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Regular Article

Self-assembling properties of ionisable amphiphilic drugs in aqueous solution

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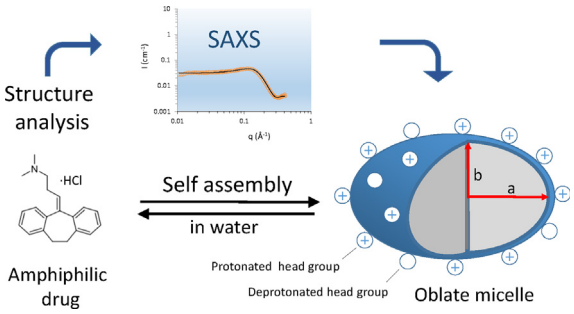
GRAPHICAL ABSTRACT



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ABSTRACT

Hypothesis: Common amphiphilic drug molecules often have a more rigid nonpolar part than conventional surfactants. The rigidity is expected to influence the self-assembling properties and possibly give rise to aggregation patterns different from that of regular surfactants.

Experiments: We have investigated self-assembling properties of the hydrochloride salts of adiphenine (ADP), pavatrine (PVT), and amitriptyline (AMT) at concentrations up to 50 wt% using small-angle x-ray scattering, dynamic light scattering, cryo-transmission electron microscopy, and surface tension measurements.

Findings: All drugs form small micelles of oblate spheroidal shape at concentrations above the critical micelle concentrations (CMC). The micelles grow weakly in size up to about 20 wt%, where the aggregation number reaches a maximum followed by a slight decrease in size at higher drug concentrations. We observe a correlation between the decrease in micelle size at high concentrations and an increasing charge of the micelles, as the degree of ionization increases with increasing drug concentration and decreasing pH. In contrast to what has previously been reported, the aggregation behavior of all studied drugs resembles the closed association behavior of conventional surfactants with a short aliphatic chain as hydrophobic tail group *i.e.* the micelles are always small in size and lack a second CMC. CMC values were determined with surface tension measurements, including also lidocaine hydrochloride (LDC) and chlorpromazine hydrochloride (CHL).

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1. Introduction

Amphiphilic drug molecules are the pharmaceutically active ingredients in many tranquilizers, antidepressants, antispasmolytic, anaestethic, and antihistaminic medicals [1]. They constitute a broad class of molecules, including substances used for the treatment of cancer, e.g., doxorubicin. The amphiphilic properties are expected to be an important factor determining their pharmacological activity and properties related to toxicity and haemolysis. In connection with drug delivery the amphiphilic properties determine their possibility to be solubilized, their bioavailability, incorporation into and effect of lipid membranes, transport properties and release rate from formulations [2].

The self-assembling properties of several classes of amphiphilic drugs have been investigated in some detail, but mainly in dilute aqueous solutions and by means of classical experimental techniques such as static light scattering, conductivity, NMR spectroscopy (chemical shifts), and measurements of colligative properties [1]. This has revealed relationships between the structure of drug molecules and the nature of aggregates. In general, the hydrophobicity derives largely from aromatic ring structures which can make the molecules less flexible than conventional surfactants with an aliphatic chain as hydrophobic tail group. This may have an impact on their self-assembling properties.

Attwood and others have recognized three main types of behavior for amphiphilic molecules depending on the molecular structure [1]. 1) Molecules with less rigid hydrophobic groups, like adiphenine hydrochloride (ADP) [*cf.* Fig. 1], have well defined critical micelle concentrations and display closed aggregation behavior similar to conventional surfactants, meaning that there is an optimal aggregation number. Moreover, the standard free energy of transferring the hydrophobic parts from water to the micelle is in agreement with expectations for conventional surfactants, as is the effect of salt on the critical micelle concentration (CMC).

2) Molecules with rigid ring structures, like pavatrine hydrochloride (PVT), often lack a well-defined CMC and have been suggested to display open association patterns (continuous growth with no optimal aggregation number), possibly with the molecules arranged in stacks. A rigid ring structure appears to be necessary for open association but not the only requirement, since most molecules behaving in that way have a hydrophilic group substituted directly on the ring structure. Furthermore, molecules with a charged group attached directly to the aromatic structure resemble organic dyes with a delocalized charge and are believed to aggregate into stacks, like methylene blue.

3) Molecules with rigid ring structures, but with the charged group at the end of a hydrocarbon chain, have been suggested to form pre-micelles below a well-defined CMC. One example from that category is chlorpromazine hydrochloride (CHL), a tranquillizer belonging to a family of tricyclic phenothiazine derivatives. In pure water CHL has been proposed to form small premicellar stacks which associate at the CMC into micelle-like globular aggregates of multi-stacks [3]. These are, in turn, believed to start growing dramatically at a second CMC at a much higher concentration. Moreover, there are indications that large amounts of salt can induce growth resembling the transition from globular to rod-like micelles observed for some regular surfactants. Addition of salt has also been reported to induce growth of the tricyclic antidepressant drug amitriptyline hydrochloride (AMT) [4]. In all cases the behavior in solution has almost exclusively been extracted out from combination of thermodynamic data, static light scattering measurements and simple models of self-assembly [1]. There are very few studies providing structural information from small-angle scattering techniques [5]. A recent exception is a thorough study by Clulow et al. of the self-assembling properties of the amphiphilic antimalarial drug OZ439 Mesylate Salt [6].

In the present study we have investigated aqueous solutions of ADP, PVT, AMT, and the local anaesthetic lidocaine hydrochloride (LDC), by means of small-angle X-ray scattering (SAXS). As complimentary techniques, we have also studied the systems using cryo-transmission electron microscopy (cryo-TEM), dynamic light scattering (DLS), and surface tensiometry, the latter investigation including also CHL. The aim was to determine the size and shape of micelles in a wide concentration range with or without added salt (physiological conditions, 0.15 M NaCl), to improve the understanding of the self-assembling properties of amphiphilic drugs.

2. Experimental section

2.1. Materials

Adiphenine hydrochloride (>99%, diphenylacetic acid 2-(diethylamino)ethyl ester), amitriptyline hydrochloride (≥98%), chlorpromazine hydrochloride (≥98%, 2-Chloro-10-(3-dimethylaminopropyl)phenothiazine hydrochloride), and lidocaine hydrochloride monohydrate (≥98%, 2-diethylamino-N-(2,6-dimethylphenyl)acetamide hydrochloride monohydrate), were purchased from Sigma Aldrich, Sweden, while pavatrine hydrochloride (96%, 2-diethylaminoethyl 9H-fluorene-9-carboxylate hydrochloride) was purchased from BOC Sciences, USA. All amphiphilic drugs were in powder form and used without further purification. The molar mass of the drugs equal M = 347.9 g mol^{-1} (ADP), M = 313.9 g mol^{-1} (AMT) and M = 345.9 g mol^{-1} (PVT). The molecular volumes were estimated to v = 477 Å^3 (ADP), v = 493 Å^3 (AMT), v = 466 Å^3 (PVT) [11].

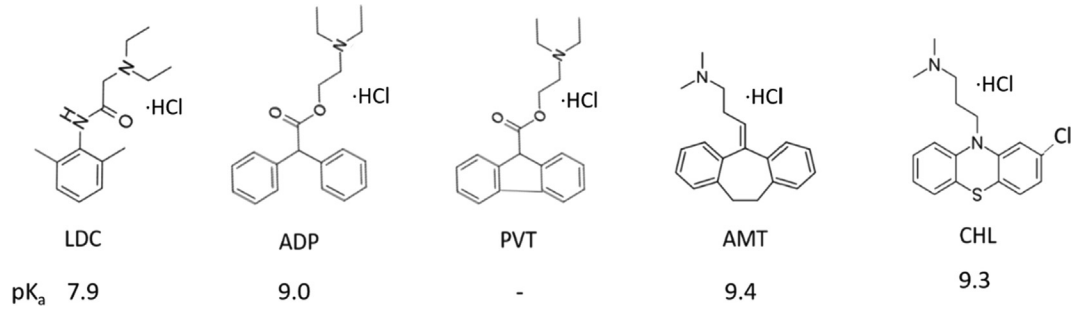

Fig. 1. Chemical structure of hydrochloride salts of lidocaine (LDC), adiphenine (ADP), pavatrine (PVT), amitriptyline (AMT), and chlorpromazine (CHL). The cationic forms are in equilibrium with the neutral base forms, the proportions determined by the pK$_a$ values (indicated in the figure) for LDC [7], ADP [8], AMT [9], and CHL [10], respectively; no literature value found for PVT.

2.2. Preparation of drug aqueous solutions

30 mL of amphiphilic drug solutions were prepared in 50 mL VWR centrifuge tubes by dissolving the drug in water (Millipore Synergy with UV ultrapure, type 1, water purification system, 18.2 MΩ cm at 25 °C, USA) under hand shaking conditions for about 20 s at room temperature. The solutions were equilibrated for at least 2 h before beginning the experimental measurements and stored at room temperature for maximum 7 days prior to use. The pH of drug solutions were measured with a Metrohm 744 pH Meter.

2.3. Surface tensiometry

Surface tension measurements were performed at room temperature using a Sigma 703D force tensiometer (Biolin Scietific, Finland) equipped with a Wilhelmy plate as a probe, which was ethanol-flushed and flame-dried before each measurement. The test solutions covered a wide range of concentrations up to 70 wt% prepared by diluting a concentrated amphiphilic drug aqueous solution with water. For each sample, the tensiometer experiments were repeated two times and the data were used to construct a plot of surface tension (γ) vs. log concentration (ln C) [*cf.* Fig. 2].

2.4. Small-Angle X-ray scattering (SAXS)

SAXS experiments were conducted using a Bruker AXS instrument comprising an Excillium gallium metal jet X-ray source with pinhole geometry and a home-built scatterless slits in front of the sample [12]. The samples were placed in 1 mL Eppendorf tubes and loaded through an automatic injection system into home-built quartz capillaries housed in stainless steel holders at 25 °C. The exposure time was 30 min and the quartz capillaries were cleaned and dried before each experiment.

The raw scattering data were analyzed and computed using the SuperSAXS software by Oliveira and Pedersen [13]. Specifically, scattering data were normalized to absolute scale by the use of water as a standard and scattering intensities derived from the water background with or without added salts were subtracted from the raw scattering data. The final intensities $I(q)$ were displayed as a function of the scattering vector modulus $q = \frac{4\pi\sin(\theta/2)}{\lambda}$ where λ is the X-ray wavelength equal to 1.34 Å and 2θ is the scattering angle. For micellar solutions the scattered intensity can be described by [14]:

$$I(q) = \frac{d\sigma(q)}{d\Omega} = (\Delta\rho)^2 \phi_{mic} V_{mic} P(q) S(q) \quad (1)$$

$\frac{d\sigma(q)}{d\Omega}$ is the differential scattering cross section (ratio of the number of scattered photons per unit solid angle Ω and time to the number of photons in the incident beam per unit area and time) per volume of the sample, $\Delta\rho$ is the scattering length density difference between the micellar solution and water (with or without salt), ϕ_{mic} is the volume fraction of micelles, V_{mic} is the micelle volume, $P(q)$ is the form factor, and $S(q)$ is the structure factor. For $P(q)$ we used the models for homogeneous sphere or ellipsoids of revolution as described in the Supplementary material S1. The structure factor was taken into account by using the Hayter-Penfold model in the Rescaled Mean-Spherical Approximation (RMSA), taking into account the electric double layer repulsion between micelles by means of a screened coulomb potential [15].

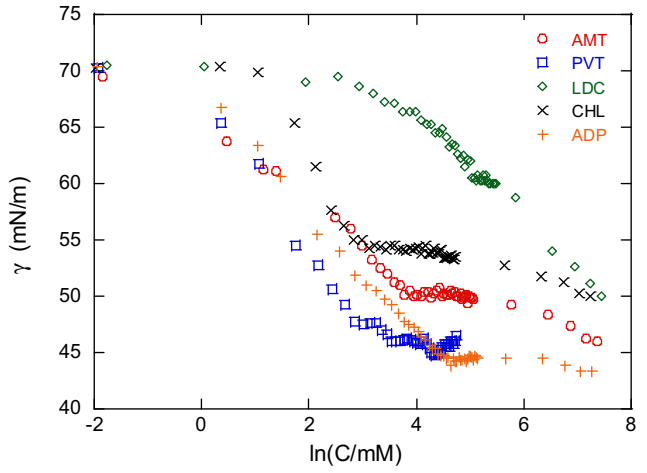

Fig. 2. Surface tension (γ) versus concentration on a logarithmic scale for the amphiphilic drugs CHL, AMT, PVT, ADP, and LDC, dissolved in aqueous solutions at 25 °C.

2.5. Cryo-Transmission electron microscopy (cryo-TEM)

Cryo-TEM experiments were conducted using a Zeiss Libra 120 microscope. 100 mM NaCl was added to the amphiphilic drug solutions in order to increase the contrast. Samples were loaded onto a holey polymer film with radius 3 μm and maximum thickness 500 nm which was mounted on a copper mesh grid. The grid was coated with a carbon layer, in order to increase the sample conductivity when exposed to the electron beam. The samples were vitrified using liquid ethane and imaged at – 160 °C.

2.6. Light scattering

Light scattering experiments were conducted using a Nd-YAG laser as a light source with wavelength 532 nm, a unit of ALV/LSE-5004 light scattering electronics including an ALV-7004/USB correlator, and an ALV/CGS-3 compact goniometer system. The experiments were conducted at angle 94° and temperature 25 °C using the ALV-Correlator software. In order to avoid dust particle interference, all solutions were filtered through Fischer syringe filters (PTFE hydrophilic, 0.2 μm) and subsequently, loaded into glass cuvettes. The counting time was set at 30 s for each run.

The hydrodynamic radius of micelles was estimated based on the Stokes-Einstein equation given by Eq. 2:

$$R_h = \frac{k_B T}{6\pi\eta D} \quad (2)$$

where k_B is the Boltzmann constant, T the absolute temperature, η is the solvent viscosity, and D is the translational diffusion coefficient calculated from the intensity correlation function

$$g_2(t) = e^{-2\Gamma t} \text{where} 2\Gamma = 2Dq^2 \quad (3)$$

Γ is the relaxation rate and the magnitude of the scattering vector is defined as

$$q = \frac{4\pi n \sin(\theta/2)}{\lambda} \quad (4)$$

where n is the refractive index.

3. Results

3.1. Surface tension

Fig. 2 shows the surface tension curves for aqueous solutions of the amphiphilic drugs at 25 °C. For all tested drugs the surface tension initially decreases with increasing concentration, characteris-

tic of surface active molecules. For CHL, AMT and ADP the surface tension starts to level out at a nearly constant value at a well-defined concentration (CMC), typical of self-assembling amphiphiles. PVT displays a similar behavior at low concentrations but lacks a well-defined CMC. According to previous works [16], this has been suggested to indicate a more gradual aggregation process. LDC lacks a clearly developed plateau, but the kink in the curve where the surface tension has dropped down to just above 60 mN m^{-1} will be interpreted as the CMC. Table 1 shows the CMC values determined from the surface tension data. We will relate the CMC values and the shape of the surface tension curves to the size of the aggregates and the properties of the molecules in the discussion part of the paper.

3.2. SAXS**a

SAXS experiments were performed on aqueous solutions of AMT, ADP and PVT at a temperature of 25 °C. Figs. 3-5 show the differential scattering cross section as a function of the scattering vector q for different drug concentrations. The scattering curves for the three systems show a similar progression with increasing concentration. The typical scattering behavior of repelling interacting micelles develops above the CMC and the maximum scattering intensity increases in magnitude as well as shifts to higher q with increasing concentration. At 40 wt% AMT and ADP, the scattering intensity starts to increase with decreasing q in the low-q range, indicating the formation of large structures in addition to the rather small micelles. At this concentration the samples were more viscous than at lower concentrations. The presence of large structures is more pronounced in the SAXS data for 50 wt% ADP, and in the sample with 50 wt% AMT micelles could no longer be observed. The corresponding high concentration samples of AMT and ADP appear cloudy to the bare eye, and the concentration of micelles no longer increases with drug concentration in these samples, indicating the formation of a more concentrated phase in addition to the micellar phase. However, we observed no Bragg peaks in the investigated q range.

Close to, or below, the CMC, the presence of some larger structures appears as indicated by the increase in scattering intensity at low-q values. This scattering behavior appears to be most pronounced for PVT [*cf.* Fig. 5], but is also indicated below the CMC for AMT and ADP [*cf.* Figs. 3 and 4]. At 1 wt% PVT no micelles appear, but a sharp rise in scattering intensity at low q indicates the presence of some larger structures. A small amount of micelles is seen to coexist with the larger objects at 3 wt% PVT, whereas only micelles appears in the SAXS data above 5 wt%. The presence of larger structures was confirmed by visual inspection, showing that the samples with 3, 5 and 10 wt% ADP contained small amounts of precipitate, and the samples with 1wt% PVT was slightly turbid.

It should be noted that all our studied amphiphilic drugs are weak acids with a positively charged amine group that may donate a proton to the solvent water molecules and transform into the non-charged species of the drug molecule. This means that the amphiphilic drugs are a mixture of ionic and nonionic species that form mixed micelles in an aqueous solution. The relative amount

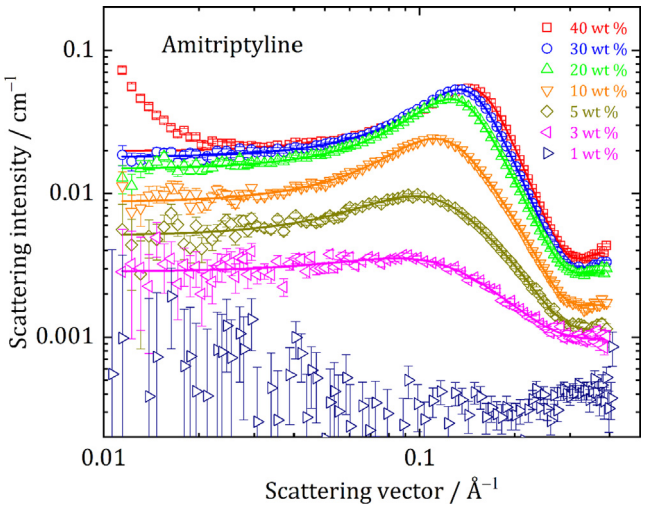

Fig. 3. SAXS data (symbols) for amitriptyline hydrochloride (AMT) in pure water at 25 °C at different drug concentrations. Solid lines represent optimized fits using a model for interacting oblate spheroidal micelles. Results from the model fitting analysis are given in Table 2.

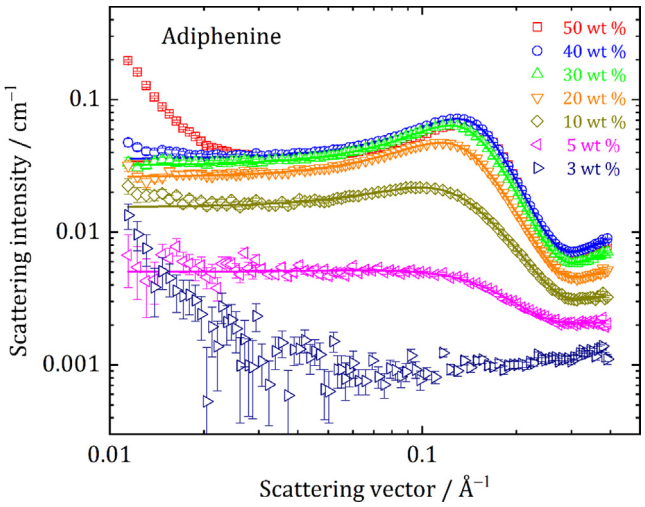

Fig. 4. SAXS data (symbols) for adiphenine hydrochloride (ADP) in pure water at 25 °C at different drug concentrations. Solid lines represent optimized fits using a model for interacting oblate spheroidal micelles. Results from the model fitting analysis are given in Table 2.

Table 1
CMC values and aggregation numbers.

Drug	CMC (wt %)(this work)	CMC (mM)(this work)	CMC (mM)(other)	N_{agg} (this work)	N_{agg} (other)
LDC	4.6	170	130[a]	–	8[a]
ADP	3.3	96	82[b]	30	10[b]
PVT	0.7–1.4	20–40	20-40[b]	32	–
AMT	1.4	43	36[c]	34	7[c], 22[d]
CHL	0.57	16	19[c]	–	12[c], 21[e]

[a] Shaik et al. [17];
[b] Attwood et al. [16];
[c] Attwood et al. [18];
[d] Al-Muhanna et al. [19];
[e] Perez-Villar et al. [5b].

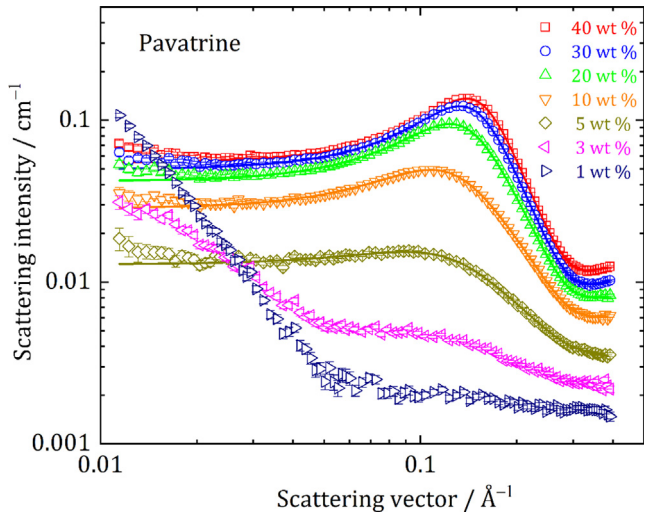

Fig. 5. SAXS data (symbols) for pavatrine hydrochloride (PVT) in pure water at 25 °C at different drug concentrations. Solid lines represent optimized fits using a model for interacting oblate spheroidal micelles. Results from the model fitting analysis are given in Table 2.

of ionic and nonionic species strongly depends on the pH in the solution with an excess of ionic species at low pH values whereas the nonionic species predominates at high pH values. For concentrations blow the CMC, the amounts of ionic and nonionic species are expected to be equal when pH equals the pK_a of the drug molecule. The CMC of the amphiphilic drugs refers to the ionic species which is the predominant form in absence of any additional acid or base. This means that below CMC, a small amount of the poorly soluble nonionic species is present that may aggregate into larger structures that can be observed in the scattering data. As the drug concentration is increased above the CMC, micelles start to form that eventually dissolve the nonionic species and, as a result, the high scattering intensity at low-q value is seen to vanish. This behavior is most pronounced for PVT and may explain the peculiar appearance in the surface tension isotherms for this particular drug [*cf.* Fig. 2]. A similar behavior was recently reported for the mesylate salt of an antimalarial drug [6], as will be further discussed below.The data of samples containing micelles were analyzed and best fitted using a model with the form factor for homogeneous oblate spheroids and a structure factor based on the Hayter-Penfold model in the Rescaled Mean-Spherical Approximation (RSMA) that takes into account repulsive electrostatic double layer force interactions [*cf.* Figs. 3-5]. The very highest concentrations of AMT and ADP, for which an increase in scattering intensity is seen at low-q values, were fitted in the regime q > 0. 20 Å^{-1}, where the larger structures are not expected to influence the scattering behavior. The parameters determined from the model fitting analysis are given in Table 2. Two fitting parameters are related to the form factor, *i.e.* the equatorial semi-axis (a) and the polar semi-axis (b) of a spheroidal micelle. $V_{mic} = 4\pi a^2 b/3$ is the total volume of the micelle and N_{agg} is the aggregation number calculated as the ratio between V_{mic} and the volume v of the drug molecule, including the volume of the head group. In principle, the value should be considered as an upper limit, since the possibility that water molecules are incorporated in the micelles is not taken into account [6]. It was not possible to distinguish between prolate and oblate shapes of the comparatively small micelles by simply comparing the quality of the fits. However, the growth behavior of the micelles makes more sense assuming oblate shape, since it was seen that the shorter semi-axis is rather constant whereas the longer semi-axis increases as the aggregation number of the micelles increases. When prolate shape was assumed, on the other hand, it was seen that the aggregation number increased mainly as a result of an increasing shorter semi-axis, in contradiction to what is expected for elongated rod-like micelles. For all drugs the aggregation number increases slightly with increasing concentration up to about 20 wt%, where it reaches a maximum, and then start to decrease [*cf.* Fig. 6]. The drugs can be ranked roughly in the following way according to aggregation number: AMT > PVT > ADP. In comparison with regular surfactants the aggregation numbers appear to be rather small. Included in Table 2 are also three fitting parameters related to the structure factor, *i.e.* the effective degree of ionization (α), the effective hard-sphere volume fraction (ϕ_{HP}) and the ionic strength (I). The hard sphere-radius was always set to the volume equivalent radius of the micelles. As expected, it is seen that both ϕ_{HP} and I increase with increasing concentration of drug. The limited number of samples examined precludes accurate determination of CMC from plots of ϕ_{HP} vs. C. However, the data in Table 2 are not in conflict with the CMC values obtained from the surface tension measurements. At the highest drug concentrations the Hayter-Penfold model, based on a screened coulomb potential, is less accurate. In this range the values of α, ϕ_{HP}, and I reported inTable 2 should be regarded as in-model estimates, the absolute values of which may not be realistic.

The charge of the micelles in terms of the degree of ionization appears to be significantly larger for ADP as compared to AMT and PVT, which may explain why ADP forms somewhat smaller micelles than PVT with a similar chemical structure. Moreover, α for all three drugs is seen to increase with increasing drug concentration. This behavior is consistent with the fact that all three drugs are, in their hydrochloric forms, weak acids and that the pH is lowered as increasing amounts of drugs are added to the solutions. This was confirmed by measurements showing that, as the drug concentration increased from 0.5 to 40 wt%, the pH decreased from 6.3 to 4.2 for AMT, from 4.6 to 2.3 for ADP, from 4.1 to 2.6 for PVT, from 5.9 to 3.0 for CHL, and from 6.3 to 4.6 for LDC (Supplementary material S2). Lowering pH increases the fraction of the ionic species of the drug and, as a result, increases the charge density of the micelles. An increase in charge density is expected to raise the spontaneous curvature of the micelles and lower their aggregation number, in agreement with our observations of a decreasing N_{agg} at very high drug concentrations [*cf.* Fig. 6]. DLS measurements in pH regulated solutions confirmed this (see below).

Fig. 7 shows SAXS data recorded at drug concentration 30 wt% in the presence of 0.15 M NaCl. The curves are less influenced by intermicellar interactions than the corresponding ones in the absence of added salt, as expected since the addition of salt lowers the electrostatic double layer repulsion between micelles. The results from the corresponding model fits (solid curves) are included in Table 2. Due to the small influence of the structure factor at high ionic strengths, it was not possible to determine all fitting parameters in the Hayter-Penfold model; the result is essentially for a hard-sphere model. It is seen that the addition of salt has only minor effects on the aggregation number for all drugs.

3.3. Dynamic light scattering (DLS)

DLS experiments were performed on aqueous solutions of AMT and ADP at 25 °C without the addition of salt as well as in the presence of 0.1 M NaCl. DLS experiments could not be conducted on the PVT systems since the drug molecule absorbed light at the laser wavelength (532 nm). Table 3 shows the apparent hydrodynamic radii of micelles calculated from the measured diffusion coefficients using the Stokes-Einstein equation. The hydrodynamic radius of micelles determined in the salt-free solutions was clearly

Table 2
Results from SAXS data analysis of amphiphilic drugs in water at 25 °C.

Drug	C (wt %)	[NaCl] (M)	b (Å)[1]	a (Å)[2]	V_{mic} (nm^3)	N_{agg}	α[3]	ϕ_{HP}[3]	I[3]
AMT	3.0	0	13.8 ± 2.4	17.0 ± 2.0	16.7	34 ± 8	–	–	–
AMT	5.0	0	13.5 ± 0.8	17.4 ± 0.7	17.2	35 ± 3	0.35 ± 0.07	0.025 ± 0.002	0.08 ± 0.02
AMT	10	0	13.6 ± 0.3	18.2 ± 0.3	19.0	38 ± 2	0.43 ± 0.03	0.049 ± 0.001	0.12 ± 0.01
AMT	20	0	14.1 ± 0.2	18.8 ± 0.2	20.8	42 ± 1	0.48 ± 0.02	0.082 ± 0.001	0.22 ± 0.02
AMT	30	0	13.6 ± 0.2	18.6 ± 0.2	19.7	40 ± 1	0.55 ± 0.03	0.099 ± 0.002	0.32 ± 0.03
AMT	40	0	12.4 ± 0.3	18.3 ± 0.3	17.2	35 ± 1	0.68 ± 0.05	0.117 ± 0.002	0.49 ± 0.04
AMT	30	0.15	11.9 ± 0.3	18.4 ± 0.3	16.9	34 ± 1	–	0.057 ± 0.001	–
PVT	5.0	0	13.7 ± 0.7	16.1 ± 0.6	14.9	32 ± 3	0.51 ± 0.26	0.015 ± 0.002	0.20 ± 0.09
PVT	10	0	13.4 ± 0.3	17.2 ± 0.3	16.6	36 ± 1	0.40 ± 0.03	0.036 ± 0.001	0.16 ± 0.02
PVT	20	0	13.6 ± 0.2	17.9 ± 0.2	18.2	39 ± 1	0.40 ± 0.02	0.070 ± 0.001	0.21 ± 0.01
PVT	30	0	13.4 ± 0.2	18.0 ± 0.2	18.2	39 ± 1	0.43 ± 0.02	0.092 ± 0.002	0.27 ± 0.02
PVT	40	0	13.0 ± 0.2	17.9 ± 0.2	17.5	38 ± 1	0.45 ± 0.02	0.106 ± 0.002	0.32 ± 0.02
PVT	30	0.15	12.6 ± 0.2	18.0 ± 0.2	17.2	37 ± 1	–	0.060 ± 0.001	–
ADP	5.0	0	10.5 ± 4.9	18.6 ± 4.3	15.2	32 ± 11	–	–	–
ADP	10	0	9.5 ± 1.8	19.0 ± 1.9	14.4	30 ± 3	1.1 ± 0.6	0.032 ± 0.002	0.40 ± 0.16
ADP	20	0	11.8 ± 0.4	19.0 ± 0.4	17.8	37 ± 1	0.68 ± 0.09	0.062 ± 0.002	0.37 ± 0.05
ADP	30	0	10.8 ± 0.5	19.5 ± 0.5	17.2	36 ± 2	0.66 ± 0.07	0.081 ± 0.002	0.41 ± 0.05
ADP	40	0	–	–	14.2	30 ± 10	0.76 ± 0.07	0.097 ± 0.002	0.48 ± 0.05
ADP	50	0	–	–	13.7	29 ± 10	1.1 ± 1.0	0.109 ± 0.007	0.87 ± 0.73
ADP	30	0.15	–	–	14.6	31 ± 15	–	0.054 ± 0.001	–

1) Polar semi-axis (oblate spheroid). 2) Equatorial semi-axis (oblate spheroid). 3) Effective degree of ionization (α), effective hard sphere volume fraction (ϕ_{HP}) and ionic strength (I) determined from the Hayter-Penfold RMSA model

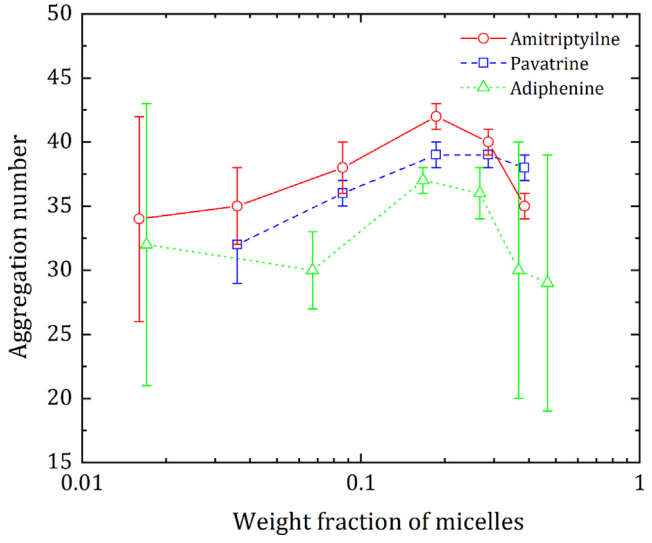

Fig. 6. Aggregation number plotted against weight fraction of micelles ($C - CMC$) for AMT, ADP and PVT in pure water at 25 °C.

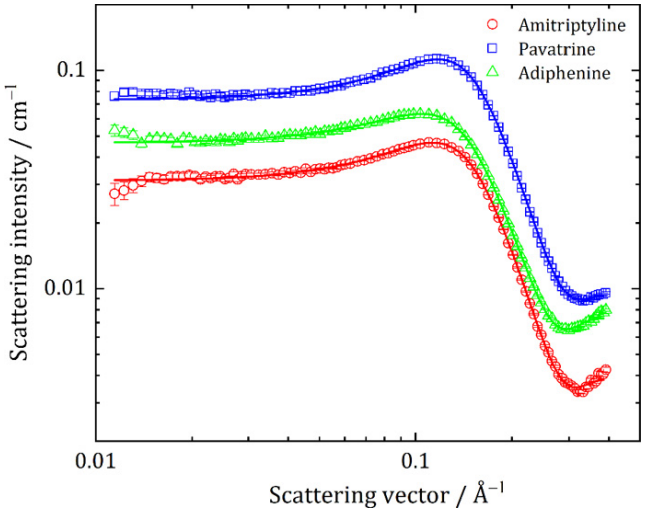

Fig. 7. SAXS data and model fits for the amphiphilic drugs AMT, PVT, and ADP in [NaCl] = 0.15 M at 25 °C. Results from the model fitting analysis are given in Table 2.

smaller than obtained from SAXS. This can be attributed to well-known electrostatic effects on self-diffusion coefficients of charged colloids measured in the absence of screening electrolyte. At the lowest drug concentrations, the radii obtained in the presence of 0.1 M NaCl were slightly larger than the micellar size determined with SAXS. The reason for this is most probably that DLS is more sensitive for the diffuse layer of counter-ions outside the charged micelles. This may also explain why the decrease in micellar size at high drug concentrations, which was observed with SAXS, is not evident in the DLS results. As the micelles become more charged at higher drug concentrations, the amount of counter-ions in the micelles increases. Likewise, ADP micelles have a higher hydrodynamic radius than AMT micelles, although the aggregation numbers determined with SAXS was smaller, probably due to a higher surface charge and amount of counter-ions.

The dependence of hydrodynamic radius, as determined with DLS, on pH for micelles formed by AMT at two drug concentrations is shown in Fig. 8. pH was regulated with hydrochloric acid while keeping the ionic strength of the aqueous solvent constant at a value corresponding to physiological concentration [NaCl] = 0.15 M. It is evident that the size of the micelles decrease significantly

Table 3
Results from DLS. Hydrodynamic radius of micelles at different concentration of drug in aqueous solutions with or without 0.1 M NaCl at 25 °C.

C (wt %)	Drug	R_H (Å)no salt	R_H (Å)0.1 M NaCl
3	AMT	7.4	–
5	AMT	6.6	14.1
10	AMT	6.5	14.1
20	AMT	7.6	16.1
30	AMT	8.9	18.0
40	AMT	10.0	19.4
5	ADP	8.8	14.5
10	ADP	9.6	15.4
20	ADP	10.5	17.3
30	ADP	12.3	19.9
40	ADP	14.0	22.5

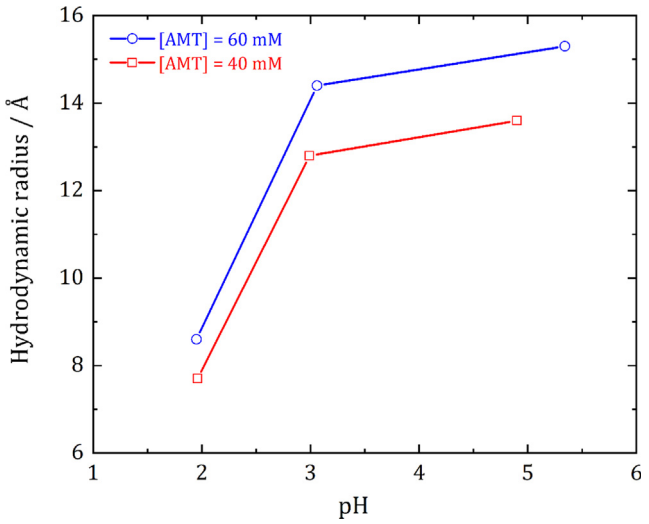

Fig. 8. The hydrodynamic radius of micelles formed by AMT at different pH and fixed physiological ionic strength.

as pH is lowered and the fraction of ionized AMT in the micelles is increased.

3.4. Cryo-TEM

Cryo-TEM experiments were conducted on the studied amphiphilic drug systems, in order to investigate a possible presence of larger aggregates. Fig. 9 and Fig. S1c, e, and f show that AMT, ADP, PVT, and CHL form small globular micelles at 30 mM above the CMC of each drug, in agreement with the SAXS data. No worm-like micelles or other large aggregates appeared at high drug concentrations, in contrast to other surfactant systems, as studied by e.g. Bergström et al. [20] and Sakya et al. [21], indicating the absence of a second CMC [22] for amphiphilic drugs even at rather high concentrations.

Cryo-TEM experiments were also conducted below CMC for AMT (Fig. S1b, [AMT] = 3.2 mM (0.1 wt%)) and PVT (Fig. S1d, [PVT] = 17 mM (0.6 wt%)), in order to investigate the possible presence of a premicelle state. The cryo-TEM images for those concentrations were compared with 100 mM NaCl free from drug. In agreement with our SAXS results, no pre-micelles could be detected neither for AMT nor PVT.

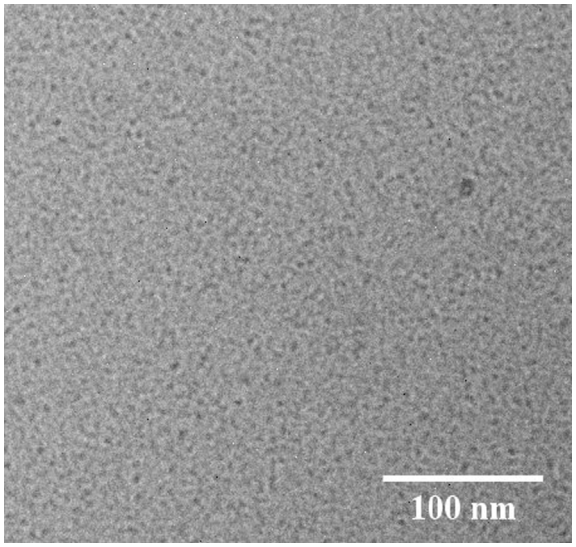

Fig. 9. Cryo-TEM image of AMT-HCl with concentration of 70 mM (2.2 wt%) in aqueous solution showing the presence of small globular micelles.

4. Discussion

The present investigation shows that AMT, ADP and PVT form globular micelles at concentrations up to at least 40 wt% (>1.1 M). The aggregation numbers increase weakly with increasing drug concentration in the dilute regime, reaches a maximum at about 20 wt%, and then decrease at higher concentrations. The results indicate that there is an optimum aggregation number at each concentration ('closed association'), in similarity to the behaviour of conventional surfactants with an aliphatic chain as tail group. The amphiphilic drugs investigated here mainly differ from conventional surfactants in that they have a more or less rigid non-polar group. From previous static light scattering measurements and theoretical modelling, it has been concluded that the micelles formed by PVT grow in size gradually with increasing concentration ('open association'), in a cooperative fashion [16]. In contrast to this early study, our SAXS data analysis demonstrates that the aggregation number increases weakly but gradually at low concentrations, very much similar to the behavior of conventional surfactants below a second CMC [22]. The apparent absence of a second CMC, where the micelles begin to grow strongly into rod-like or wormlike micelles, is clearly in conflict with an open association process.

All our studied amphiphilic drugs show similar aggregation patterns in the range of concentrations studied by SAXS. Nevertheless, the surface tension measurements show that PVT behaves differently from ADP and AMT at low concentrations [*cf.* Fig. 2]. Rather than displaying one well-developed plateau, as for ADP and AMT (and conventional surfactants), the surface tension curve of PVT appears to have two or three small plateaus. These are located in the concentration range where the static light scattering data by Attwood et al. [16] deviated from the behavior expected for micelles with a constant aggregation number. Comparison of the SAXS data of the three studied drugs, demonstrates that PVT mainly differ from AMT and ADP in that a significant amount of large structures appear below CMC and coexist with micelles in the regime 3–5 wt% [*cf.* Fig. 5]. Clulow et al. [6] showed that the amphiphilic drug OZ439 Mesylate Salt formed a turbid dispersion of free base aggregates when added in small amounts to water. At higher concentrations, the aggregates coexisted with cationic micelles. With increasing total drug concentration, the concentration of micelles increased and the fraction in aggregates decreased, leading eventually to complete dissolution. The crystalline state of the aggregates was evidenced by Bragg peaks in the scattering curves. It is likely that similar events take place for PVT at concentrations below and around the CMC. PVT has lower CMC than ADP and AMT. The solubility of the base form of PVT is therefore expected to be lower than the base forms of the other two, and thus present in aggregates to a larger extent at the CMC. We propose the following mechanism to explain the peculiar shape of the surface tension curve for PVT. In the presence of undissolved base the concentration of dissolved free base monomers is constant. However, above the CMC it forms mixed micelles together with the cationic form, and as the total drug concentration increases, more and more of the undissolved form ends up in micelles. As long as there is undissolved material left, the composition of the micelles and therefore the free concentration of the cationic form is nearly constant. This gives rise to a plateau in the surface tension curve. When the free base form is completely dissolved, the mole fraction of the cationic form in the micelles increases and by that the free concentration in equilibrium with the micelles. This decreases the surface tension. However, the sur-

face tension will approach a new plateau as the concentration reaches the CMC in solutions without undissolved material. The proposed mechanism gives rise to two plateaus in the surface tension curve. Interestingly, Clulow et al. [6] observed phase transitions in the dispersed aggregates. If that is the case also for PVT, it would give rise to additional plateaus in the surface tension curves [*cf.* Fig. 2]. As already noted, the scattering at low q in Fig. 5 indicates that large aggregates are present at low PVT concentration, and that the aggregates dissolve at higher drug concentrations. However, since our solutions were only slightly turbid, the fraction material in aggregates was small, which can be one reason why we did not observe any Bragg peaks. For ADP and AMT, the scattering data indicated that the aggregated fraction was even smaller than for PVT. This is in agreement with the surface tension curves displaying only one plateau above the CMC.

CHL and other tricyclic drug molecules have been proposed to form small pre-aggregates at concentrations below the CMC [1]. The surface tension curve for CHL in Fig. 2, displaying a section with high slope followed by a section with lower slope before the CMC is reached, gives support to that. However, the overall shape of the curve resembles more that of ADP and AMT than that of PVT, supporting the idea that the latter molecule behaves differently from the other. Above the CMC, CHL forms micelles with aggregation numbers comparable to that of ADP and AMT, as shown by previous SANS measurements [5b] [*cf.* Table 1]. This is in agreement with the well-developed plateau above the CMC in the surface tension curve [*cf.* Fig. 2]. In general, amphiphiles forming micelles with aggregation numbers of that magnitude are expected to have well-defined CMC values and to show small variations of the surface tension in a concentration range above the CMC. LDC is interesting in this respect, since it is believed to form small aggregates [*cf.* Table 1]. Our attempt to determine the aggregation number with SAXS failed due to weak scattering, but SANS experiments (not published) show that it forms small micelles with aggregation number close to the literature value [17] given in Table 1. Shaikh et al. [17] reported a CMC for LDC of 0.13 M based on density measurements, whereas others [23] have claimed that the molecule lacks a CMC. Our measurements show that there is no well-developed plateau in the surface tension curve. However, as noted above, there is a small but distinct domain at around 0.17 M with a smaller slope than at both lower and higher concentrations [*cf.* Fig. 2]. For LDC, the slope of the curve is almost the same after and before the plateau. For ADP, AMT and CHL the curve bends downward after the plateau but the effect is much smaller than for LDC.

For non-ionic amphiphiles, a decrease of the surface tension above the CMC would mean that the concentration of free molecules in equilibrium with the micelles increases. To investigate the situation for the present drugs, we have calculated theoretical surface tension curves for LDC and AMT. The calculations are based on the following relationship derived from the Gibbs adsorption equation [*cf.* Supplementary material S4]

$$\gamma = \gamma_{CMC} - 2RT\Gamma_{\mathrm{CMC}}\ln\left(\frac{a_s}{CMC}\right) \tag{4}$$

where γ_{CMC} is the surface tension at the CMC, Γ_{CMC} is the surface excess at the CMC, a_s is the mean activity of the amphiphilic salt calculated from the Poisson-Boltzmann (PB) cell-model of micelle equilibrium [24]. At equilibrium between micelles and free monomers we have:

$$RT\ln x_1^b = \Delta\mu^0 + \gamma a + \mu_{el} + \mu_{mix} \tag{5}$$

where x_1^b is the mole fraction of free monomers at the cell-border, $\Delta\mu^0$ is a standard free energy of transferring the non-polar part of the molecule from water to the micelle interior, a is the area per molecule in the head group region, γ is a proportionality constant with units of surface tension describing the non-electrostatic free energy of forming the surface of the micelle, μ_{el} is the contribution from the electrostatic free energy, and μ_{mix} is the contribution from the free energy of mixing the micelles. The derivation of Eq. (4) and a description of the PB calculations are given in Supplementary material S5. Eq. (4) requires that Γ_{CMC} has the same value at and above the CMC, a reasonable assumption considering that the surface tension curves have constant slope in a range just below the CMC. For simplicity, the aggregation number was assumed to be constant and equal to 8 for LDC and 34 for AMT [*cf.* Table 1]. Fig. 10 shows the calculated curves plotted together with the experimental data for LDC and AMT taken from Fig. 2. The PB theory captures the main features of the surface tension curves above the CMC. The elevation of the activity of the drug salts above the CMC is responsible for the lowering of the surface tension. The thermodynamic quantities derived from the PB-cell model show that the increase of the counter ion activity is mainly responsible for the effect. For AMT, the activity of the drug cation has a maximum value just above the CMC, but after that decreases rapidly. For LDC, the activity of the drug cation continues to increase up to concentrations well above the CMC before it reaches a (local) maximum value. Thus, for LDC also the increased activity of the drug cation contributes to the lowering of the surface tension. An analysis shows that the entropy of mixing has a large influence on the variation of the chemical potential of the LDC cation because of its low aggregation number. For AMT and regular surfactants with large aggregation numbers that contribution is small. We have included in Fig. 10 a calculated curve for a hypothetical surfactant with the same CMC as LDC but with aggregation number 55. The presence of a well-developed plateau demonstrates that it is the entropy of mixing that is responsible for the shape of the surface tension curve for LDC rather than its high CMC.

The aggregation numbers of AMT and ADP determined with SAXS (and supported by DLS) are significantly larger than values reported from previous studies [*cf.* Table 1]. Nevertheless, our results show that micelles formed by the amphiphilic drugs are rather small and the self-assembling properties, with an absence of a second CMC, are similar to conventional surfactants with a fairly short aliphatic chain as hydrophobic part. It is interesting to compare AMT with conventional cationic surfactants of the alkyltrimethylammoinium chloride series, which have identical head group to AMT. Our determined aggregation numbers for AMT (molecular volume equal to 493 Å^3) is similar to, or slightly less than, C_{12}TAC [25a] (478 Å^3) and significantly less than C_{14}TAC [25a] (532 Å^3) and C_{16}TAC [25a] (586 Å^3). Hence, we may

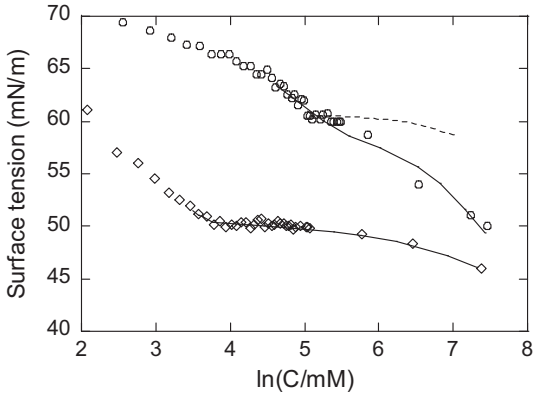

Fig. 10. Theoretically calculated surface tension (solid curves) compared with experimental data for AMT (diamonds) and LDC (circles), respectively. Dashed curve: Theoretically calculated surface tension for hypothetical amphiphile with CMC = 0.19 M and N = 55.

conclude that micelles formed by amphiphilic drug surfactants with a rigid hydrophobic part resembles conventional surfactants with a short aliphatic chain as tail group. Moreover, the acidic properties of the hydrochloric salts of the drug molecules have the consequence that self-assembled micelles consist of a mixture of an ionic and a non-ionic species, where the fraction of ionic species increases as the drug concentrations is increased and pH is lowered. As a result, the micelles are observed to start decreasing in size as the drug concentrations is raised above about 20 wt%.

The observation in this work, that the drug micelles remain small and discrete even at concentrations as high as 50 wt%, shows that they have no pronounced tendency to grow out indefinitely in one or two directions. In this respect, they resemble surfactants with a short aliphatic chain as hydrophobic part, e.g. dodecyltrimethylammonium chloride, forming globular (slightly elongated [26]) micelles arranged in a cubic liquid crystalline phase [27] at that concentration. It is interesting that we have found no indication of ordered phases in the drug systems, not even at the highest concentration (70 wt%) in the tensiometer study, where, e.g., a micellar cubic phase, which is solid-like, would have been difficult to miss. For comparison, at 50 wt%, both sodium dodecyl sulphate [28] and cetyltrimethylammonium bromide [29] form 'infinitely' long rod-like micelles ordered in hexagonal phases.

The growth behavior of surfactant micelles has been rationalized from the recently developed General Micelle Model [30], according to which small micelles grow weakly in size at low concentrations whereas they start to grow strongly into rod-like and worm-like micelles above a certain concentration, defined as the second CMC. The General Micelle Model is based on bending elasticity theory combined with solutions thermodynamics and it has been demonstrated that the second CMC increases with increasing spontaneous curvature (H_0) as well as with decreasing bending rigidity (k_c) and decreasing saddle-splay constant ($\bar{k}_c$) [31]. Hence, from our present SAXS study we are able to conclude that the amphiphilic drugs, which grow weakly in size in the entire range of concentrations in the isotropic L_1 phase, must have either higher H_0, lower k_c or lower $\bar{k}_c$, or a combination of all, as compared to conventional surfactants with a long flexible aliphatic chain.

Most interestingly, from previous theoretical evaluations of bending elasticity properties of micelles, it was found that the spontaneous curvature is expected to be considerably larger, and the bending rigidity smaller, for ionic surfactants with a rigid hydrophobic part as compared to surfactants with a flexible hydrophobic part of similar size [32]. Increasing H_0 and decreasing k_c are both expected to lower the micelle aggregation number as well as raising the second CMC, in agreement with the observations from our SAXS results. The difference is a direct consequence of the presence of a free energy contribution due to the conformational entropy of aliphatic chains for conventional surfactants that is absent for amphiphilic drugs with a rigid hydrophobic part. As a result of the presence of chain conformational entropy, the thickness of a micellar monolayer becomes only slightly dependent on the curvature of the micelles. This means that the hydrocarbon/water interfacial area per aggregated monomer must substantially increase with increasing curvature of the surfactant layer as the size of the micelles decreases. Micelles formed by surfactants with a rigid tail (like amphiphilic drugs), on the other hand, are free to adjust its monolayer thickness (and, as a consequence, the hydrocarbon/water interfacial area per aggregated monomer) in order to minimize the interfacial free energy upon changing its curvature. As a result, the spontaneous curvature increases and the bending rigidity decreases for surfactants lacking a chain conformational entropy contribution.

5. Conclusions

The results of the present investigation show that ADP, PVT, and AMT form small micelles in a wide concentration range above their respective CMCs in aqueous solution. According to our SAXS data analysis, all drugs form oblate spheroidal micelles that grow weakly in size up to ca. 20 wt%, where a maximum in aggregation number is reached followed by a slight decrease in micelle size at higher drug concentrations. The aggregation numbers are always rather small, *i.e.* in the range 30–40 with AMT > PVT > ADP. A phase separation could be observed for AMT above about 40 wt% and for ADP above about 50 wt%. However, a liquid (L_1) phase was observed to be present up to at least 70 wt% for ADP, PVT, AMT, which is a higher concentration as compared to conventional surfactants.

For PVT, the concentration for onset of micelle formation was not as distinct as for ADP, AMT, and CHL. This has previously been rationalized by proposing PVT to display an 'open association' aggregation pattern [1]. In contradiction to these previous results, our SAXS and DLS data show that PVT, as well as ADP and AMT, displays a 'close association' pattern typical of regular ionic surfactants. From our SAXS data it is evident that PVT mainly differ from ADP and AMT in that higher amounts of larger structures are present below and about the CMC, and that eventually become dissolved by PVT micelles at concentrations above the CMC. In agreement with what has been shown by others in a related system [6], we suggest that the large structures formed at low concentrations are particles of the free base as a result of the amphiphilic drugs being weak acids. The effect is pronounced for PVT, which has a large fraction of drug in its nonionic form, giving rise to the peculiar behavior in the surface tension isotherm of that particular drug.

The degree of ionization and surface charge of the amphiphilic drug micelles are sensitive to pH and, since the pH decreases with increasing drug concentration, the micelles become increasingly more charged with increasing drug concentrations, giving rise to the observed decrease in aggregation number with increasing drug concentration above about 20 wt%. ADP is observed with SAXS to be more charged, and to form smaller micelles, than AMT and PVT.

The investigated amphiphilic drug molecules differ from conventional ionic surfactants mainly in the sense that they consist of a more rigid hydrophobic part. Comparing AMT with the conventional cationic surfactants with identical head groups in the alkyltrimethylammonium chloride (C_nTAC) series, shows that AMT resemble conventional surfactants with a short aliphatic chain as tail group ($n \leq 12$), but differ from surfactants with a longer chain in that they form significantly smaller micelles and lack a second CMC in the entire concentration range of the isotropic (L_1) phase. An interesting extension of the present work is to investigate the effects of the studied drug molecules on the curvature of lipid bilayers, important in drug delivery applications.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcis.2021.05.049.

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