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Nanostructures from self-assembling triazine tertiary amine *N*-oxide amphiphiles

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A new set of amphiphilic tertiary amine N-*oxides has been prepared and their self-assembly properties observed in aqueous solution by tensiometry, dynamic and static light scattering. X-ray crystallographic analysis of parent amines and sulfoxide congeners indicates the formation of hydrogen bonded dimers as the primary assembly unit for formation of vesicles in preference to the compact micelles typical of lauryl dimethylamine* N-*oxide (LDAO). 6-Benzyloxy*-N,N'-*bis(5-diethylaminopentylamine oxide)[1,3,5]triazine-2,4-diamine forms a 1 μm vesicle observed to entrap fluorescein. The [1,3,5]triazine core thus allows variation of the new self-assembled structures from nano- to micrometre length scales.*

Introduction

Molecular structures that display subtle and controllable properties for manipulation of proteins,[1] delivery of nucleic acids and other biomedical applications[2] are in strong demand. Approaches to their development include the synthesis of symmetrical and higher order structures such as triangular oligoethyleneglycols,[3] facial,[4] rigid tandem[5] and tripodal amphiphiles,[6] amphiphilic nanotubes,[7] gold dodecylmalonic acid coated nanoparticles,[8] and mimicry of natural osmolytes.[9] The last have been grouped[10] as 'methylamines' (which include for example trimethylamine *N*-oxide (TMAO),[11] non-detergent sulfobetaines[12]), polyols (including trehalose[13]) and amino acids. Although the detailed mechanisms remain closely studied,[1c, 10, 14] molecules such as these have been shown to suppress those interactions leading to non-productive folding or aggregation pathways[1a] more effectively than classic additives. Matching the most appropriate to a given protein[1b] is arduous and has led to the commercial development of factorial screening kits that, together with automated screening procedures, are applicable to most non-membrane proteins.[15]

The greatest challenge is posed by the manipulation of membrane-associated proteins and significant advances in methods for their solubilisation and crystallisation are currently being made.[16] Facial and rigid tandem amphiphiles bearing tertiary amine *N*-oxide and glycosides offer highly desirable qualities, including enhanced crystallisation of integral membrane proteins.[2a, 17] We wished to explore whether amphiphiles that can self-assemble using the aromatic and hydrogen bonding interactions associated with a heterocyclic core[18] might offer emergent features that extend these properties.[19] In particular we sought to use the [1,3,5]triazine core as an accessible platform from which to: vary molecular structure,[20] explore the role of polar functionality on self-assembly,[21] and ultimately the effects of these amphiphiles on protein solubility and folding.

The TMAO kosmotrope[22] is known to counteract the effects of urea on proteins,[11b, 23] and the same moiety[24] appears in amphiphiles for protein crystallisation,[6] membrane protein solubilisation[25] and other applications[24c, 25-26] which led us to design a set of tertiary amine *N*-oxide and analogous sulfoxides to investigate their supramolecular structure.[19] We expected their properties to depend on: (i) the nature of the hydrophobic portion (Figure 1: R = $C_{12}H_{25}$ or benzyl), (ii) the potential for diamino[1,3,5]triazine to engage in hydrogen bond or π-interaction mediated self-assembly[18a, 27] and (iii) the number and nature of strongly dipolar functions (amine *N*-oxide or sulfoxide) in the headgroup.[28] We here demonstrate their aggregation behaviour is consistent with our design, and report their properties assessed by surface tensiometry, dynamic and static light scattering.

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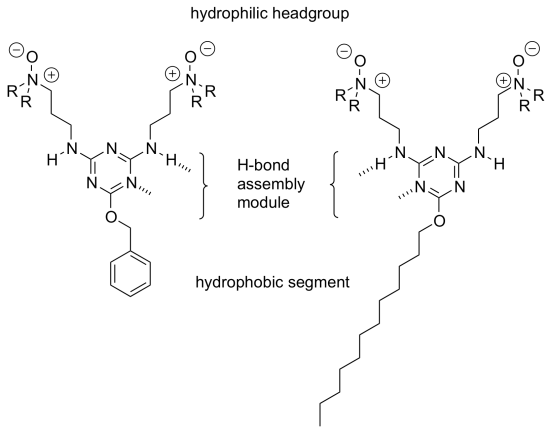


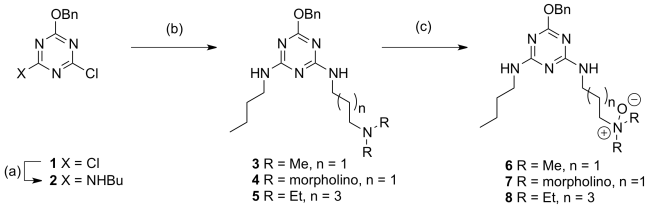
Figure 1. Design of self-assembling amphiphiles.

Results and Discussion

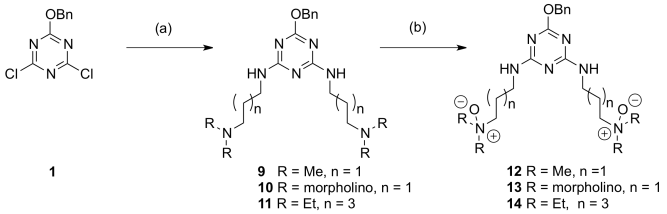
Synthesis of amphiphiles

Preparation of amphiphiles bearing the smaller hydrophobic segment was initiated by stepwise substitution from 2-benzyloxy-4,6-dichloro[1,3,5]triazine,[29] **1** (Scheme 1). Selective monosubstitution of **1** was achieved using either butylamine in tetrahydrofuran (THF) to give 4-benzyloxy-6-chloro[1,3,5]triazin-2-yl)butylamine **2** *en route* to unsymmetrical amphiphiles **3** – **5**. Replacement of the final chlorine atom required higher temperature[20a] to give tertiary amines which were oxidized to their cognate *N*-oxides **6**-**8** using *m*-cpba, in moderate yield. 2-Methylpropene was bubbled through the reaction mixture at -78 °C in order to remove excess oxidant.[20c] Symmetric double-headed derivatives were obtained by substitution of both chlorines at 60 °C (Scheme 2), with moderate yields of compounds **9** – **11** being achieved.

Crystals suitable for X-ray analysis of compound **10** were prepared by the slow diffusion of petroleum ether (60-80 °C) into compound **10** dissolved in chloroform (Figure 2) showing a symmetric hydrogen-bonded dimer structure involving one triazine nitrogen and amine NH donor.



Scheme 1. (a) Butylamine, *N*,*N*-diisopropylethylamine, THF, 1 h, 0 °C, (68%); (b) 3-(dimethylamino)propylamine, *N*,*N*-diisopropylethylamine chloroform, 16 h, 60 °C, **2** (53%); 4-(3-aminopropyl)morpholine, *N*,*N*-diisopropylethylamine, THF, 16 h, 60 °C, **3** (62%); *N*,*N*-diethylpentane-1,5-diamine[30] *N*,*N*-diisopropylethylamine, chloroform, 16 h, 60 °C, **5** (58%); (c) *m*-CPBA, K_2CO_3, CH_2Cl_2, 2 h, -78 °C; 2-methylpropene 5 min., **6** (40%), **7** (52%), **8** (52%).



Scheme 2. (a) 3-(Dimethylamino)propylamine, *N*,*N*-diisopropylethylamine, chloroform, 16 h, 60 °C, **9** (37%); 4-(3-aminopropyl)morpholine, *N*,*N*-diisopropylethylamine, THF, 16 h, 60 °C, **10** (59%); *N*,*N*-diethylpentane-1,5-diamine, *N*,*N*-diisopropylethylamine, chloroform, 16 h, 60 °C, **11** (36%); (b) *m*-CPBA, K_2CO_3, CH_2Cl_2, 2 h, -78 °C; 2-methylpropene 5 min., **12** (62%), **13** (72%), **14** (52%).

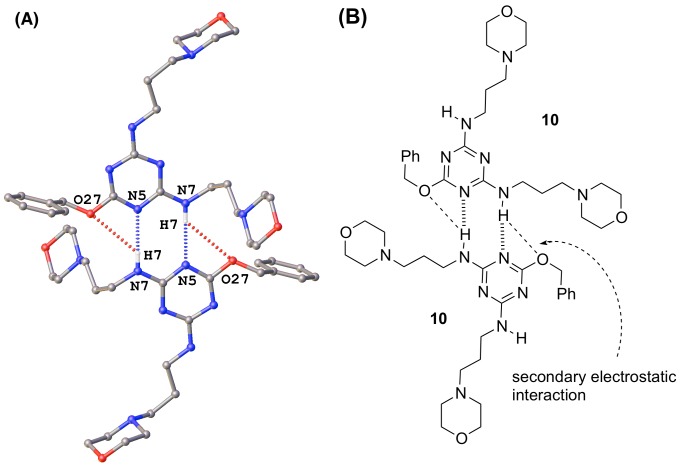
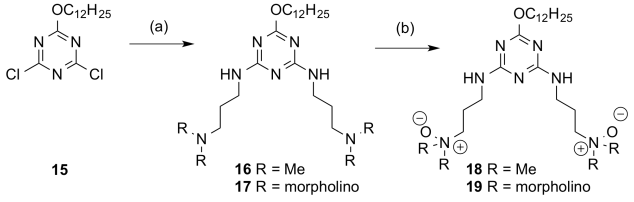


Figure 2. (**A**) Dimer of **10** showing H-bonding of triazine nitrogen (N(5), N(5A)) with the NH protons (N(7), N(7A)) and oxygen (O(27), O(27A)) of the substituents, N(7)H … O(27) 2.98 Å; H(10) … O(27) 2.89 Å. (**B**) Structure emphasizing the proximity of triazine NH protons and oxygen (O(27), O(27A)) for attractive secondary electrostatic interactions.[31] CCDC 930465.

Preparation of 2,4-dichloro-6-dodecyl[1,3,5]triazine,[32] **15** allowed access to analogues **16** and **17**; (Scheme 3) these reactions proceeded in lower yields than the benzyloxy series, mainly due to difficulties in the aqueous work-up and purification of the strongly amphiphilic compounds.



Scheme 3. (a) 3-(Dimethylamino)propylamine, *N*,*N*-diisopropylethylamine, chloroform, 16 h, 60 °C, **16** (20%); 4-(3-aminopropyl)morpholine, *N*,*N*-diisopropylethylamine, THF, 16 h, 60 °C, **17** (58%); (b) *m*-CPBA, K_2CO_3, DCM, 2 h, -78 °C; 2-methylpropene 5 min., **18** (52%), **19** (87%).

Compound **17** was initially obtained as an oil, which partially solidified on standing. Recrystallisation of this solid from water provided crystals that were suitable for X-ray analysis (Figure 3A), again revealing a symmetric hydrogen bonded dimer. The dodecyl chains interdigitate and one of the morpholine nitrogen arms is involved in a further hydrogen bond to a triazine in the layer above, creating a self-assembled box-like structure (Figure 3B). Similarly to structure **10**, rotation around C-N and C-O bonds connecting the hydrophobe and one polar sidechain to the triazine ring allows formation of a facially amphiphilic entity (*vide infra*) reminiscent of X-ray crystallographic structures of tertiary amine *N*-oxide amphiphiles[33] and the tandem four-armed maltoside derivatives which have been successfully used to recrystallise membrane proteins.[2b, 17a]

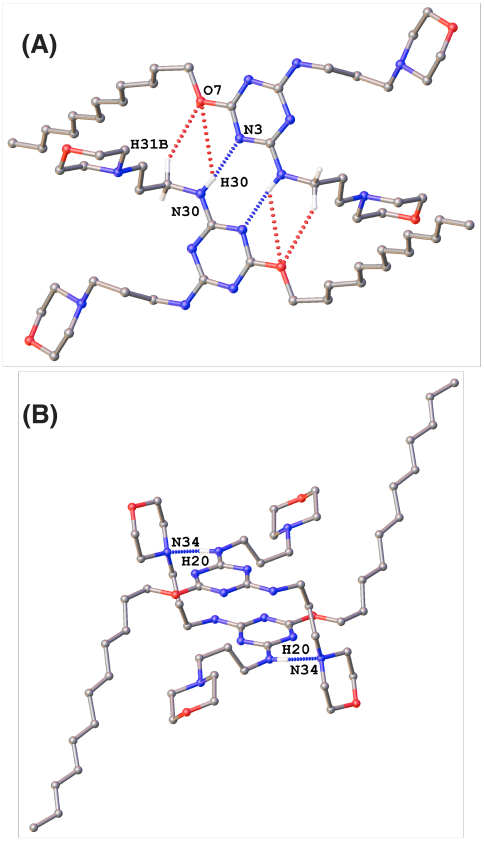
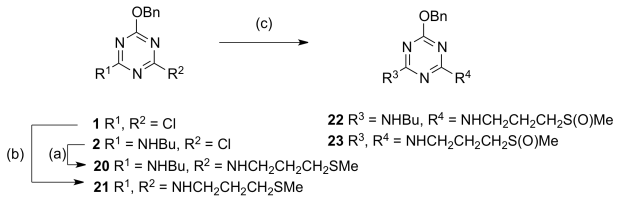


Figure 3 (**A**). The X-ray crystal structure of edge-to-edge H-bonded dimer of triazine **17**. In addition to primary H-bonds between NH(30) … N(3) there are longer contacts between O(7) … H(31B) 3.06 Å; O(7) … H(33B) 3.04 Å and reinforcing secondary electrostatic interactions O(7) … H(30) 3.14 Å; (**B**) H(20) is also involved in an additional box formation, using a reciprocal hydrogen bond to N(34A) of the morpholine ring H(20) … N(34) 2.218 Å. The two aromatic rings are parallel but too far offset for π-stacking. CCDC 930466.

Compounds isolated as solids soon deliquesced, even with reasonable care taken over their storage. Nonetheless, these tertiary amine *N*-oxides were stable and re-analysis of compounds that had been stored for two years at ambient temperature showed little or no decomposition by TLC and ^{1}H NMR.

Substitution of triazines **1** and **2** with 3-(methylthio)propylamine gave thioethers **20** and **21**, which were oxidized to the congener sulfoxides **22** and **23**. (Scheme 4).



Scheme 4 (a) 3-(Methylthio)propylamine, *N*,*N*-diisopropylethylamine, chloroform, 16 h, 65 °C, **20** (66%); (b) 2 eq. 3-(methylthio)propylamine, *N*,*N*-diisopropylethylamine, chloroform, 16 h, 65 °C, **21** (70%); (c) $NaIO_4$, acetone/water, 4 h, 0 °C, **22** (49%); **23** (40%).

Isolation of disulfoxide **23** as a yellow solid enabled preparation of crystals suitable for X-ray analysis using slow diffusion of diethyl ether into a chloroform solution (Figures 4 and 5), revealing a symmetric hydrogen-bonded dimer remarkably similar to that seen for tertiary amines **10** and **17**. Disorder of the racemic sulfoxide was evident in the structure (Supporting Information Figure S2), and close contacts were seen between the sulfoxide functional group and α-C-H of the adjacent sidechain and NH proton H(7A), with sulfoxide O(11B) adding additional stabilising interactions not seen in the amine series.

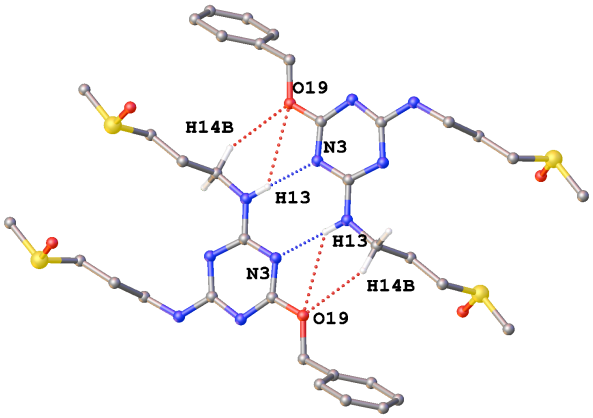


Figure 4. Dimer formed by the triazine **23** (N(3), N(3A)) and the NH protons (H(13A), H(13B)); N(3) … H(13A) 2.00 Å. H-bonding interactions involving O(19) and O(19A) add stabilising secondary electrostatic interactions O(19) … H(13A) 2.86 Å. Additional short contact O(19) … H(14A). CCDC 930464.

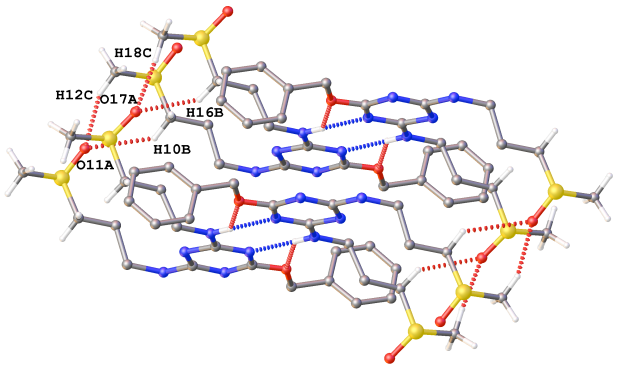


Figure 5. The H-bonded dimer formed by the triazines **23** crystallize as an infinite stack running along the *y* axis. Each dimer is connected with the dimer above by sulfoxide-sulfoxide interactions, O(17) … H(16) 2.50 Å, O(17) … H(18) 2.43 Å, although the triazines themselves are off-set and not involved in π-stacking. Disorder and H atoms not involved in these interactions are removed for clarity. CCDC 930464.

Self-assembly and aggregation

Lauryl dimethylamine *N*-oxide (LDAO, also know as *N*,*N*-dimethyldodecylamine *N*-oxide, DDAO) is a well-characterised amphiphile[34] with multiple uses in chemical biology[24c] and personal care settings.[26a, 35] It forms compact micelles with a critical aggregation concentration (CAC) of 1.7 mM[34a]. Analogous double-headed C10 – C16 dimethylamine *N*-oxide amphiphiles (Figure 6) have recently been observed to form micelles with hydrodynamic radii of 1.8 – 2.4 nm at CACs of 33 – 0.15 mM respectively.[28, 36]

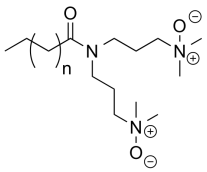


Figure 6. Double–headed *tert*-amine *N*-oxide amphiphiles, n = 10, 12, 14 prepared in reference [28].

We initially used surface tensiometry to determine the CACs of the tertiary amine *N*-oxides and our measured value for LDAO of 1.7 mM, coincided well with the literature value (Table 1). Dodecyloxy derivatives had a CAC 10-fold lower than that of LDAO and more than 100 times less than some of the compounds bearing the benzyloxy group. Amphiphiles containing only one amine oxide functional group (**6** – **8**) were observed to have CAC values at least twice those of the corresponding difunctional compounds (**12** – **14**; **18**, **19**). Aggregate size was determined by dynamic light scattering whilst molecular weights were determined by static light scattering (Table 1). In both cases an excess of electrolyte was added (0.2 M NaCl) to give a better defined aggregate species.[37] Most of the new compounds studied appear to form structures with typical dimensions of a vesicle (taken here as aggregate diameter ≥ 100 nm) rather than the much smaller 5.0 nm micelle formed by LDAO, however **7**, **8** and **19** appeared to be in equilibrium with smaller aggregates, probably micelles. Contrastingly, compound **14** was mostly present as a *ca.* 1 μm assembly, large enough to be visualised using confocal fluorescence microscopy, by entrapment of fluorescein within the vesicle (Figure 7 and Supporting Information three-dimensional *z*-stack reconstruction).

The shape and size of a vesicle or similar bilayer assembly minimises the energy of the inner and outer leaflets and is influenced by molecular shape, non-covalent interactions and concentration of electrolyte or osmolyte. In the case of tertiary amine *N*-oxides there may also be more complex effects due to interfacial pH,[34d, 38] or shared protonation between functional groups which requires further investigation using potentiometric and NMR titration.

Table 1. Summary of physicochemical parameters for the new amphiphiles.

Compound	CAC /mM	Surface tension /mN m^{-1}	Aggregate diameter /nm	Aggregate molecular weight	Aggregation number (*N*)
LDAO	1.7 lit.[34a] 2.0	30.8 ±0.2	5.0±0.5	18000±400	79±2 (lit.[34a] 78)
6	6.0	27.9±0.9	400±40	242500±2500	650±5
7	25.0	28.4±1.5	13±1 100±15	14000±700	335±5
8	10.0	27.6±0.2	15±1 100±15	226000±4500	525±10
12	12.0	40.4±0.2	180±9	110000±2000	260±5
13	20.0	39.9±0.3	190±10	220500±1000	420±5
14	1.4	39.1±0.6	195±10 1140±280	988000±9900	1850±20
23	2.8	60.6±0.2	131±3	66000±2000	150±5
18	0.2	40.0±0.2	270±10	494000±12000	1000±25
19	0.2	40.3±0.3	6.0±0.1 240±50	70000±300	120±5

X-ray data for the precursor triazine γ-alkylamines (Figures 2, 3), analogous sulfoxides (Figures 4, 5) and literature X-ray crystallographic data for diamino[1,3,5]triazines[39] and associated supramolecular structures,[27b, 27c, 40] suggest that hydrogen bond mediated dimerisation of the triazines is an important interaction in the relatively non-polar vesicle environment.[41] Figure 9(A) shows how two triazines might associate to form a dimer consistent with the symmetric structures seen in the solid state for precursor triazine γ-alkylamines **10** and **17**. Whilst π-π interactions could play a similar role, it seems likely that a hydrogen bond mediated self-assembly process[18] in addition to the hydrophobic effect,[41b] is leading to vesicle-like assemblies for several compounds. What is clear is that whether the major hydrophobe is benzyloxy, or the longer dodecyloxy function, significantly larger aggregates are observed for all of these amphiphiles than the simple single chain LDAO. Importantly, the literature data from double-headed analogues such as dicephalic *N*,*N*'-bis[3,3'-dimethylamino)propyl]alkylamine di-*N*-oxide surfactants (Figure 6) lacking the triazine group show hydrodynamic radii comparable to LDAO, rather than the significantly larger hydrodynamic diameters presented for the new amphiphiles in Table 1. Figure 8(B) depicts a bilayer structure that might be formed by any of the double headgroup amphiphiles through interdigitation of either

benzyl or dodecyl groups. Further ribbon-like assemblies due to triazine hydrogen bonding are not observed in the X-ray structures herein. It is however possible that these, or additional π-π interactions are strengthening the assembly process leading to the preference for vesicles rather than compact micelles seen for LDAO.

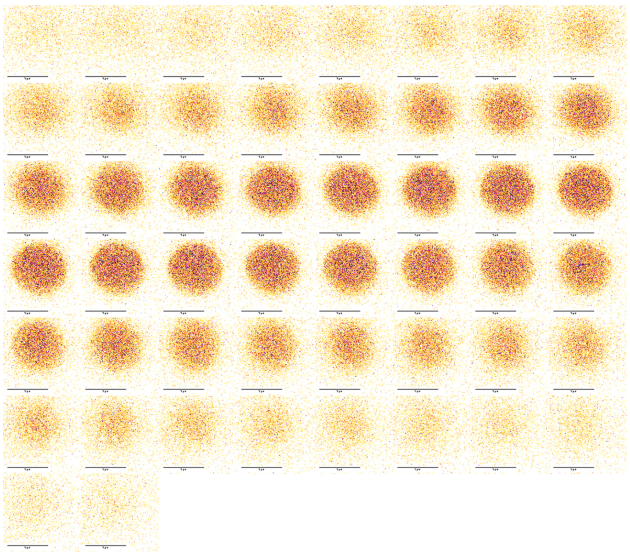


Figure 7. Confocal microscopy false colour image montage (*z*-stack slices, top left to bottom right, scale bar = 1 μm) of fluorescein entrapment by compound **14** (25 mM) dispersed in 0.2 M NaCl (aq). The *z*-stack slices shown can be combined to produce three-dimensional images of the 1 μm approximately spherical vesicle (Supporting Information).

Notably, compound **14** which forms a 1 μm vesicle (Figure 7) possesses a benzyloxy segment, adding the possibility of additional aromatic interactions to stabilise the less highly curved architecture observed herein. The amphiphile assemblies are of interest for several nanotechnological applications and we note that dynamic control over aggregate assembly has previously been achieved by varying pH in LDAO solutions.[34d, 38, 42] In summary, since double-headed, single-tailed tertiary amine *N*-oxide amphiphiles have previously been shown to form relatively compact micelles,[28] the presence of the triazine moiety is key to the formation of the larger structures observed herein.

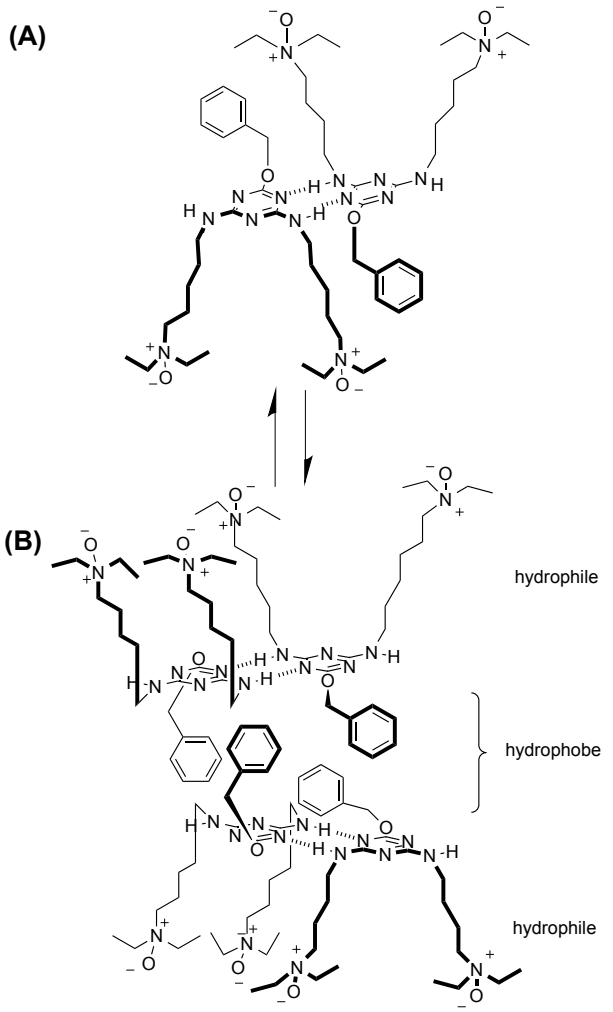


Figure 8. (A) Hydrogen bond mediated dimerisation of compound **14**; **(**B) proposed self-association to bilayer aggregate in water.

Conclusion

The new self-assembling amphiphiles provide a ready platform from which to control the balance of hydrophobic and kosmotropic headgroup properties. We have shown how the versatile [1,3,5]triazine core enables the systematic variation of supramolecular structures that are stable in water over nanometre to micrometre length scales. Coupled with an efficient screening process that can reveal detailed biomolecular structure, such as NMR-based tools[43] we are now beginning to explore how they might be used with a range of biopolymers. By measuring amphiphile molecular parameters and matching them against protein properties, we wish to move towards more predictive selection of additives, in particular for manipulation of membrane associated proteins.[44]

Experimental Section

See Supporting Information for synthesis of amphiphiles.

Amphiphile solutions were prepared using distilled water with a conductance of 18 MΩ from an Elgastat Maxima Ultra Pure Water purifier. After making up a stock solution (20 ml) for each compound (LDAO, 25.00 mM; **6**, 12.50 mM; **7**, 50.00 mM; **8**,

12.50 mM; **12**, 25.00 mM; **13**, 12.50 mM; **14**, 6.25 mM; **23**, 12.50 mM; **18**, 1.56 mM; **19**, 1.56 mM) the surface tension was recorded in duplicate. The stock solution was then diluted by 50% using distilled water and the measurement once again recorded. The process was repeated until the concentration of the compound no longer had an affect on the surface tension *i.e.* the surface tension was in accordance with that of pure water, which at 25 °C has a value of 72.8 mN m^{-1}.[37] The CAC is taken as the point at which the two lines intersect, just after the slope for surface tension has ceased. Determination of error is therefore dependent on a distinct inflexion point.

Surface tension measurements were recorded on a NIMA Dynamic Surface Tensiometer Type: DST9005 (Coventry, England), using the DuNuoy ring method. After finding the meniscus of a given solution, the platinum ring was submerged 2.0 mm below the surface, before gently raising. In each instance raising the ring a distance of 5.0 mm above the surface of the solution was sufficient to break the meniscus. After recording the surface tension at each concentration the glass sample container was washed using fresh distilled water and dried. The platinum ring was cleaned by flaming with a butane gas burner before being allowed to cool.

Tabulated surface tension results and charts are available in Supporting Information S2.

Samples for dynamic light scattering were prepared at 15 x CAC (LDAO, 25.95 mM; **6**, 85.00 mM; **7**, 375.00 mM; **8**, 138.80 mM; **12**, 173.40 mM; **13**, 302.5 mM; **14**, 20.76 mM; **23** 42.20 mM; **18**, 2.88 mM; **19**, 2.61 mM) in 5 ml of 18 MΩ ultra pure water that had been filtered five times through a 0.22 μm filter. Sodium chloride (Fisher) was added to each 5 ml sample to give a final concentration of 0.2 M.[45]

Dynamic light scattering experiments were recorded in a polystyrene cuvette on a Malvern Instruments Zetasizer 3000 H_{SA} spectrometer equipped with a 5-mW helium-neon laser. Each sample was repeated separately five times, with each of these taken as an average of ten data points. All data were collected out at an angle of 90° at 25 °C with the determination of the hydrodynamic radius being carried out using the CONTIN algorithm.

Stock solutions for static light scattering were prepared for each of the samples (LDAO, 40.00 mM; **6**, 154.00 mM; **7**, 144.00 mM; **8**, 139.00 mM; **12**, 47.70 mM, **13**, 67.50 mM; **14**, 113.00 mM; **23**, 56.00 mM; **18**, 16.50 mM; **19**, 62.00 mM) in 18 MΩ ultra pure water (5 ml) that had been filtered five times through a 0.22 μm filter. Sodium chloride was added to each sample to give a final concentration of 0.2 M. After performing the static light scattering measurement (see below) on the stock solution, the sample was diluted with 0.2 M NaCl (1.5 ml) and the measurement repeated. The dilution was repeated three more times resulting at a total of five concentrations for each sample being recorded.

Static light scattering experiments were recorded in a polycarbonate cylindrical cuvette on a Malvern Instruments Autosizer 4800 spectrometer equipped with a 5-mW helium-neon laser. Each measurement was carried out using a Debye plot function on the Autosizer 4800 program. A baseline consisting of 0.2 M NaCl in 18 MΩ ultra pure water, was run prior to the measurement of the samples. All sample run measurements were taken from an average of 10 repeats carried out at an angle of 90° at 25 °C. Once data had been acquired for all concentrations the aggregate's molecular weight was calculated using the following equation:

$$\frac{Kc}{R} = \frac{1}{M_w P(\theta)} + 2A_2 c + 3A_3 c^2$$

With the following constants:

A_2: Second viral coefficient
A_3: Third viral coefficient
Standard refractive index (η):1.330
Refractive index (η) of dispersant: 1.330
Wavelength: 532.0 nm
Change in refractive index as a function of concentration, dn/dc: 0.110 ml/g
Rayleigh Ratio (R): 1.40 x 10^{-5}

Confocal laser scanning microscopy measurements were performed using a Zeiss Axiovert LSM 510 confocal laser-scanning microscope, operating at 494 nm excitation wavelength and 520 nm emission wavelength. A 5 ml sample containing **14** (20.8 mM in 0.2 M NaCl) and fluorescein (5.0 mM) was prepared using 18 MΩ ultra pure water (previously filtered five times through a 0.22 μm filter). The solution was shaken vigorously until complete dissolution and allowed to settle before transferring to a small petri dish. The microscope was submerged into the sample prior to visualisation, with the UV light switched on.

Supporting Information

Preparation and characterisation of new compounds. Tensiometry, static and dynamic light scattering data, additional X-ray structures and Quicktime format movies of three-dimensional *z*-stack reconstructions from confocal microscopic imaging.

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Keywords: ((self-assembly · hydrogen bond · amphiphile · tertiary amine *N*-oxide · protein folding))

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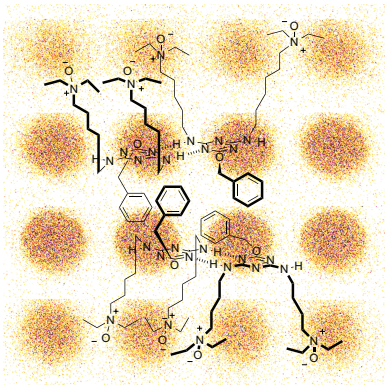
ARTICLES

Strengthened through self-assembly: Amphiphiles with subtle properties for chemical biology applications are in much demand. γ-Tertiary amine *N*-oxide [1,3,5]triazines form stable aqueous assemblies: picture shows a *z*-stack montage of slices through a 1μm diameter fluorescein loaded vesicle. The new assemblies are very different from the small micelles formed by single headed, or even double headed analogues lacking the triazine ring.



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Nanostructures from self-assembling triazine tertiary amine *N*-oxide amphiphiles