Published in final edited form as:
J Phys Chem B. 2010 April 15; 114(14): 4836–4840. doi:10.1021/jp100458x.

Self-assembly behaviour of colistin and its prodrug colistin methanesulfonate: implications for solution stability and solubilization

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Abstract

Colistin is an amphiphilic antibiotic that has re-emerged into clinical use due to the increasing prevalence of difficult-to-treat Gram-negative infections. The existence of self-assembling colloids in solutions of colistin and its derivative prodrug, colistin methanesulfonate (CMS) was investigated. Colistin and CMS reduced the air-water interfacial tension, and dynamic light scattering (DLS) studies showed the existence of 2.07 ± 0.3 nm aggregates above 1.5 mM for colistin, and of 1.98 ± 0.36 nm aggregates for CMS above 3.5 mM (mean ± SD). Above the respective critical micelle concentrations (CMC) the solubility of azithromycin, a hydrophobic antibiotic, increased approximately linearly with increasing surfactant concentration (5:1 mol ratio colistin:azithromycin), suggestive of hydrophobic domains within the micellar cores. Rapid conversion of CMS to colistin occurred below the CMC (60 % over 48 hr), while conversion above the CMC was less than 1 %. The formation of colistin and CMS micelles demonstrated in this study is the proposed mechanism for solubilization of azithromycin and the concentration-dependent stability of CMS.

Keywords

Micelle; dynamic light scattering; amphiphilic peptide antibiotic; azithromycin

Introduction

Self assembly of pharmacologically active compounds in solution is an interesting, important and frequently understated phenomenon. Amphiphilic structure is often a prerequisite for pharmacological activity, and can be key to the interaction between drug molecules and biological membranes.[1] Colistin (also known as polymyxin E, Figure 1A) is an old antibiotic that exhibits membrane permeabilizing and bactericidal activity against Gram-negative

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bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*.[2] The escalating incidence of antibiotic resistance[3,4] and the lack of new antibacterial compounds under development[5] has resulted in a renewed interest and resurgence in the clinical use of colistin. Colistin became available for clinical use in 1959 and has never been subjected to modern drug development scrutiny, including a full assessment of its pharmacological and physicochemical properties.

Colistin is a complex, multi-component antibiotic mixture. The main constituents accounting for approximately 85% of this mixture are colistin A and colistin B (Figure 1a).[6] The structure of colistin A contains an octanoic acyl hydrophobic moiety, while colistin B contains a heptanoic acyl residue. The headgroup of colistin A and B is a pentacationic decapeptide, possessing five diaminobutyric acid (Dab) residues that are positively charged at physiological pH. The hydrophobic fatty acyl tail and hydrophilic cationic headgroup renders colistin amphiphilic.

In the clinical setting, colistin is administered in the form of a less toxic and non-active prodrug,[7] the sulfomethyl derivative, colistin methanesulfonate (CMS) (Figure 1b). CMS is a polyanionic compound resulting from the sulfomethylation of the five Dab residues of colistin. All five sulfomethyl groups of the inactive prodrug CMS must be cleaved from the peptide structure to form the active parent compound colistin.[7] Conversion of CMS to colistin has been shown to be rapid at low, clinically relevant concentrations in plasma and urine *in vivo*[8] and in buffer solutions *in vitro* (<100 μg/mL at 37 °C).[9] In contrast, more concentrated solutions of CMS (>70 mg/mL), such as those relevant to commercial pharmaceutical formulations, have been shown to be stable for extended periods with respect to formation of colistin.[10] The self assembly of CMS monomers into association colloids at a critical concentration offers a potential explanation for the concentration-dependent stability of CMS.[10] The formation of micelles or colloidal aggregates has been reported to influence the stability of other antibiotics, including the penicillins.[11–16] Other authors have suggested the existence of colloidal species in solutions of polymyxin B (sulfate, Figure 1c)[17] and colistin (sulfate),[18] however no direct evidence has been published to confirm this phenomenon, or to indicate the concentration at which self assembly occurs.[18,19] The self assembly of CMS is therefore of interest from the perspective of the stability of pharmaceutical formulations of CMS. Combination antibiotic treatments are becoming an important approach for minimizing development of bacterial resistance; hence the potential for colistin and CMS to provide micellar solubilization of poorly water-soluble antibiotics, such as macrolides,[20] is also of interest.

The primary aim of this investigation was to characterize the self-assembly behaviour of both colistin and CMS in solution. The potential solubilizing capacity of colistin and CMS solutions was investigated using a poorly water-soluble macrolide antibiotic, azithromycin (Figure 1e). In addition, we investigated whether micellization could be a possible mechanism for the observed concentration-dependent stability of CMS.

Materials and Methods

Materials

Triton X-100®, polymyxin B nonapeptide hydrochloride (PMBN), sodium chloride, potassium chloride, sodium phosphate, sodium hydroxide, potassium dihydrogen phosphate and 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) were obtained from Sigma (St Louis, MO). Colistin sulfate was from Zhejiang Shenghua Biok Biology Co. Ltd (Huzhou, China), CMS sodium from Alpharma (Copenhagen, Denmark), azithromycin dihydrate EP from Kopran Pty Ltd (Mumbai, India). Water was purified using a Milli-Q® water purification system (Millipore Corp., Bedford, MA). All analytical reagents were of HPLC grade and all chemicals were used as received. All CMS and colistin solutions were freshly prepared.

Characterization of colistin and CMS self assembly

Dynamic light scattering—Colloidal aggregation in aqueous solutions of colistin and CMS was investigated by dynamic light scattering (DLS) (Malvern Zetasizer Nano S, Malvern Instruments, Worcestershire, UK).[21,22] This DLS instrument uses a 4 mW He-Ne laser (λ = 632.8 nm) with detection at 173° and a thermostatted sample chamber set to 25 °C. The viscosity and refractive index of water at 25 °C, 0.8937 cP and 1.333 [23], respectively, were used for all measurements. In the absence of self-assembling colloidal aggregates, the intensity of back-scattered light is comparable to that of the solvent. In the presence of aggregates, the intensity of back-scattered light increases with increasing concentration of aggregates.

Solutions were prepared in Milli-Q water by dilution from stock solutions and were filtered through a 0.02 μm Anotop® filter (Whatman, Maidstone, UK) to remove dust prior to DLS measurements in a low-volume polystyrene cuvette. The back-scattered light from the solvent ($S173_{solvent}$) was subtracted from that of each sample (S173) and was plotted as a function of concentration. Each sample was prepared in triplicate and was measured three times to ensure reproducibility. Triton X-100® served as a 'positive control' and PMBN (Figure 1d), which is not amphiphilic, was used as a 'negative control'.

Size information was obtained from the correlation function by two means on the Zetasizer Nano DS using the software package DTS Nano v5.10. For z-average diameters, a single exponential was fitted to the correlation function to yield a cumulant analysis. To obtain the distribution of particle sizes, a multiple exponential was fitted to the correlation function.

Surface tension—Surface tension measurements were carried out on solutions of colistin and CMS using a NIMA DST 9005 automatic tensiometer (Nima Technology Ltd, UK) fitted with a platinum Du Nouy ring (ring diameter 20.6 mm, wire diameter 500 μm). Solutions were allowed to equilibrate to 25 °C before the ring was immersed 5 mm below the surface of the solution. The tensiometer was automatically retracted from the solution at a rate of 5 mm/min. Between measurements, the ring was rinsed with Milli-Q® water and ethanol, and further cleaned by flaming. Solutions were prepared in triplicate.

Solubilization experiments—The solubility of azithromycin (aqueous solubility < 200 μg/mL)[24] in CMS and colistin solutions was investigated, and compared to that in water and pH 7.4 phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 2 mM KH_2PO_4). Excess azithromycin was added to dissolution medium in 50-mL polypropylene tubes and shaken in a water bath at 25 °C; each solution was conducted in three replicates. Samples were collected to determine the time at which no further dissolution of azithromycin occurred, deemed to be the equilibrium solubility. Azithromycin suspension samples were filtered through a 0.2 μm regenerated cellulose Minisart® filter (Sartorius, Göttingen, Germany) to remove any undissolved azithromycin (preliminary studies showed negligible adsorption to the membrane). Filtered samples (200 μL) were stored at −20 °C and diluted 1:1 with methanol (MeOH) prior to high-performance liquid chromatographic (HPLC) analysis. The HPLC system (Shimadzu, Kyoto, Japan) comprised of a SIL-10A controller, two LC-10AD pumps, a SIL-10AD auto-injector, a CTO-2A column oven, a DCH-14A degasser and a SPD-10A UV detector connected to a multi-instrument data acquisition and data processing system (Class-VP, Shimadzu, Kyoto, Japan). Azithromycin was separated and quantified on a Waters C_8 Symmetry® column and a mobile phase of MeOH:0.02 M KH_2PO_4 (80:20 v/v). The KH_2PO_4 solution was adjusted to pH 7 with 10 % NaOH before mixing with MeOH. Sample was eluted at a flow rate of 1.0 mL/min with UV detection at 210 nm. The retention time of azithromycin was approximately 5.2 min. Good linearity was achieved (> 0.998) over the range 0.1 – 2 mg/mL. Levels of accuracy and reproducibility were less than 4.5 % and 6 %, respectively. The lower limit of quantification was 0.05 mg/mL.

Stability of CMS in solution—The degradation of CMS in solution over time was assessed by measuring the extent of colistin formation. CMS solutions were prepared at 0.052, 0.52, 5.2 and 52 mM in 0.9% (154 mM) NaCl. A 50-mL aliquot of each solution was dispensed into 50-mL polypropylene tubes and stored in the dark at 4°C or 25°C for 120 hr (n = 3 for each concentration at each temperature). Samples (1 mL) were collected at 0, 2, 4, 8, 12, 24, 48 and 120 hr and stored at −20 °C pending analysis for colistin concentration by a validated HPLC assay[25]. Briefly, samples (150 μL) were reacted with FMOC-Cl to produce fluorescent derivatives of colistin, which were subsequently analyzed using reversed-phase HPLC. The amount of colistin formed was then used to calculate (on a molar basis) the amount of CMS remaining in solution. Acceptable linearity was achieved (> 0.994) over the range 1 to 60 μg/mL. Inter-day accuracy was within 4.5 % and reproducibility was within than 8 %. The lower limit of quantification was 1 μg/mL.

Results

Figure 2 shows the intensity of light scattered by solutions of CMS, colistin and PMBN, as a function of concentration. Inflections in the scattering patterns are observed for colistin and CMS at approximately 1.5 mM and 3.5 mM, respectively, where the scattering pattern increases above that of the solvent. Conventionally, this point is identified as the CMC.[15] In contrast, solutions of PMBN showed negligible scattering above that of water (Figure 2), and no reliable size data could be obtained from DLS measurements up to 20 mM. Colistin and CMS micelles had z-average diameters of approximately 2.07± 0.30 and 1.98 ± 0.36 nm (mean ± SD), at concentrations above the CMC, respectively, while for Triton X-100 the z-average was 8.17 ± 0.59 nm (Figure 3). Z-average data (Figure 4) indicated no change in micelle size with increasing concentration for both CMS and colistin micelles.

The surface tension measurements on solutions of colistin and CMS clearly show that both colistin and CMS are surface active, reducing the surface tension to approximately 35–40 mN/m at 10 mM (Figure 5), however, a definitive CMC could not be deduced from these data.

The data presented in Figure 6 shows the capacity of colistin and CMS solutions to solubilize azithromycin. The solubility of azithromycin increased approximately linearly with colistin or CMS concentrations above their respective CMCs.

Figure 7 shows the stability of CMS presented as the percentage of CMS remaining, at various initial concentrations of CMS, over 120 hr. Rapid conversion of CMS to colistin occurred in the CMS solution with an initial concentration of 0.052 mM. Less rapid conversion to colistin occurred in the CMS solution with an initial concentration of 0.52 mM, while, degradation of CMS at 5.2 and 52 mM (well above the CMC) was negligible.

Discussion

There have been a limited number of reports suggesting the self assembly of colistin in aqueous solution. While studying the interaction of colistin with phospholipids using two probes that fluoresce in a hydrophobic environment (e.g. within the interior of a micelle), Mestres et al. found that both probes exhibited moderate fluorescence intensity when incubated in colistin solutions in the absence of phospholipids.[18] It was concluded that this fluorescence indicated the existence of micelles or aggregates. However, neither the fluorescence data nor the colistin concentrations at which these experiments were carried out were reported. In another study, the interactions between polymyxin B (Figure 1c) and liposomes were investigated. The authors indicated that they had identified a CMC for polymyxin B, however these data were not reported.[19]

In the present study, DLS measurements have directly established the existence of aggregates in solutions of colistin and CMS, but not those of the non-amphiphilic PMBN. The slight deviation from linear scattering intensity vs. concentration for both colistin and CMS (Figure 2) is typical of charged compounds and has been observed in light scattering studies of other charged, micelle-forming drug molecules.[26–28] The inflection points in the curves indicated a CMC for colistin at 1.5 mM and for CMS at 3.5 mM. The value for the CMC of colistin (polymyxin E) was substantially lower than that suggested by Lawrence et al. for polymyxin B, although it is not clear how the CMC was measured in that case.[19]

CMS micelles are apparently slightly smaller than colistin micelles (Figure 3). Although colistin and CMS have the same distribution of lengths for the hydrophobic tails (Figure 1a and 1b), CMS is a larger molecule because of the bulkier sulfomethylated head group. However, it is not unreasonable to expect colistin sulfate to form slightly larger micelles than CMS sodium by virtue of the divalent sulfate counter ion, reducing head group electrostatic repulsion.[15] Other drug molecules have also been observed to form micelles of a similar size,[29] for example gramicidin, a membrane-permeabilizing peptide comprising 15 amino acids[1,30].

The size of colistin and CMS micelles remained the same with increasing concentration (Figure 5). Based on this observation, it is likely that colistin and CMS micelles follow a ‘closed association’ model, having a discrete number of monomers per micelle, rather than associating following the ‘open’ or ‘step-wise’ growth model[15]. Polymyxin B has been shown to exhibit flexibility in the hydrophobic region[31], which, is a structural prerequisite for the closed association model.[32]

Poorly-water soluble drugs are solubilized by the hydrophobic interior of micelles and the amount of drug solubilized generally increases linearly with increasing surfactant concentration above the CMC.[33] Such a relationship was observed for azithromycin solubilized in both colistin and CMS solutions. This strongly indicates the association of the fatty acid tails of colistin and CMS, which provides a hydrophobic solubilizing environment for azithromycin. An ion-pair interaction between ionized azithromycin (pK_a = 8.74) and anionic CMS within micelles was considered as a possible explanation for the solubilization of azithromycin; however, this kind of interaction cannot explain the solubilization of azithromycin in colistin solutions.

Strong evidence has been presented for the self assembly of colistin and CMS in aqueous environments. The marked change in the observed rates of CMS conversion to colistin between 0.52 and 5.2 mM (Figure 7) is entirely consistent with the CMC of 3.5 mM for CMS, as determined by light scattering (Figure 2). Based upon this observation, the micellisation of CMS would appear to afford some protection of the labile sulphomethyl groups from degradation. Assuming that micellisation is driven by attractions between the flexible fatty acid tails of CMS, the anionic, susceptible sulfomethyl groups would be orientated towards the exterior surface of the micelle. The activity of water at micelle surfaces has been shown to be different from that the bulk;[34] high concentrations of counter ions immobilized in the Stern layer can cause the depletion and reduced mobility of water at the micellar interface.[35] Therefore, the hydrolysis of susceptible sulfomethyl groups arranged within a micelle can be slower than that of the monomer dispersed in the bulk.

Colistin is a mixture of two major molecules with identical head groups but fatty acyl tails of different lengths, likely to impart slightly different surface properties, making elucidation of a precise CMC difficult. In addition, the characterization of CMS is complicated by the possibility that rapid conversion to colistin occurs at low concentration, changing the surface active behaviour of the solutions with time, and that the distribution of sulfomethyl groups in

CMS is unknown and likely of a polydisperse nature, i.e. the conversion may not proceed in a stepwise fashion and will be a distribution. The identification of the CMC from surface tension measurements in this system is complicated by the aforementioned polydisperse nature of colistin and CMS structures, and potentially small concentrations of other surface active impurities. Nevertheless, the interfacial activity of the colistin amphiphiles is also evident in the surface tension measurements shown in Figure 6. The reduction in interfacial tension caused by colistin after the CMC to approximately 40 dynes/cm is comparable to observed for other cationic octyl-chain surfactants.[36]

Organization of drug molecules into self-assembling colloids has been shown to affect rates of drug degradation,[11,37,38] and has been proposed as the mechanism for the enhanced stability of CMS at high concentrations.[10] The formation of colistin in pharmaceutical formulations of CMS is of concern because colistin is much more toxic than CMS (LD_{50} 217.7mg/kg and 5.43 mg/kg in mice, respectively).[39] Recently, the death of a cystic fibrosis patient following the inhalation of a solution of CMS was purportedly due to the formation of colistin in the CMS formulation prior to use,[40] however, the direct link to stability in that case has been questioned[10]. Nevertheless, the instability of CMS at low concentrations has potential to impact on the clinical use of CMS. While CMS for parenteral use is presented in freeze dried form, it is reconstituted and diluted substantially in intravenous fluids prior to administration, which has been shown to accelerate the conversion of CMS to colistin.[10]

In conclusion, the self association of the polymyxin antibiotic peptides, colistin and CMS, has been demonstrated in aqueous solution using DLS and solubilization techniques. The formation of aggregates of CMS in solution is proposed to be the mechanism for the greater stability of CMS observed at high concentrations.

Acknowledgments

The authors wish to acknowledge the valuable advice of Professor David Attwood from the University of Manchester, UK. JL is an Australian National Health and Medical Research Council R. Douglas Wright Research Fellow. The work described was supported by Award Number R01AI079330 and Award Number R01AI070896 from the National Institute of Allergy and Infectious Diseases. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health.

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Figure 1a

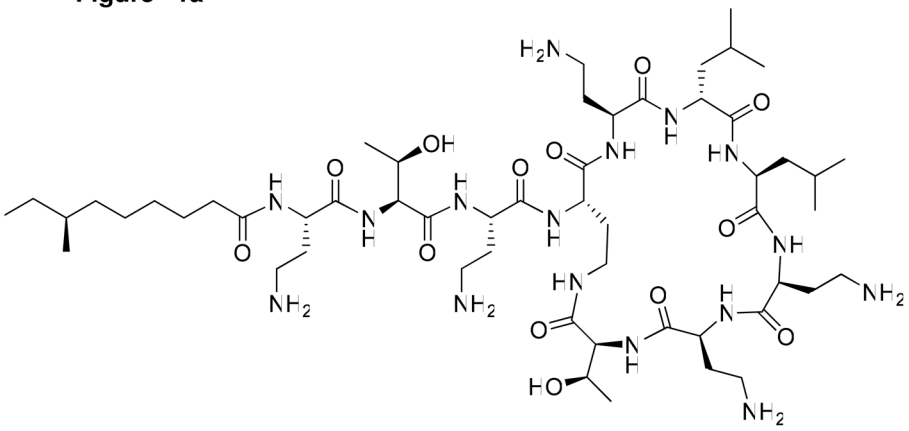

Figure 1b

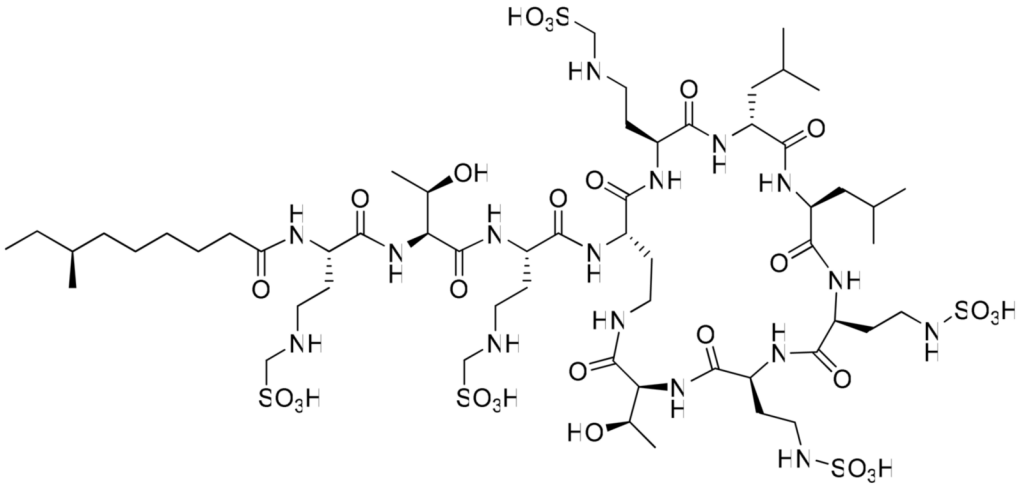

Figure 1c

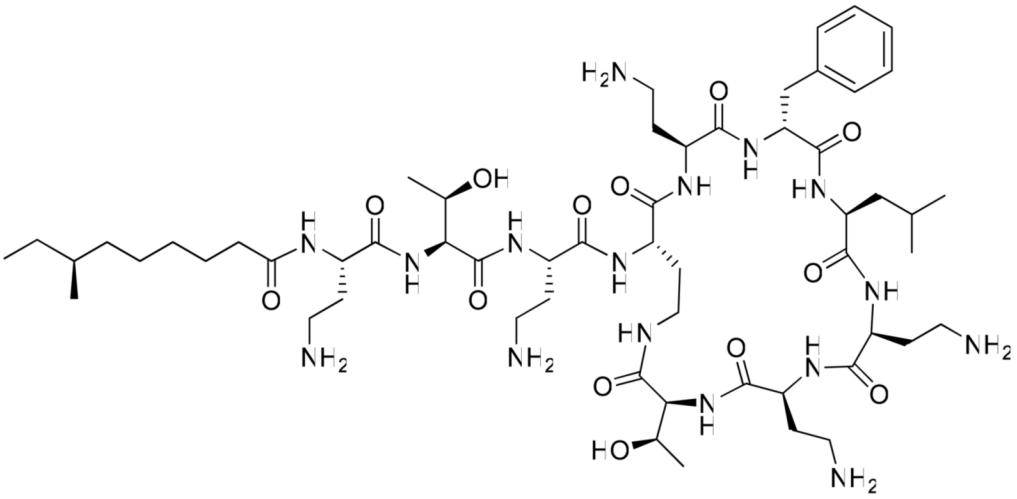

Figure 1d

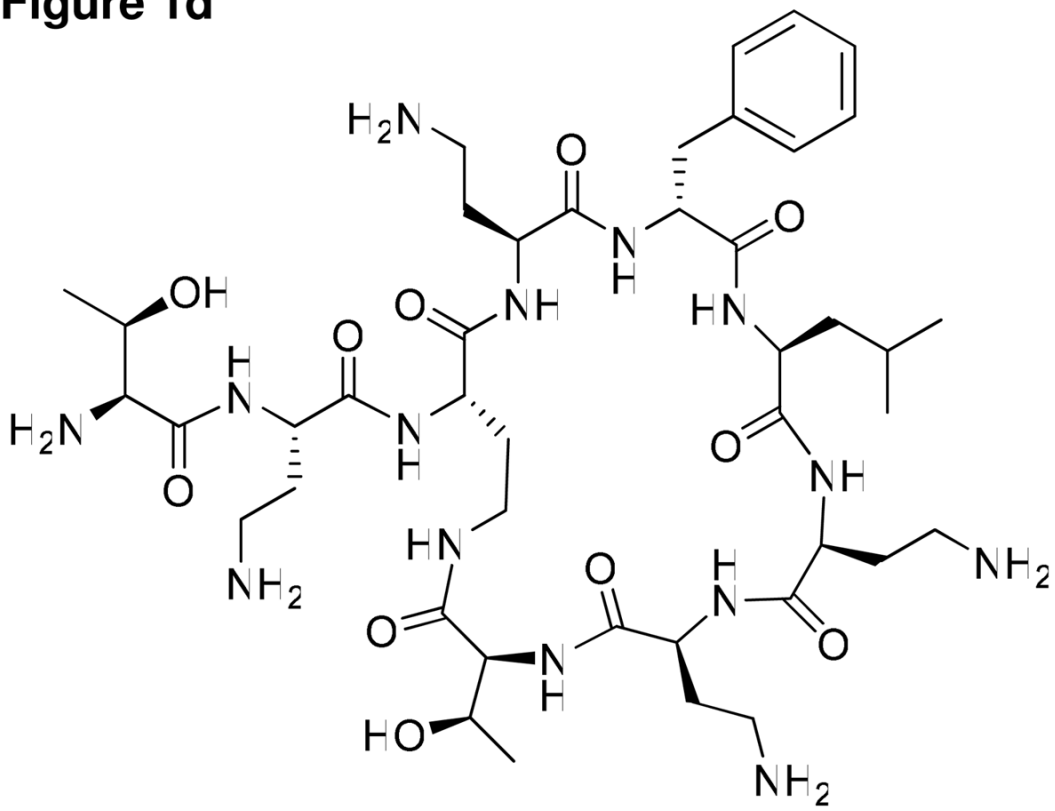

Figure 1e

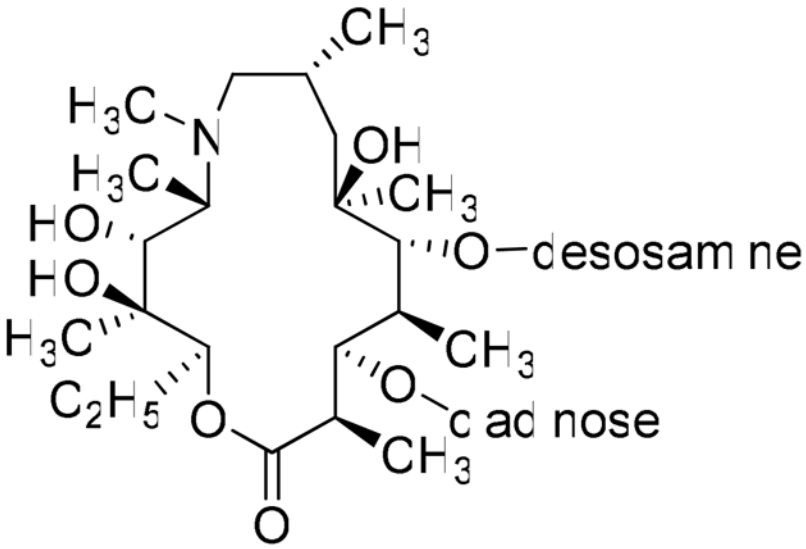

Figure 1.

Figure 1a: Colistin chemical structure: Colistin A (as shown) fatty acid is 6-methyloctanoic acid. Colistin B fatty acid is 6-methylheptanoic acid.

Figure 1b: CMS chemical structure: CMS A fatty acid is 6-methyloctanoic acid. CMS B fatty acid is 6-methylheptanoic acid.

Figure 1c: Polymyxin B chemical structure: Polymyxin B_1 fatty acid is 6-methyloctanoic acid. Polymyxin B_2 fatty acid is 6-methylheptanoic acid.
Figure 1d: Polymyxin B nonapeptide chemical structure.
Figure 1e: Azithromycin chemical structure.

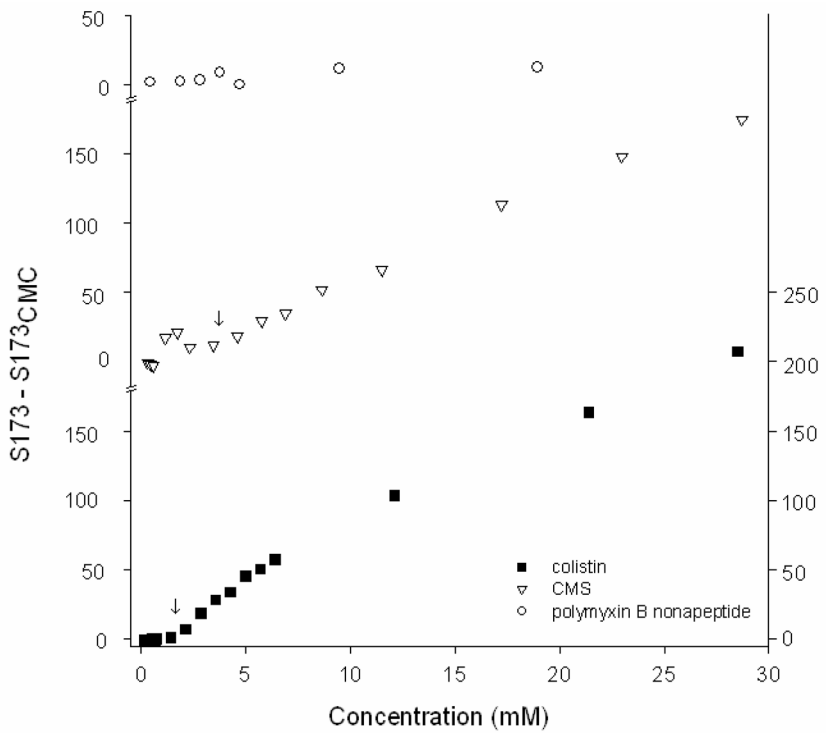

Figure 2.

Dynamic light scattering of solutions of colistin, CMS and polymyxin B nonapeptide versus concentration of the respective species. The dynamic light scattering is expressed as the intensity of light scattered at 173° (S173) for solutions of the species minus the intensity of light scattered by the solvent ($S173_{solvent}$). Arrows indicate points of inflection (CMC).

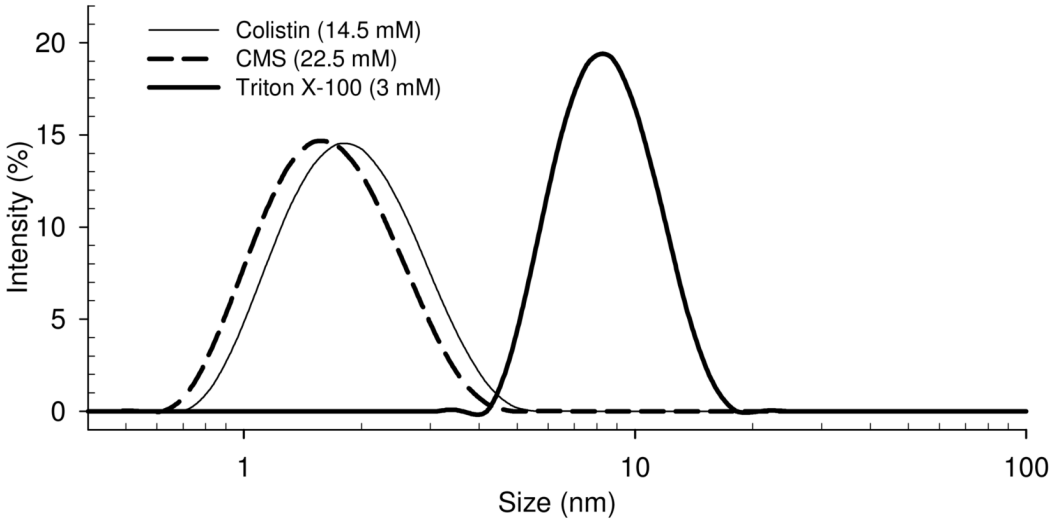

Figure 3.
Intensity size distribution profiles for colistin, CMS and Triton X-100 micelles at 25 °C.

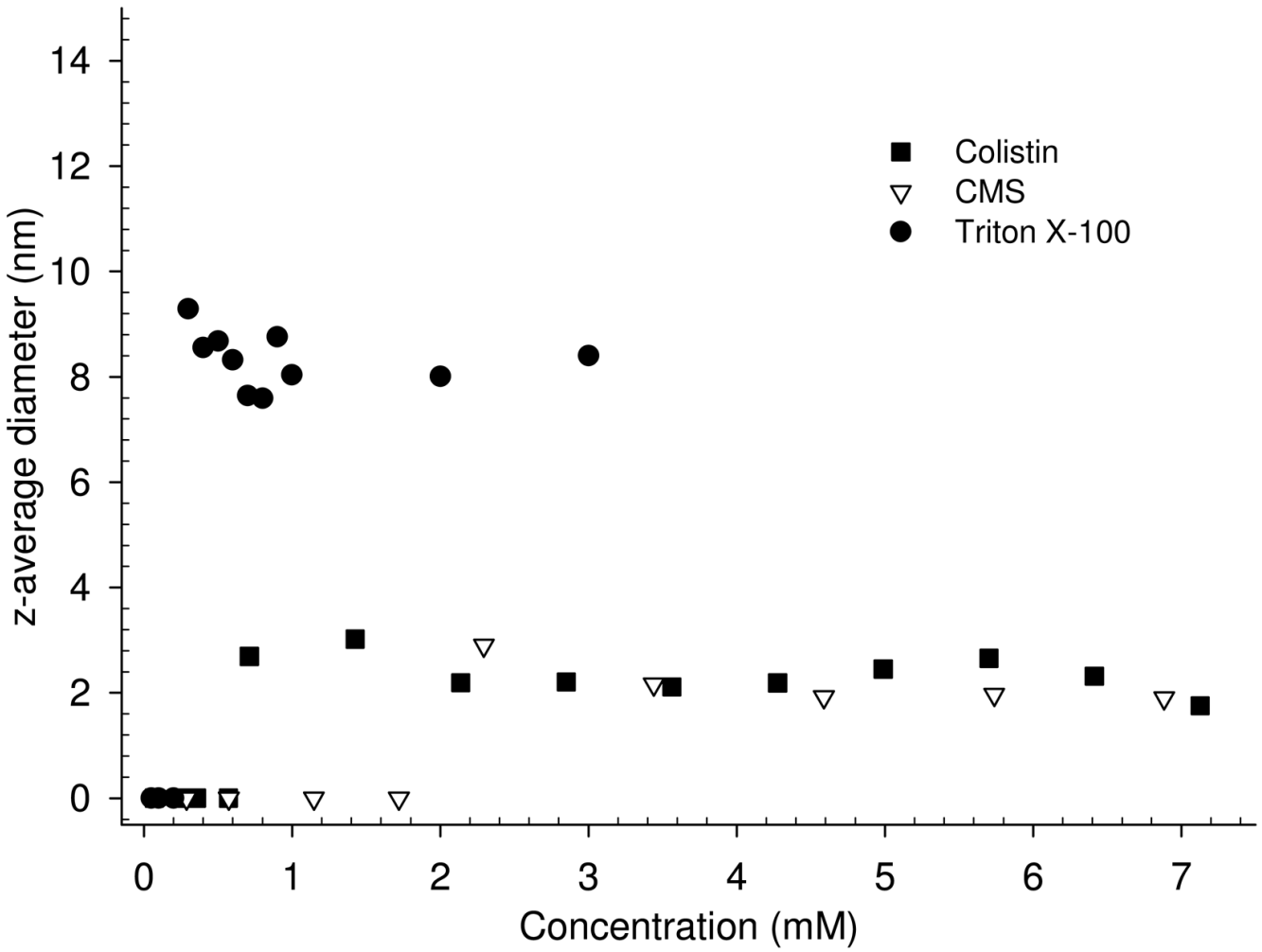

Figure 4.
The z-average diameters of colistin, CMS and Triton X-100 micelles plotted as a function of concentration.

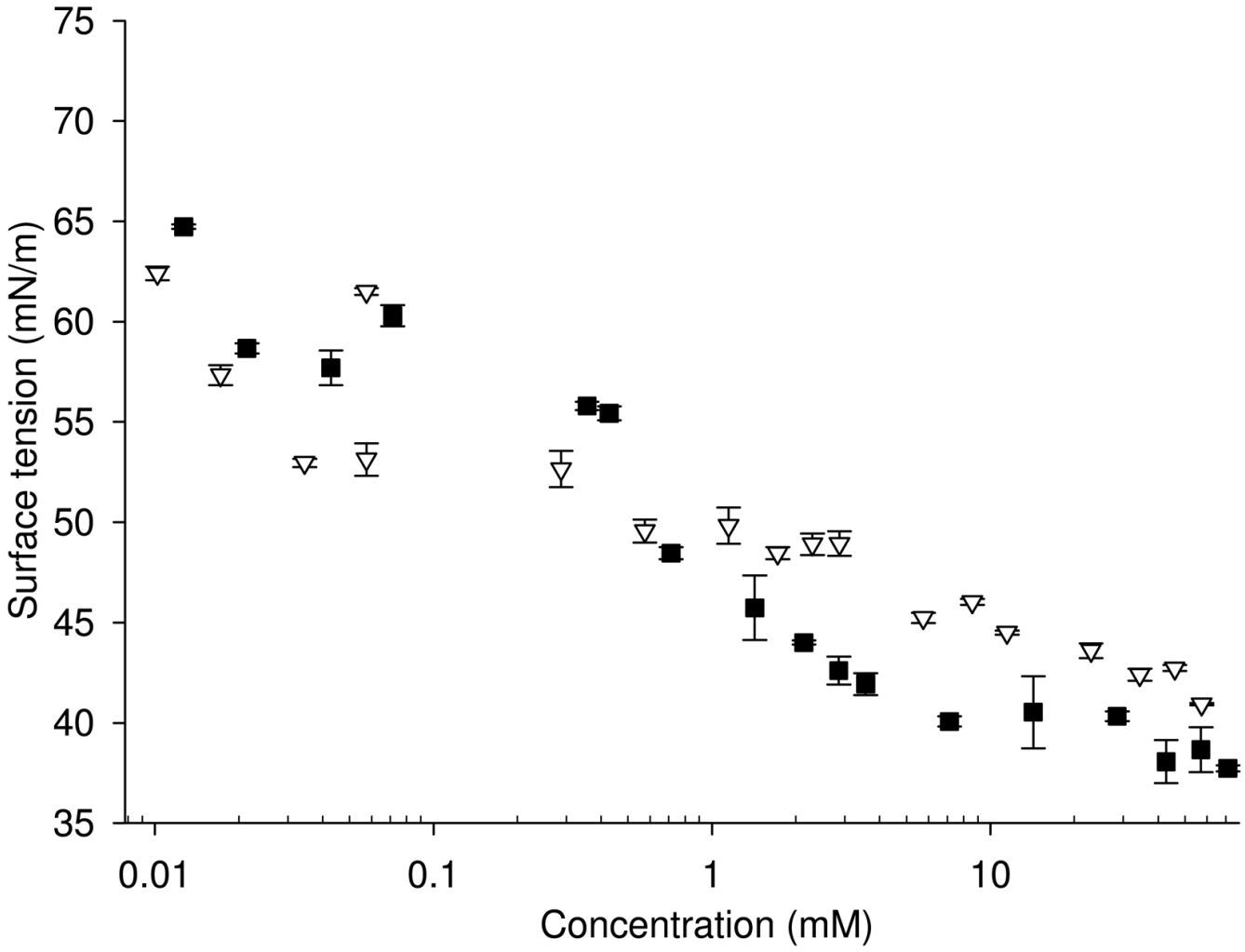

Figure 5.
Surface tension of aqueous solutions of colistin and CMS.

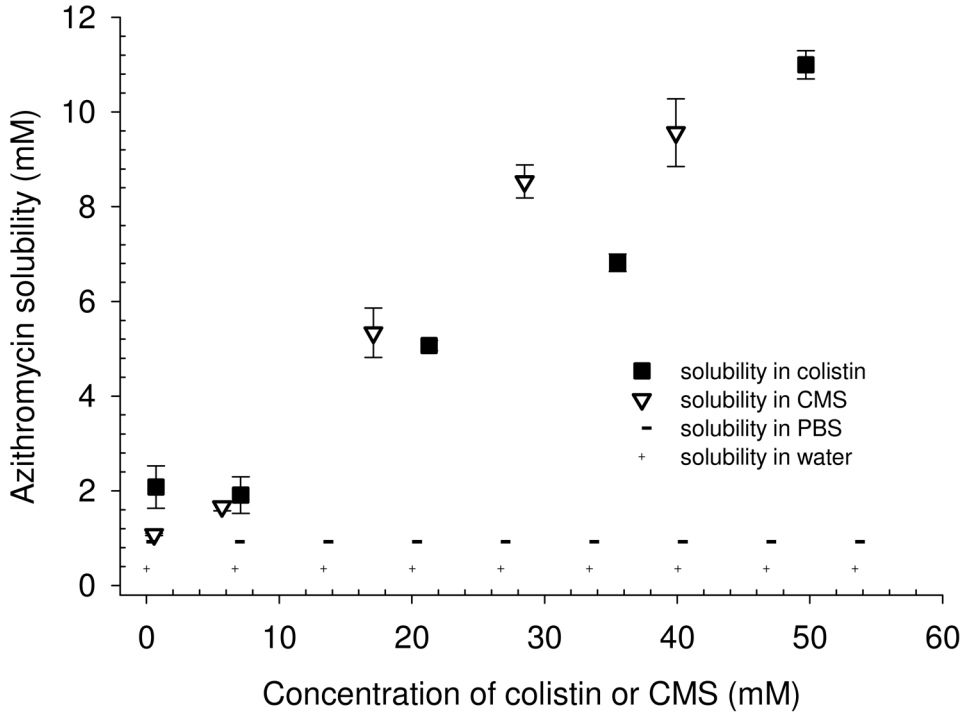

Figure 6.
Solubilization of azithromycin in colistin and CMS solutions. The broken bold line represents the solubility of azithromycin in phosphate buffered saline. The cross hashed markers indicate the aqueous solubility of azithromycin in the absence of any buffer species.

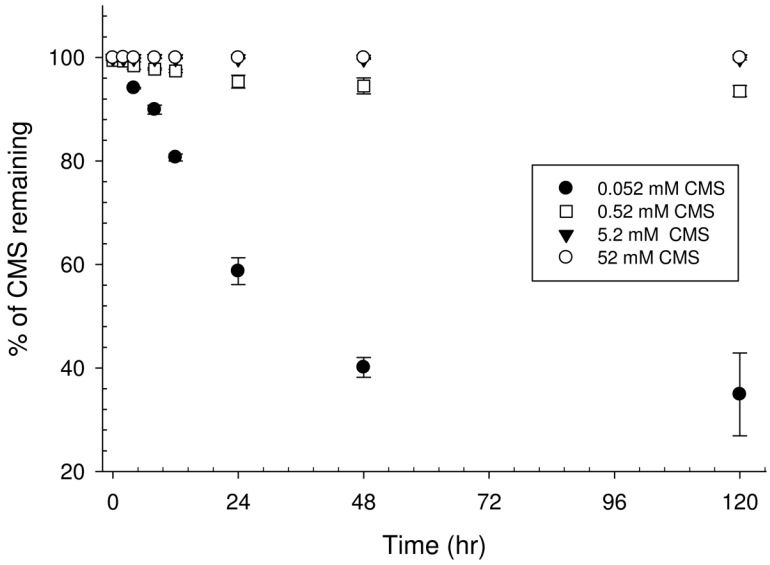

Figure 7.

Stability of CMS at increasing concentrations in 0.9% saline at 25°C, presented as the percentage of CMS remaining versus time. The percentage of CMS remaining was determined as the difference between the measured molar colistin concentration and the initial molar CMS concentration.