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RESEARCH ARTICLE

Unusual Enthalpy Driven Self Assembly at Room Temperature with Chitosan Amphiphiles

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Abstract: ***Background*:** GCPQ (N-palmitoyl-N-monomethyl-N,N-dimethyl-N,N,N-trimethyl-6-O-glycolchitosan) is a self-assembling polymer being investigated as a pharmaceutical nano-carrier. GCPQ nanoparticles shuttle drugs across biological barriers, improving drug performance. The exact chemistry of GCPQ is varied by the relative proportion of hydrophobic (N-palmitoyl) and hydrophilic (quaternary ammonium) groups and molecular weight.

***Objective*:** We hypothesised that the thermodynamics of self-assembly is controlled by the polymer molecular weight and hydrophobicity.

***Method*:** The thermodynamics of self-assembly was investigated using isothermal calorimetry.

***Results*:** GCPQs (Mw = 8-15 kDa) formed micellar aggregates at critical micellar concentrations of 1-2.4 μM at 25°C and micellisation was unusually enthalpy driven. There was a positive correlation between ΔHmic and mole% quaternary groups (Q): ΔHmic = 3.8 Q-159 (r2 = 0.93) and a negative correlation between ΔHmic and molecular weight (Mw): ΔHmic = -13.5 Mw-26.3 (r2 = 0.99).

***Conclusion*:** These findings provide insights into the positive drivers of stable self-assemblies, namely hydrophobicity and molecular weight, as both hydrophobicity and molecular weight are associated with an increased enthalpy contribution to micellisation.

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1. INTRODUCTION

Amphiphilic polymers, composed of hydrophilic and hydrophobic units self-assemble in aqueous media forming micelles [1], vesicles [2-4] or amorphous solid nanoparticles [2]. Amphiphilic polymers are of importance in pharmaceutical formulation for example, where they serve as drug carriers, improving and, in some cases, facilitating therapeutic outcomes [5, 6]. Amphiphilic polymers may be block co-polymers [3] or have hydrophilic backbones and hydropho-bic pendant groups [2, 4]. The hydrophobic units may be multicyclic [7], alkyl [2] or acyl [4] and the hydrophilic groups either anionic [8], neutral [9], cationic [2] or zwitterionic [10]. The self-assembly of low molecular weight and polymer amphiphiles in aqueous media is largely driven by hydrophobic interactions [11].

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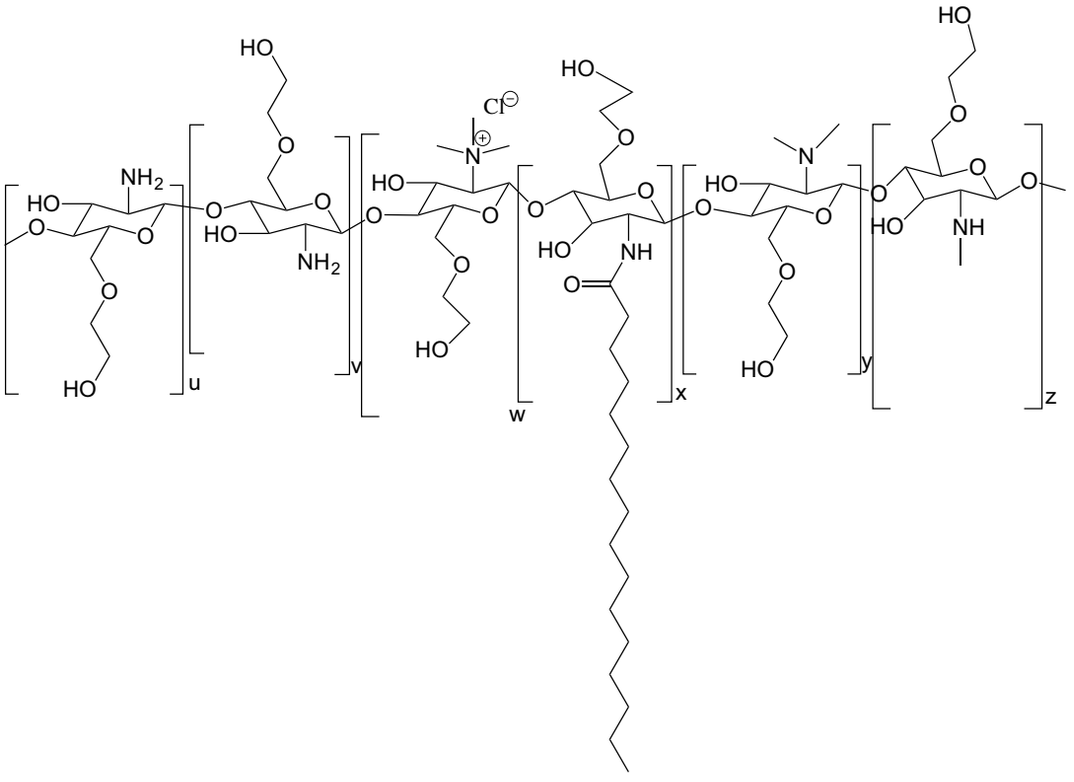


Fig. (1). N-palmitoyl-N-monomethyl-N,N-dimethyl-N,N,N-trimethyl-6-O-glycolchitosan (GCPQ).

The self-assembly of amphiphiles into micelles may be studied by monitoring changes in the bulk properties of an amphiphile dispersion (*e.g.* conductivity, surface tension, enthalpy or the polarity around a hydrophobic fluorophore) [12-14]. Amphiphile self-assembly into micelles is quantified by measuring the critical micelle concentration (CMC); *i.e.* the concentration at which the amphiphiles first start to self-assemble into micelles [12]. Micelles are spherical nanometre sized objects with a hydrophobic core and hydrophilic surface and micellisation is affected by the chemistry of the amphiphile and environmental conditions such as temperature or the presence of ions [12].

We used isothermal calorimetry to measure the enthalpy change of micellization (ΔH_{mic}) and in turn determine the CMC, Gibbs free energy change of micellization (ΔG_{mic}) and the product of the temperature entropy change of micellization ($T\Delta S_{mic}$) of a chitosan amphiphile (GCPQ, N-palmitoyl-N-monomethyl-N,N-dimethyl-N,N,N-trimethyl-6-O-glycolchitosan, Fig. **1**). GCPQ (Fig. **1**) is being investigated as a drug delivery system and the synthesis, purification and chemical characterisation of this polymer has been reported previously [15]. GCPQ self assembles into spherical micelles and GCPQ encapsulates hydrophobic and amphiphilic drugs within nanoparticles and delivers these biologically active compounds across biological barriers, such as the gastrointestinal epithelial barrier, significantly enhancing the oral bioavailability of drug compounds in preclinical models [13, 16, 17]. GCPQ also delivers peptides via the nose to brain route [18]. GCPQ thus alters drug biodistribution in therapeutically favourable ways. Drug encapsulation and colloidal stability in high dilution are required for GCPQ to be effective as a drug delivery system and these two properties are driven by the molecule's aggregation thermodynamics. Micelle formation at room temperature is usually driven by the entropy gain following micellization [19-21], with a switch to being enthalpy driven as the temperature rises [20]. However, here, we report an unusual enthalpy driven micellization at room temperature, associated with negative $T\Delta S_{mic}$ values. Others have reported enthalpy driven precipitation events in aliphatic amines in response to deprotonation of the amine groups [22]. However, enthalpy driven micellization at room temperature is unusual.

2. EXPERIMENTAL

2.1. Materials

All reagents and chemicals were obtained from Sigma Aldrich, U.K. unless otherwise stated and were used without further purification. GCPQ was obtained from Nanomerics Ltd and had been purified according to an established protocol involving exhaustive dialysis [15]. The polymer is isolated as a hydrochloride salt with a solution pH of 4-4.5 and has a pKa of 6.5 [15]. A MicroCal iTC200 (Malvern Instruments, Malvern, UK) was used for the isothermal calorimetry titrations

2.2. Polymer Nomenclature

The various GCPQ polymers were named using the following nomenclature: GC(Mw in kDa) P(Mole% palmitoyl groups)Q(Mole% quaternary ammonium groups) and so a polymer with a molecular weight of 9 kDa and containing 9 mole% palmitoyl groups and 36 mole% quaternary ammonium groups would be named as follows: GC9P9Q36. The ratio of hydrophilic (quaternary ammonium groups) versus hydrophobic (palmitoyl groups) substitution is given by the quaternary to palmitoyl ratio (QPR, Equation 1).

$$QPR = \frac{Q}{P} \quad (1)$$

where Q = mole% quaternary ammonium groups and P = mole% palmitoyl groups.

2.3. Isothermal Titration Calorimetry

The enthalpy change (ΔH_{demic}) on demicellisation was measured (Figs. **S1** and **S2**) using a MicroCal iTC200 calorimeter (Malvern Instruments, Malvern UK). Prior to each experiment, 200 μL of deionised water was added to the sample cell (which has a capacity of 280 μL). GCPQ (1 mg mL^{-1}) in water was added to the injection syringe which holds 40 μL. Prior to each experiment, a water-water run was carried out to ensure a good baseline was obtained (Fig. **S2**). At regular intervals (120 s), GCPQ dispersions (0.8 - 2 **μ**L) were injected into the sample cell and the heat flow measured as a function of time with constant stirring at 1000 rpm. Data analysis was carried out using the MicroCal Origin version 7.0 and Graphpad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA). Demicellisation experiments were performed at room temperature (25°C). All experiments were conducted in triplicate and the mean ± s.d. reported. The ΔH_{demic} and CMC of sodium dodecyl sulphate was also measured at 15°C in order to ensure that the instrument was performing as expected.

2.4. Transmission Electron Microscopy

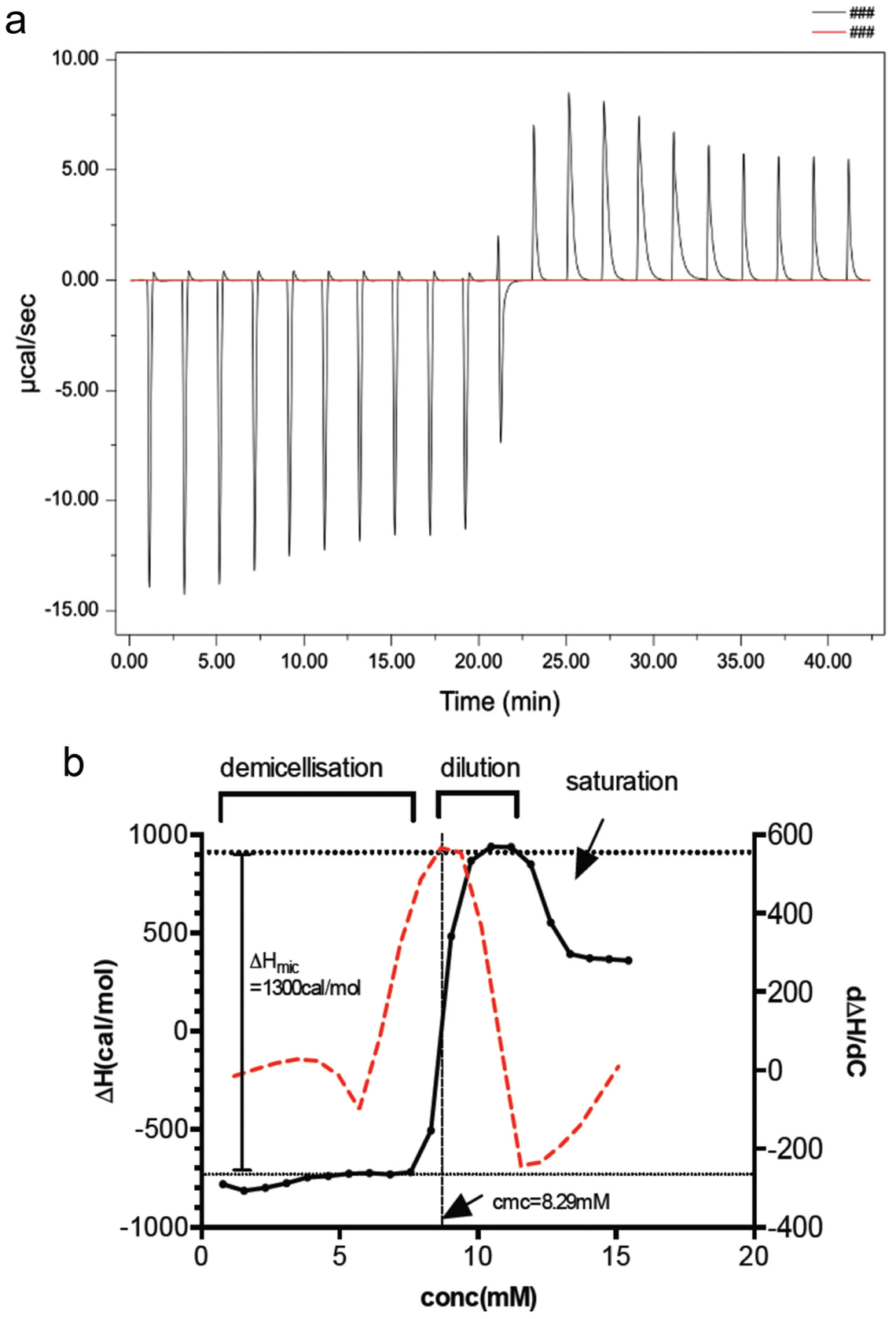
Transmission electron microscopy (TEM) with negative staining was carried out using methods previously described [13].

3. RESULTS AND DISCUSSION

Isothermal calorimetry (ITC) was used to determine the various thermodynamics parameters of the micellisation events. Dilution enthalpograms are shown in Figs. (**2**, **S1** and **S2**) and an example of the micellar system is shown in Fig. (**3**). On the first injections of the polymer micelle dispersion into the water containing sample cell, the micelles are diluted below their CMC values and the dilution enthalpograms reflect the resultant demicellisation of the micelles, as well as dilution of the resulting monomers (Fig. **2b**). During the second set of injections the dilution enthalpograms reflect the end of the demicellisation events as the CMC is reached (Fig. **2b**). The final stage of the enthalpograms indicates very little change in heat flow as more micelles are added to the sample cell and the micelles remain intact (Fig. **2b**). Large changes in enthalpy per injection volume, are an indicator that demicellisation is occurring; while the abrupt change in enthalpy events indicates that demicellisation is complete and any additional micelles are simply diluted in the sample cell [19]. To detect the abrupt change in enthalpy events, the first derivative of the change in heat flow per injection volume was determined and plotted, with the peak in this plot used to determine the CMC [19].

The enthalpy of demicellisation (ΔH_{demic}) is calculated from the difference between maximum and minimum heat change [19] (Fig. **2a**). According to the law of mass action, the standard Gibbs free energy change of demicellisation (the transfer of one molecule of GCPQ from the micelle to water) is calculated from Equation 2 [12].

$$\Delta G_{demic} = -RT \ln k \quad (2)$$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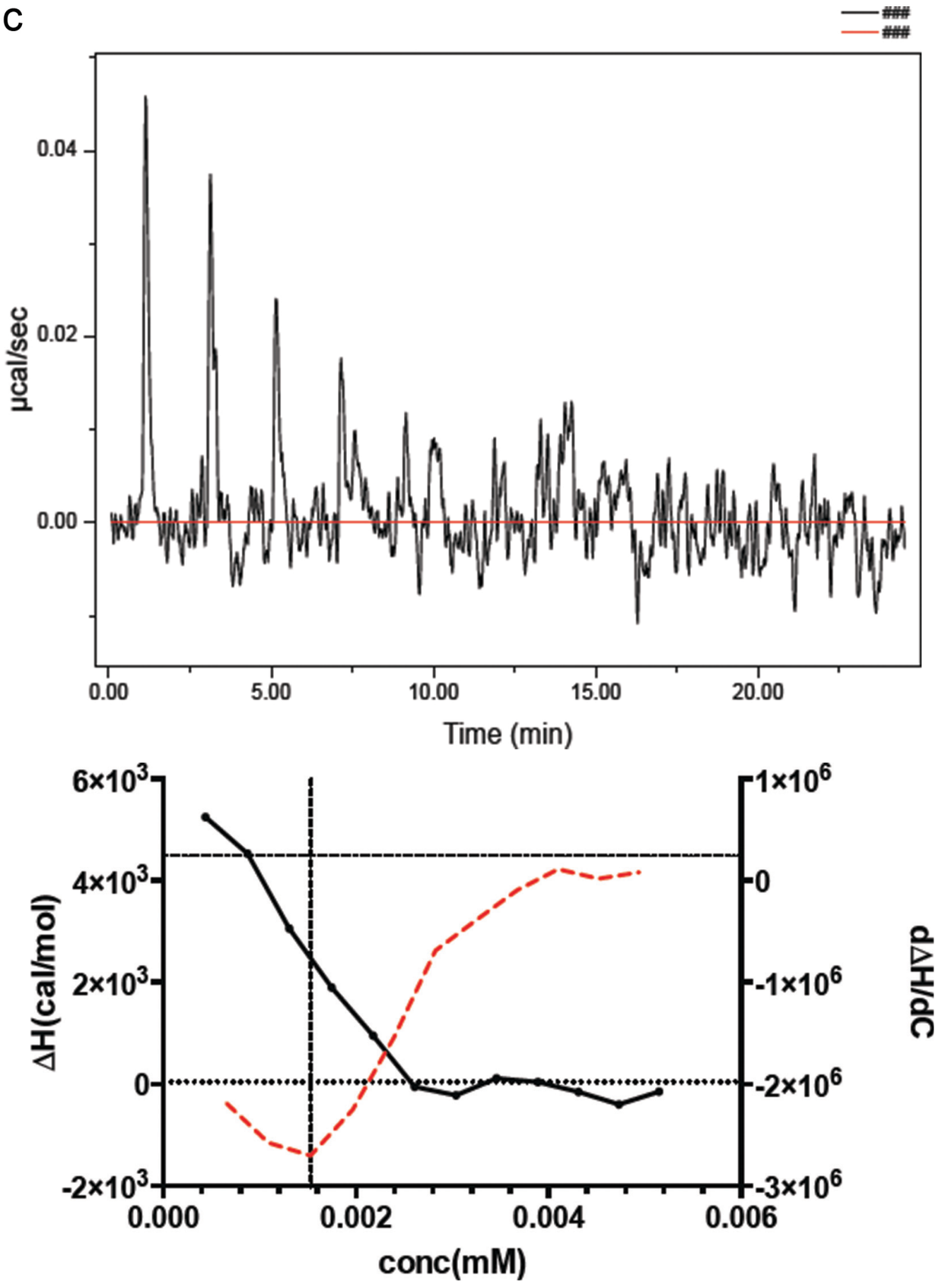
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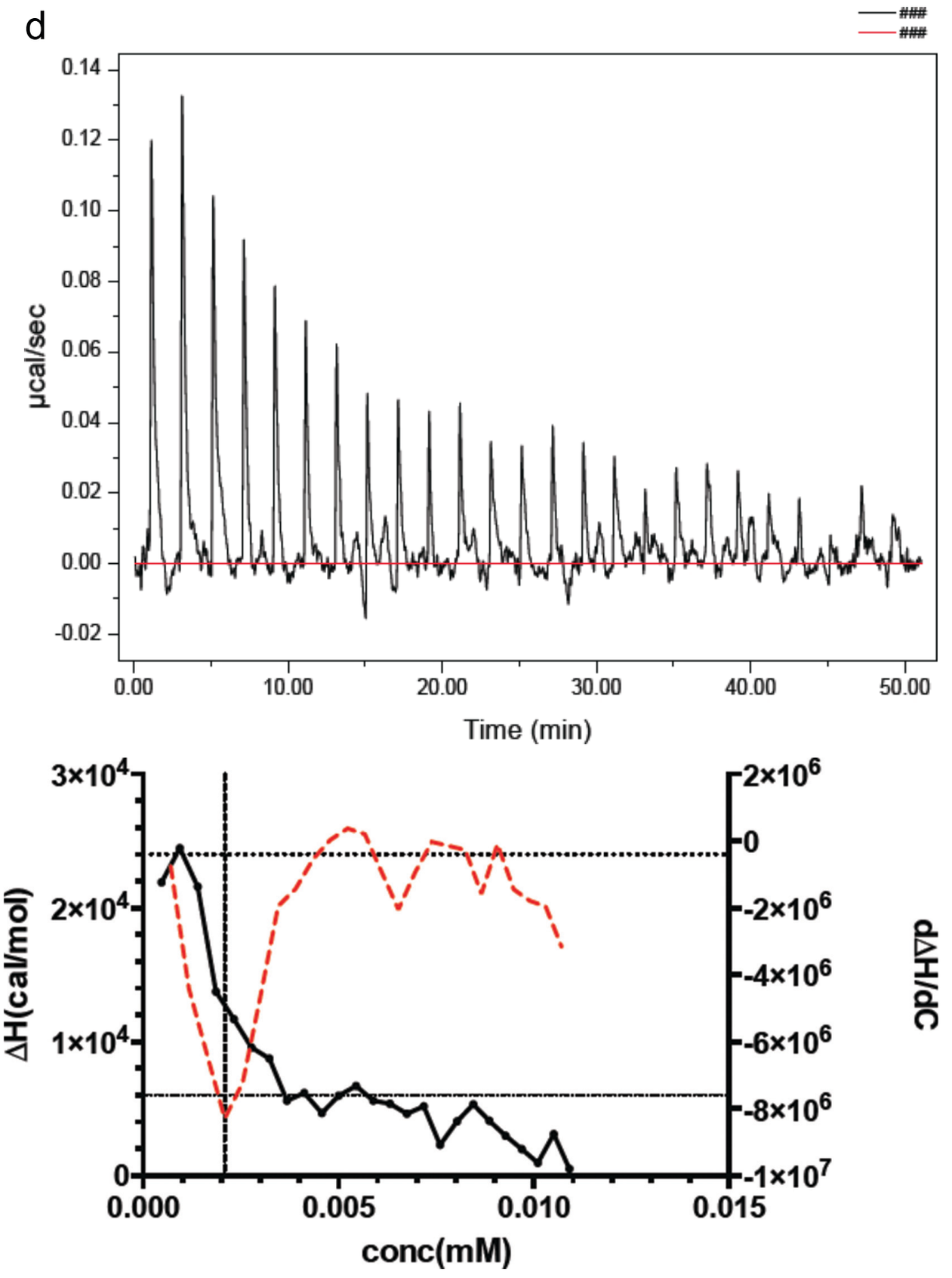
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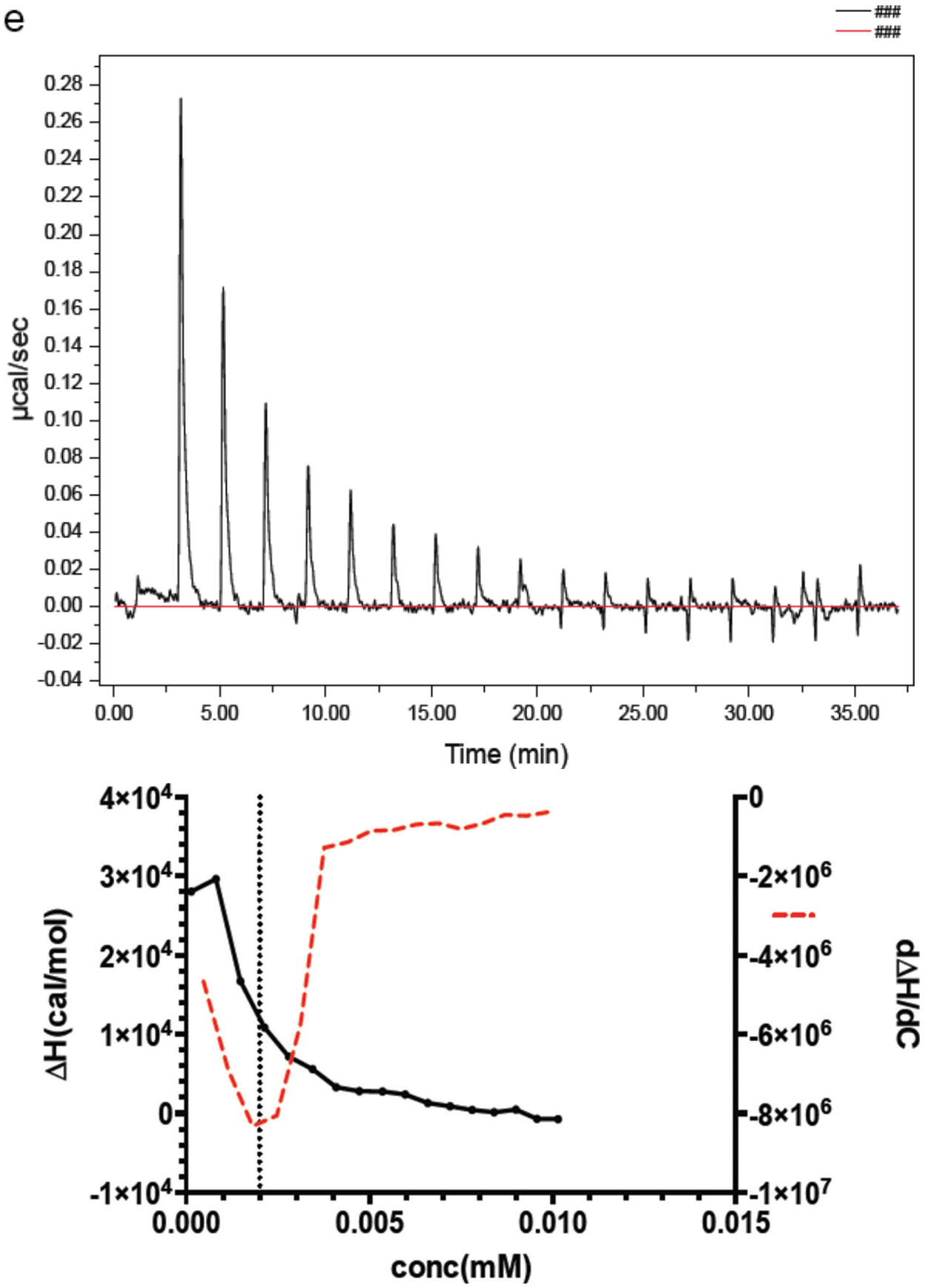
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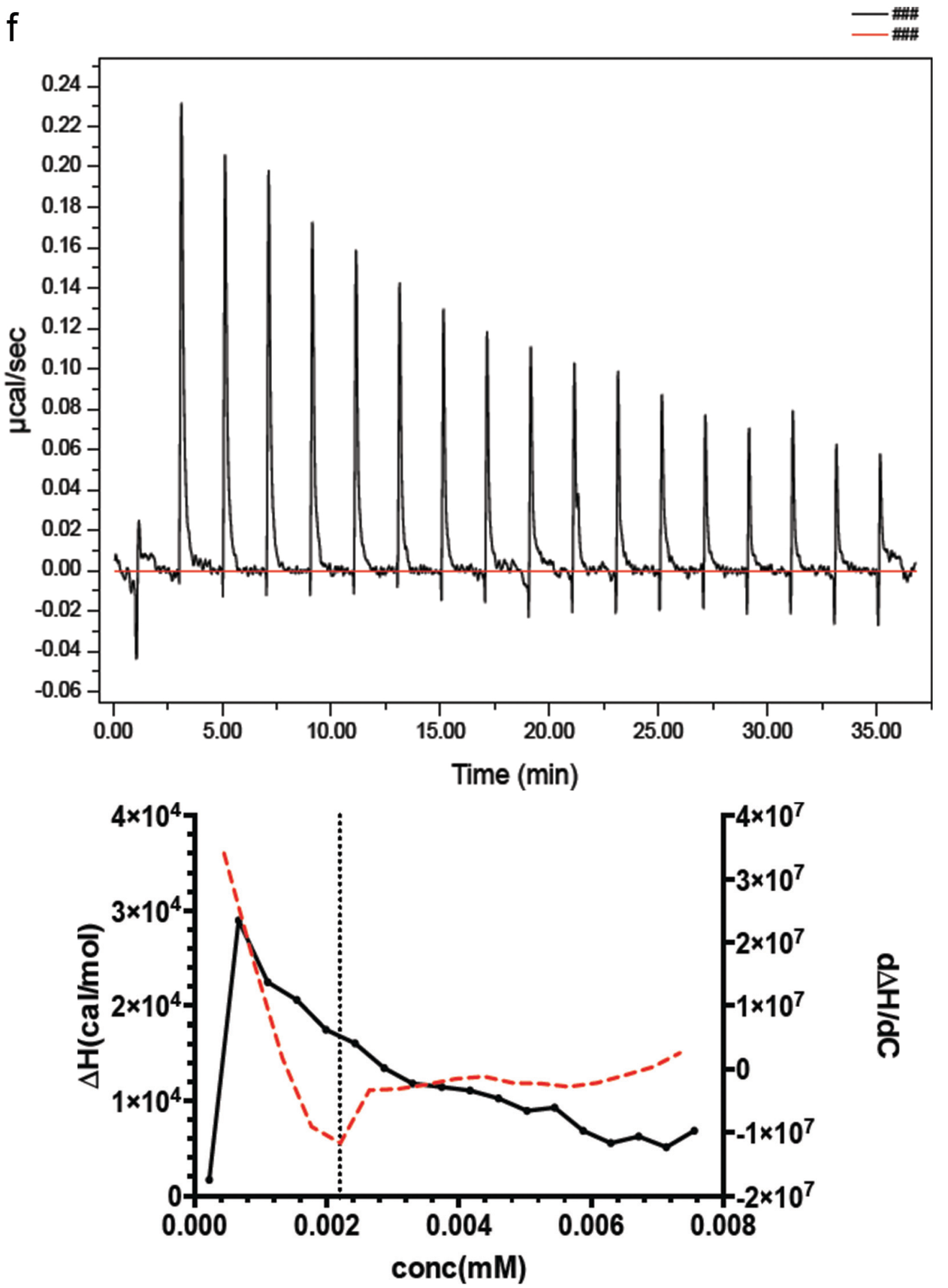

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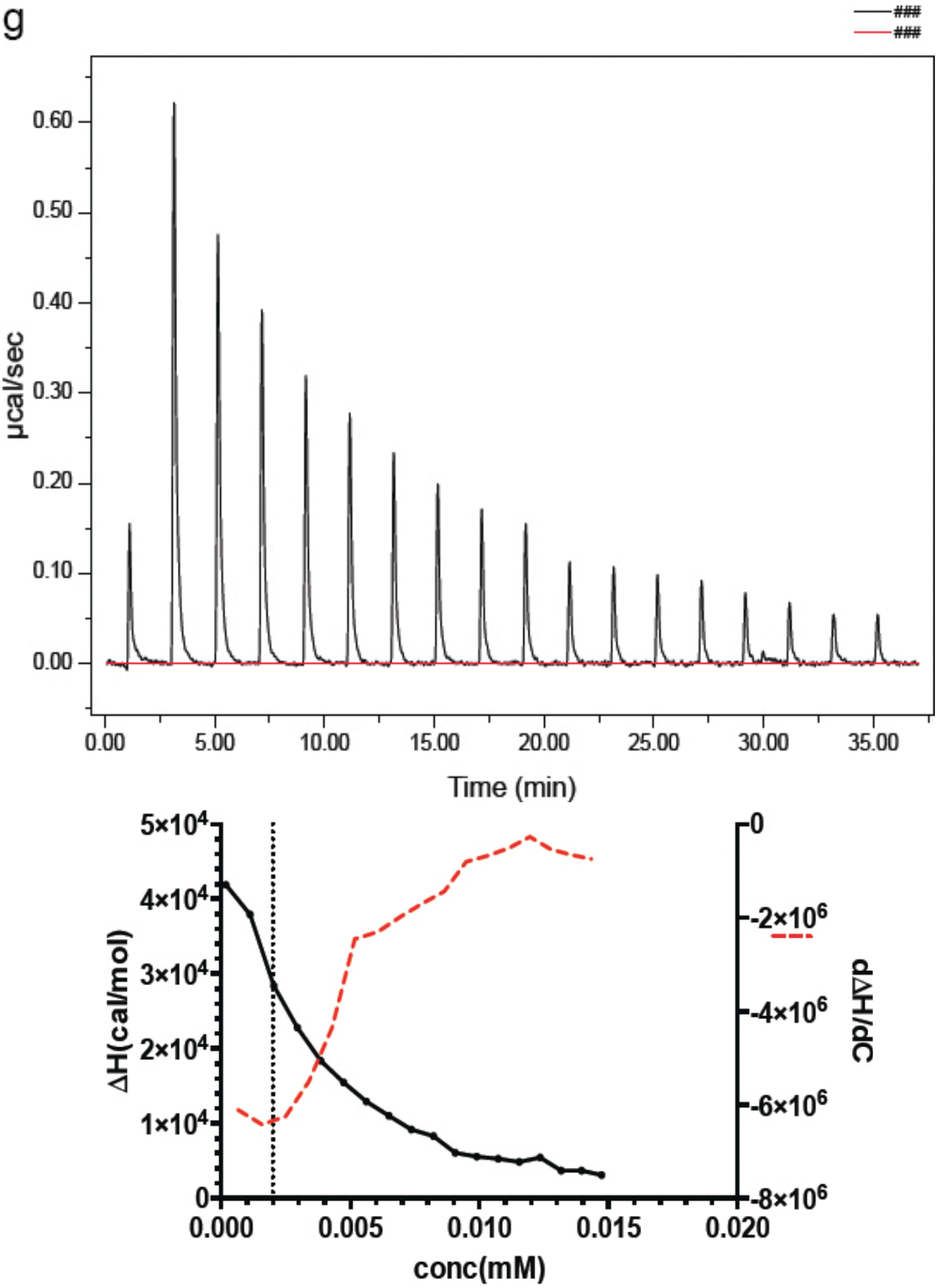
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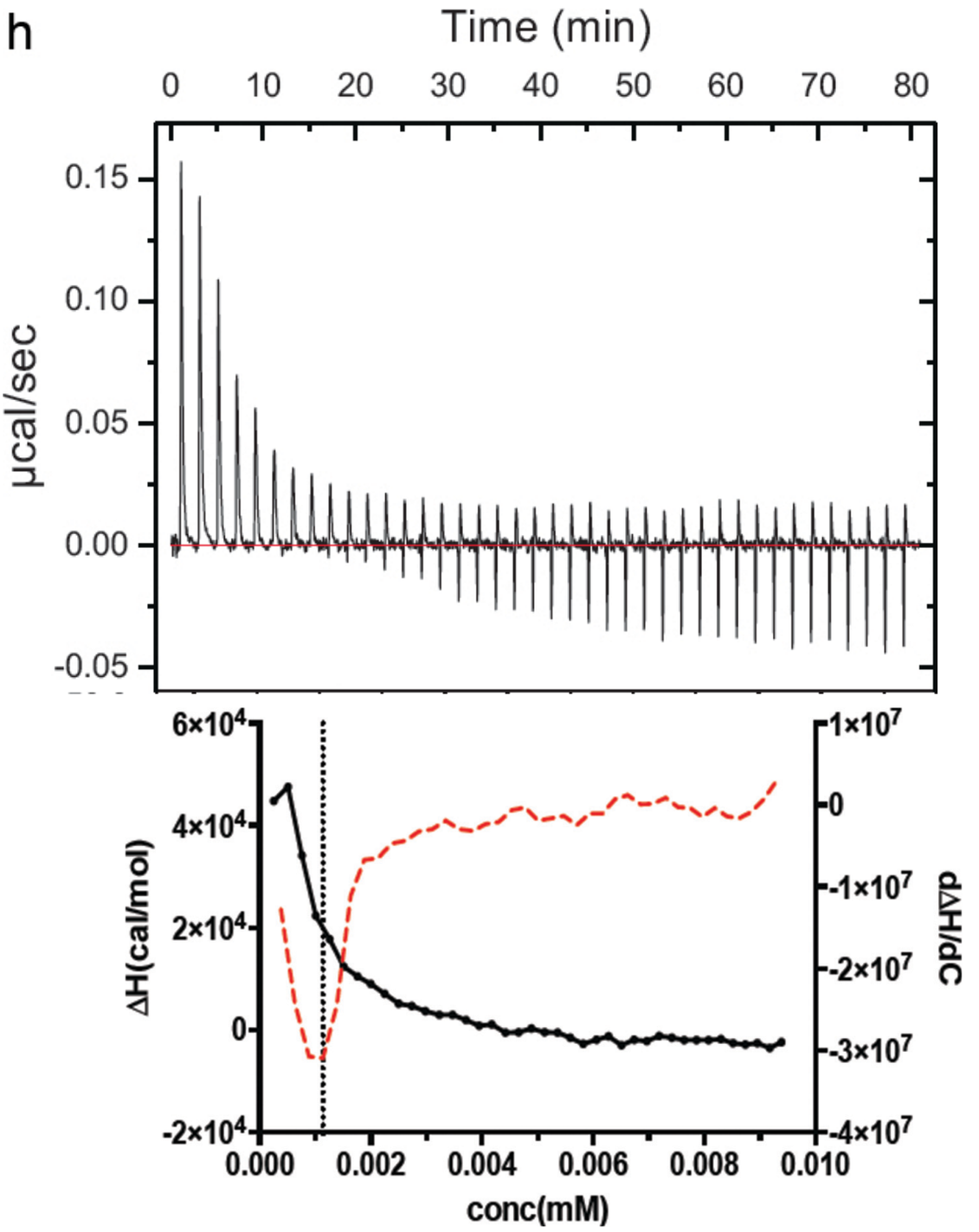

Fig. (2). Dilution (demicellisation) enthalpograms in water and the first derivative of the enthalpy change with concentration (red line) at 298°K (288°K for sodium dodecyl sulphate) for aqueous dispersions of GCPQ and sodium dodecyl sulphate (top and bottom panels in relevant plots respectively): **a**) and **b**) sodium dodecyl sulphate, starting concentration in the syringe = 160 mM, injection volume = 1µL per injection, **c**) GC9P9Q36, starting concentration in the syringe = 113.5 µM, injection volume = 0.8 µL per injection, **d**) GC12P19Q9, starting concentration in the syringe = 81 µM, injection volume = 1.2 µL per injection, **e**) GC14P12Q9, starting concentration in the syringe = 69.9 µM, injection volume = 2 µL per injection, **f**) GC9P19Q12, starting concentration in the syringe = 114.9 µM, injection volume = 0.8 µL per injection, **g**) GC10P19Q10, starting concentration in the syringe = 96.1 µM injection volume = 2 µL per injection, **h**) GC15P4Q11, starting concentration in the syringe = 65.7 µM, injection volume = 0.8 µL per injection.

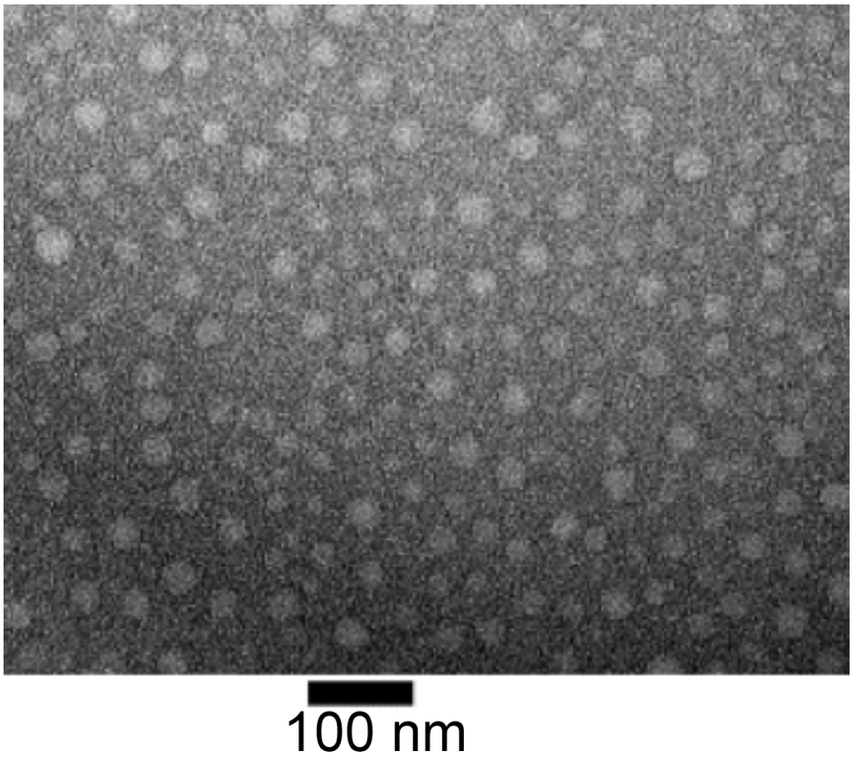

Fig. (3). Transmission electron micrograph of an aqueous dispersion (concentration = 10 mg mL^{-1}) of GC10P19Q10.

where R is the gas constant (8.3144 J mol-1 K-1), T is the temperature in °K and k is the CMC in mole fraction units. The product of temperature and the entropy change of demicellisation ($T\Delta S_{demic}$) may then be calculated from the Gibbs free energy equation (Equation 3)

$$\Delta G_{demic} = \Delta H_{demic} - T\Delta S_{demic} \quad (3)$$

All micellisation parameters (X_{mic}) are equal in magnitude but opposite in sign to the demicellisation parameters (Equations 4 and 5).

$$\Delta G_{mic} = - \Delta G_{demic} \quad (4)$$

The Gibbs free energy equation of micellisation is given by Equation 5.

$$\Delta G_{mic} = \Delta H_{mic} - T\Delta S_{mic} \quad (5)$$

where ΔG_{mic}, ΔH_{mic}, ΔS_{mic} and T are the free energy change, enthalpy change, entropy change and temperature of micellisation respectively.

At the start of the study, the CMC of sodium dodecyl sulphate (SDS) was determined at 288°K (8.29 mM, Fig. **2a** and **2b**) from the dilution enthalpograms and found to be in agreement with values in the literature using ITC: 8.37 mM at 288°K [23]. The ΔH_{mic} of SDS at 288K was 5.43 kJ mol^{-1} which is also comparable to 5.2 kJ mol^{-1} literature value [23]. Table **1** shows the critical micellar concentrations (CMCs) and micellisation thermodynamics parameters of the various GCPQs. The micellisation of all polymers is spontaneous as shown by the negative ΔG_{mic} values (Table **1**) and the CMCs are in the very low micromolar range. The fact that we could measure heat flows in very dilute samples (< 1 μM) allowed us to capture the very early heat flow data.

There are some notable findings. On addition of an amphiphile to the aqueous media, the initial transfer of the hydrophobic unit into the water phase results in the destruction of unrestricted hydrogen bonding by the water molecules, as a cavity is formed within which the hydrophobic moiety sits, with the water - hydrophobic moiety interface, akin to the water - vapour interface [11]. The hydrophobic interactions, which drive self-assembly, involve the transfer of the hydrophobic moiety from this water cavity to the interior of the micelle and the thermodynamics of this process are best described using the Gibbs Free energy equation (Equation 5). The hydrophobic cavity has water molecules unable to hydrogen bond freely in all

Table 1. GCPQ CMC values and micellisation thermodynamic parameters (mean ± s.d.).

Polymer	Mw (kDa)	CMC (μM)	Temp. (°K)	ΔH_{mic} (kJ mole^{-1})	ΔG_{mic} (kJ mole^{-1})	$T\Delta S_{mic}$ (kJ mole^{-1})	QPR
GC9P9Q36	8.8	1.5 ± 0.2	298	-23.8 ± 1.1	-42.3 ± 0.31	18.5 ± 1.08	4
GC14P12Q9	14.3	1.9 ± 0.4	298	-106.2 ± 19.7	-41.8 ± 0.05	-64.5 ± 19.7	0.75
GC9P19Q12	8.7	2.2 ± 0.1	298	-94.3 ± 9.1	-41.2 ± 0	-53.0 ± 9.1	0.63
GC10P19Q10	10.4	1.9 ± 0.4	298	-124.4 ± 28.31	-41.8 ± 0.52	-82.7 ± 28.3	0.53
GC12P19Q9	12.4	1.8 ± 0.3	298	-138.7 ± 20.2	-41.8 ± 0.35	-95.9 ± 20.5	0.47
GC15P4Q11	15.2	1.2 ± 0.2	298	-180.7 ± 17.7	-43.1 ± 0.7	-137.6 ± 17.0	2.75

directions [24-26]. On self-assembly, the Gibbs free energy associated with the removal of the hydrophobic unit from the water phase to the interior of the micelle (ΔG_{mic}) is either driven by the positive change in entropy or the negative change in enthalpy. The micellisation is entropy driven when the predominant free energy change originates from the freeing of these water molecules from the hydrophobic cavity and their ability to hydrogen bond in all directions or is enthalpy driven when the freed water molecules form new bonds (including hydrogen bonds) [11, 25, 27].

The micellisation events for the more hydrophilic 8-12 kDa polymer (GC9P9Q36) and the more hydrophobic 8-12 kDa polymers (GC14P12Q9, GC9P19Q12, GC10P19Q10 and GC12P19Q9) are clearly enthalpy driven. However the more hydrophilic 8-12 kDa polymer GC9P9Q36), with a positive entropy component, causes the freed water molecules to now enjoy additional hydrogen bonding opportunities and compensate for the entropy loss associated with GCPQ aggregation, whereas the more hydrophobic polymers suffer an entropy deficit (presumably due to aggregation of the hydrophobic polymer molecules) and an enthalpy gain associated with the formation of new hydrogen bonds by the water molecules freed from the hydrophobic cavity. It is conceivable that the GCPQs, by virtue of their comb shaped structure (Fig. **1**), will have a high surface area of hydrophobic content and thus the water molecules within the hydrophobic cavity, prior to micellisation, will have fewer hydrogen bonds overall and once freed from the cavity will be able to make more stable hydrogen bonds thus contributing to the enthalpy gain seen. We have previously carried out coarse grained modelling on the aggregation of GCPQ molecules and found that simulated micellization could be achieved with only 8 polymer chains (forming two micelles) and that micellization was extremely rapid and complete within 3 ns [28]. These simulations complement the experimentally determined very low CMC values reported here.

The formation of new hydrogen bonds on micellisation is further supported by the fact that the enthalpy contribution increases as the polymers become more hydrophobic (Fig. **4a**) and increases as the polymer chains become longer (Fig. **4b**) as both an increase in hydrophobicity and an increase in molecular weight will result in the release of additional water molecules, per mole of polymer, from the hydrophobic cavity on self-assembly.

It has been reported that immobilised amine cations in close proximity to the hydrophobic units strengthen the hydrophobic interactions [29] and since GCPQ comprises amine cations and hydrophobic units in close proximity to each other, we examined the effect of quaternary amine content on the thermodynamics of self-assembly. We would have expected to see a marked change in CMC as the polymers became more hydrophilic (have a higher QPR), however there is no correlation between CMC and QPR with the 8-12 kDa polymers (r^2 = 0.49, data not shown). There is good negative correlation, in the 8-12 kDa

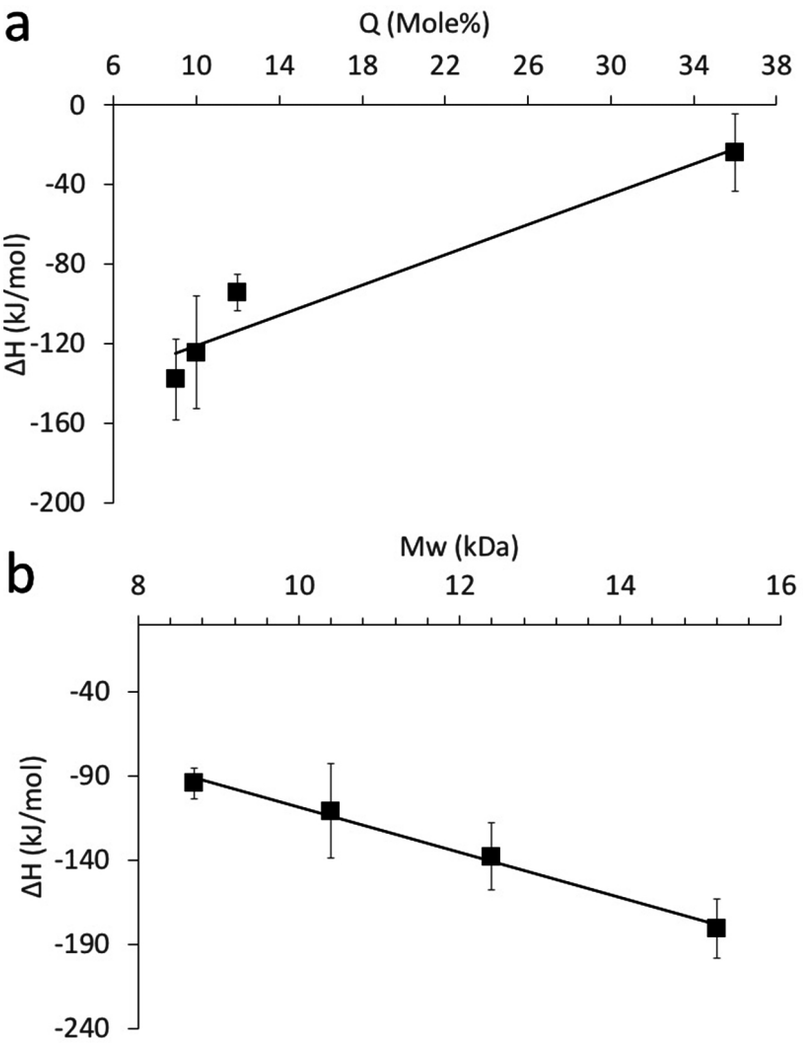

Fig. (4). The variation in ΔH_{mic} with variation in: **a**) mole% quaternary ammonium groups (Q) within the 8 – 10 kDa polymers all with a palmitoylation (P) level of 9 - 19 mole% (ΔH_{mic} = 3.80 Q -159, r^2 = 0.93) and **b**) molecular weight (Mw) within the polymers having a range of Q (9 – 12 mole%) and P (4 – 19 mole %) substitutions (ΔH_{mic} = -13.5Mw – 26, r^2 = 0.99). Each data point represents the mean and standard deviation of three separate experiments.

polymers, between the level of quaternary ammonium groups (Q) and ΔH_{mic}, when the level of palmitoyl groups is fixed at 9-19 mole% (Fig. **4a**). This strong negative correlation between Q and ΔH_{mic}, when the palmitoyl units are fixed between 9 and 19 mole%, indicates that the quaternary ammonium groups have a negative influence on the micellisation. This negative influence on micellisation by the quaternary ammonium groups is expected as these amines are hydrophilic. However, despite this polymers with high Q levels (GC9P9Q36 and GC15P4Q11) self-assemble at low CMCs.

This enthalpy driven micellisation at room temperature is unusual as micellisation is actually normally entropy driven at room temperature [19-21], with a switch to being enthalpy driven as temperature rises above ambient [20]; the latter due to an increased level of disorder around the hydrophobic cavity at elevated temperature [30], presumably as the molecules gain more kinetic energy as the temperature rises. Even when micellisation is reported to be exothermic, however, the entropy contribution is hugely dominant and drives the self-assembly [31, 32].

Quite clearly the chemistry of these polymers is responsible for the unusual exothermic reaction accompanying removal of the hydrophobic units from their water cavities. The quaternary ammonium groups may contribute to the exothermic na-

ture of the micellisation as even though the micellisation of quaternary ammonium compounds is found to be entropy driven, the demicellisation is endothermic and the micellisation is exothermic [33]. It is plausible that a reorganization of the molecules, as occurs with micellisation, would offer the water molecules an opportunity to hydrogen bond with the quaternary ammonium units as well as with each other, although these new water-amine bond formations do not drive the self-assembly. It appears as if the pendant nature of the hydrophobic groups (resulting in a high surface area of hydrophobic units) is what contributes to the very low CMCs when compared to amphiphilic polymers of similar molecular weight (MW) such as the pluronic block copolymers [34] with P104 (MW = 5900 Da) having a CMC of 0.34 mM at 25°C and F108 (MW = 14,600 Da) having a CMC of 2.7 mM at 25°C. These values are 100 - 1000 times higher than the CMCs reported here (Table **1**).

CONCLUSION

The self-assembly of a group of amphiphilic chitosans is spontaneous and occurs at low micromolar concentration (1-2.4 μM at 25°C) giving rise to highly stable aggregates. At room temperature aggregation is unusually enthalpy driven (with a negative entropy component) for the more hydrophobic chitosan amphiphiles. We attribute this unusual thermodynamic behaviour to the polymer architecture, which supports the formation of entropically unfavourable aggregates, but which releases water molecules from the hydrophobic cavity and allows these freed water molecules to form bonds with each other. Both hydrophobicity and molecular weight favour polymer aggregation.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author, [Dr. R.G.], upon request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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