter, we investigated the supramolecular polymerization mechanism of oxosquaramide and thiosquaramide-based bolaamphiphiles using a concentration-based experiment. Moreover, the effect of the mixing of both monomers and their potential for co-assembly or self-sorting in aqueous solution is examined.

In **Chapter 3**, a new approach for the synthesis of tripodal squaramide-based supramolecular polymers by multicomponent reaction will be disclosed. The Ugi reaction will be examined for the synthesis of tripodal scaffolds using nitrilotriacetic and squaric acid as the acidic component of the reaction. In this

chapter, the optimal reaction conditions such as reagent concentration, temperature and substitution of the components will be examined.

In **Chapter 4**, the synthesis of a small family of tripodal squaramide-based supramolecular polymers is described and their self-assembly in water was investigated. The monomers have varied hydrophobic and hydrophilic ratios that affect their final self-assembly properties. Spectroscopic techniques (UV-vis, fluorescence), AFM and mechanical properties were used to understand the effect of the various monomer designs on these aggregates over various length scales.

In **Chapter 5**, the synthesis of dityrosine outfitted monomers with and without carbamate linkages and investigation of their self-assembly and visible light mediated crosslinking in the presence of photoinitator is described. In the previous chapter, we found that even removal of one squaramide moiety from the core and further modulating the hydrophilic-hydrophobic balance can result in gels. Hence, we opted for removal of one squaramide arm because of the solublility of the tyrosine and bridged its attachment to the squaramide core with a tetraethylene glycol chain. The self-assembly was investigated by spectroscopic techniques (UV-vis, fluorescence) and by AFM in presence and absence of two different photoinitiators (FMN or $Ru(bpy)_3^{2+}$/SPS).

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