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Evaluation of a cationic calix[4]arene: Solubilization and self-aggregation ability

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ABSTRACT

Water-soluble calixarenes are promising macrocyclic compounds which have found numerous applications in chemistry and biology. However, these compounds have been less studied in regard to their behavior in aqueous solutions and mechanisms of drug solubilization. The present work is devoted to the evaluation of the solubilizing properties and estimation of self-aggregation ability of positively charged 5,11,17,23-tetrakis(trimethylammoniomethyl)-25,26,27,28-tetrapropoxy-calix[4]arene tetrachloride (aminocalix), including comparisons with a series of pharmaceutically relevant cyclodextrins. Phase-solubility measurements of the drugs with aminocalix and various cyclodextrins were carried out. Aminocalix showed a solubilizing ability comparable to the cyclodextrins. The drug solubility enhancement caused by the aminocalix was studied and was found to be maximal for steroid drugs. An attempt to understand the solubilizing mechanism of aminocalix was undertaken based on correlation analysis between physical and physico-chemical properties of the drugs from one side and the solubilizing ability of aminocalix from the other. Correlation analysis supports the supposition that the solubilizing effect of aminocalix is based on interaction of the drug with aminocalix aggregates rather than on inclusion complexation. UV-absorbance, osmolality and surface tension concentration dependences of aminocalix showed an inflection at 1% (w/v) which was initially related to the transition from monomers to micelles. However, dynamic light scattering and transmission electron microscopy measurements revealed that likely vesicles of diverse size exist at 0.1% (w/v) concentration. Thus the 1% (w/v) inflection point was interpreted to be spontaneous reordering of the vesicles between two different size populations.

1. Introduction

Modern drug discovery techniques, such as high-throughput screening, produce numerous drug candidates possessing favorable properties for optimum biological activity, such as chemical structure, biological target affinity and selectivity. However, their poor aqueous solubility frequently hampers their full development into marketable drug product. Solubilization of poorly soluble physiologically-active compounds continues to be one of the main stumbling blocks of formulation of new chemical entities. The problem is traditionally solved by means of various solubilizing additives, such as polymers, salts and other excipients, whose action is governed by, for example, salting-in effects, charge-transfer and inclusion "guest–host" complex formation. One of the most efficient novel groups of drug solubilizers are cyclodextrins (CDs). During the last decades CDs have been successfully employed in food, cosmetic and pharmaceutical product development. CDs are doughnut shaped with a relatively lipophilic central cavity that favors inclusion of lipophilic drugs, or rather their lipophilic moieties, to form inclusion complexes. Recently, enhanced understanding of CDs solubilizing effect, their ability to form non-inclusion complexes and nanoparticles, has opened up new possibilities of their utilization in drug delivery (Loftsson et al., 2002, 2004; Messner et al., 2010). However, CDs still have some critical disadvantages. Some of them are too expensive for industrial scale usage, whilst others have insufficient solubilizing effect or cause too much increase in the formulation bulk. Thus, the search for novel excipients with improved properties is continuously ongoing.

Calix[n]arenes (CAs) are often compared to CDs and these two chemical groups do indeed possess a number of structural similarities, particularly their macrocyclic nature and cage-like shape. The parent CAs were unsuitable for use as solubilizers due to their limited aqueous solubility and high flexibility leading to complete ring inversions (Gutsche, 1998). Attachment of polar moieties at the CAs wider rim increases their hydrophilicity, whereas rela-

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tively bulky hydrocarbon moieties (larger than ethyls (Iwamoto et al., 1991)) at the narrow rim are known to stabilize their conformations. CAs are relatively nontoxic and do not provoke immune responses (Gutsche, 1998; Yang and de Villiers, 2004a; Lalor et al., 2008), although their pharmaceutical applications have not yet been approved by the FDA. CAs have found numerous applications such as complexing agents of anions and cations in waste remediation and chemical catalysis (Abraham, 2002; Quinlan et al., 2007; Park et al., 2008), as well as in recognition of small neutral organic molecules (Arena et al., 2000). Recently, interest has focused on their use in biological systems. Proposed applications include their use as mimics of ion channels, enzyme mimics, agents for the surface recognition of proteins, platforms for magnetic resonance imaging agents, gene transfection vectors, drug delivery systems, and antimicrobials (Yang and de Villiers, 2005; Coleman et al., 2007; Jose and Menon, 2007; Lalor et al., 2007; Bagnacani et al., 2008). Although a small number of studies on the ability of CAs to achieve solubilization of drugs have been reported these have been limited to the commercially available anionic sulphonic derivatives of CAs, namely 4-sulphonic calix[n]arenes (Millership, 2001; Yang and de Villiers, 2004a,b, 2005, 2006; Yang et al., 2008). To the best of our knowledge no studies on the solubilizing effects of cationic calix[n]arenes, or their ability to deliver drugs through biological membranes, have been performed, except the use of aminocalixarenes as complexing agents for nucleotids and nucleic acids (Shi and Schneider, 1999). The purpose of this study was to evaluate the solubilizing properties and complexation capabilities of positively charged 5,11,17,23-tetrakis(trimethylammoniomethyl)-25,26,27,28-tetrapropoxy-calix[4]arene tetrachloride (aminocalix) (Arimori et al., 1995) and to compare these properties with those of some common CDs. In this study CDs were used as reference compounds and the methods applied were those normally used to evaluate complexation abilities and physicochemical properties of CDs (Loftsson et al., 2005a).

2. Materials and methods

2.1. Materials

Dexamethasone (DX) and hydrocortisone (HC) were purchased from Fagron group (the Netherlands), ketoprofen (KET) and ketorolac (KTR) tris salt were purchased from Sigma–Aldrich (USA), lidocaine (LID) was purchased from ICN Biomedicals Inc. (USA), paracetamol (PAR) was purchased from Norsk Medisinaldepot (Norway), 17β-estradiol (ESD) was kindly donated by Pharmatech (USA), 2-hydroxypropyl-γ-cyclodextrin with molar substitution of 0.6, MW 1576 Da (HPγCD) and randomly methylated β-cyclodextrin with molar substitution of 1.8, MW 1312 Da (RMβCD) were from Wacker Chemie (Germany), 2-hydroxypropyl-β-cyclodextrin with molar substitution of 0.65, MW 1400 Da (HPβCD), was from Roquette (France), sulphobutylether β-cyclodextrin sodium salt with molar substitution of 0.9, MW 2163 Da (SBEβCD), was kindly donated by Cydex, Inc. (USA), 5,11,17,23-tetrakis(trimethylammoniomethyl)-25,26,27,28-tetrapropoxycalix[4]arene tetrachloride (aminocalix) was synthesized by research group at the School of Chemical Sciences and Pharmacy, University of East Anglia in Norwich, England using modifications of published procedures (Arimori et al., 1995). All other chemicals used were of analytical reagent grade purity. Milli-Q (Millipore, USA) water was used for the preparation of all solutions.

2.2. NMR

^{1}H NMR measurements were performed for structure verification and purity evaluation of aminocalix after lyophilization. D_2O from Sigma–Aldrich (USA) was used as solvent. The NMR spectra were recorded at 298 ± 0.1 K on an AVANCE 400 instrument (Bruker Biospin GmbH, Germany). Chemical shifts are expressed as ppm (δ).

NMR data aminocalix cone: δ_H (D_2O, 400 MHz) 1.05 (12H, t, CH_2CH_3), 2.03 (8H, m, CH_2CH_3), 2.92 (36H, s, $N^+(CH_3)_3$), 3.46 and 4.6 (4H each, d, $ArCH_2Ar$), 4.03 (8H, t, OCH_2), 4.23 (8H, s, NCH_2) and 6.99 (8H, s, ArH). These data are in agreement with those reported (Arimori et al., 1995). Two doublets at 3.46 and 4.6 ppm confirm that only the *cone* isomer was present.

2.3. Estimation of critical aggregate concentration (CAC)

Aggregate formation was examined using several independent methods. UV-absorbance of aqueous aminocalix solutions was measured at 22 °C using a UV/vis-spectrophotometer 2100 pro Ultrospec (Biochrom Ltd, England). The osmolality measurments were carried out at 30 °C using a vapour-pressure osmometer K-7000 (Knauer, Germany). The osmometer was calibrated with standard sodium chloride solutions of known osmolality. Surface tension was determined in pure water at 22 °C (ring method) using a digital tensiometer K9 (Kruss, Germany). The measured physico-chemical parameters were plotted versus concentration of aminocalix in water and the CAC value determined from the inflection point of the graphs.

2.4. DLS determinations

The size of the aggregates formed was determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS Model ZEN3500 (Malvern Instrument, UK) measuring the light scattering from a He–Ne laser source (λ = 633 nm). This technique measures the time-dependent fluctuations in the intensity of scattered light generated by the Brownian motion of the particles. The velocity of this Brownian motion is measured and the translational diffusion coefficient (D^*) determined. Then D^* is used to calculate the particle size applying the Stokes–Einstein equation:

$$d = \frac{kT}{3\pi\eta D*} \quad (1)$$

where d is the hydrodynamic diameter (m), k is Boltzmann constant (J/K), T is the temperature (K), η is the solvent viscosity (kg/(m s)), D^* is the diffusion coefficient (m^2/s). All experiments were performed at 25 °C and at a scattering angle of 173° to the incident beam. Each sample was measured three times. To obtain the size distribution of the scattering objects, the autocorrelation functions were applied using the CONTIN routine that is based on Laplace inversion. Size distribution graphs, which represent dependences of relative intensity of scattered light versus hydrodynamic diameter of particles, were drawn (not shown in the present paper). In order to estimate the mass distribution of aminocalix between size populations existing in the solution the method employed for characterization of cyclodextrins aggregation (Gonzalez-Gaitano et al., 2002) was used. The mass distribution was calculated using the following equation:

$$M_i = \frac{A_i/R_i^a}{\sum A_i/R_i^a} \times 100\% \quad (2)$$

where M_i, A_i, R_i, are respectively, the mass distribution percentage, intensity area, average hydrodynamic radius of the size population i; a is a shape parameter, which equals to 3 for spherical particles.

Five freshly prepared solutions containing from 0.1 to 1.2% (w/v) aminocalix were analyzed unfiltered. For the kinetic study the solutions were kept at room temperature (approx. 23 °C) without stirring and investigated after 1, 3 and 7 days. Thus, the formation of particles and the fate of the particles formed during storage was analyzed.

Table 1
HPLC conditions.

Drugs	Mobile phase[a]	Flow rate (ml/min)	Wavelength (nm)	Retention time (min)
Dexamethasone	MeOH:W:THF (70:29:1)	1.0	241	3.7
Hydrocortisone	MeOH:W:THF (70:29:1)	1.0	254	3.2
17β-estradiol	MeOH:W:acetic acid (70:29.3:0.7)	1.0	280	5.0
Lidocaine	MeOH:W:Et_3N (80:19.5:0.5)	1.0	264	3.4
Paracetamol	AN:acetic acid 0.05 M (15:85)	1.0	272	3.2
Ketorolac	AN:W:acetic acid pH 2.5 (39:59:2)	1.2	313	5.6
Ketorpofen	AN:acetic acid pH 1.5 (60:40)	1.2	265	2.1

[a] Volume ratios. MeOH: methanol; W: water; THF: tetrahydrofuran; Et_3N: ethyltriamine; ACN: acetonitrile; acetic acid: aqueous acetic acid solution.

2.5. TEM measurments

The morphology and size of the particles in aqueous 1.2% (w/v) aminocalix solution were analyzed by transmission electron microscopy (TEM). Samples were evaporated on a Formvar/amorphous carbon-coated copper grid (previously treated by oxygen plasma) and subjected to negative staining with uranyl acetate 2% (w/v) solution. The grid was air-dried and the morphology of the aggregates was visualized using a FEI Tecnai F20 ST microscope (field-emission gun operated at 3.8 kV extraction voltage) operating at an acceleration voltage of 200 kV.

2.6. Preparation of ketorolac free acid

Ketorolac free acid was prepared from ketorolac tris salt as described elsewhere (Malhotra and Majumdar, 1997). Briefly, the salt was dissolved in milli-Q water and 2 M HCl was added until pH 2. The precipitate of ketorolac free acid was collected by filtration and washed amply with milli-Q water till neutral pH. Finally, the precipitate was dried at room temperature under air flow for 3–4 days. The ketorolac free acid thus obtained was used for further studies.

2.7. Solubility studies

The solubility of drugs was determined in phosphate buffer saline (PBS) pH 7.4 complexation media containing 0–20% (w/v) of various excipients (CDs or aminocalix) using the heating method (Loftsson et al., 2005b). An excess amount of drug was added to the buffer complexation media and the suspension formed heated in a sealed vial in an ultrasonic bath (about 70 °C for 60 min) to promote drug saturation in the buffer complexation medium. After cooling to ambient temperature the vials were opened and a small amount of solid drug added to the vials to promote drug precipitation. The chemical stability of the compounds was also monitored during heating and in all cases less than 1% degradation was observed. After equilibration at room temperature (approx. 22 °C) in a sealed vial under constant agitation for 7 days, the suspension was filtered through a 0.45 μm membrane filter (Whatman GmbH, Germany), discarding approximately the first third of the filtrate, and the solution analyzed by HPLC after dilution with 70% aqueous methanol solution, if necessary. Phase-solubility profiles were obtained by plotting the solubility of drug versus the excipient concentration (Higuchi and Connors, 1965).

2.8. Quantitative determinations

Quantitative determinations of the drugs studied were performed using a reverse phase HPLC component system from Dionex Softron GmbH (Germany) Ultimate 3000 Series, consisting of a P680 pump with a DG-1210 degasser, an ASI-100 autosampler, a VWD-3400 UV–vis detector, and Phenomenex Luna 5 μm C18 reverse-phase column (150 mm × 4.6 mm). For other HPLC conditions see Table 1.

3. Results and discussion

Some important structural and physico-chemical characteristics of the studied aminocalix and the three most common natural CDs, which have been extensively used as pharmaceutical excipients, are displayed in Table 2.

The aminocalix molecule is characterized by upper and lower rims as well as a central annulus (Fig. 1). Whilst the central cavity is able to accommodate guest molecules, due to the nature of the substituents attached to the molecular rims, aminocalix is also likely to solubilize drugs additionally through aggregate formation. A range of drug molecules was used in this study (Fig. 2) to allow investigation of the main properties which determine drug inclusion. Thus both poorly water soluble large steroid molecules (e.g. 17β-estradiol) and smaller charged molecules with either the potential for complimentary binding interactions such as the anionic ketoprofen and ketrolac or cationically charged lidocaine were investigated.

3.1. Phase-solubility studies

The solubilization effect of macrocyles can be based on two mechanisms and moreover, it is usually achieved by a combination of them. The classical mechanism of CD's solubilization effect is thought to be inclusion "guest–host" complex formation due to the existence of a cavity suitable for binding of entire drug molecules or appropriate moieties. Briefly, the 1:1 drug/excipient inclusion complex formation is described with the following equation:

$$D + C \overset{K_{1:1}}{\rightleftharpoons} [D/C] \quad (3)$$

where D is a guest drug and C is a host excipient (in particular, CD). The strength of an inclusion complex is characterized by the apparent stability constant ($K_{1:1}$), which can be determined from the phase-solubility diagram:

$$K_{1:1} = \frac{\text{Slope}}{S_0(1 - \text{Slope})} \quad (4)$$

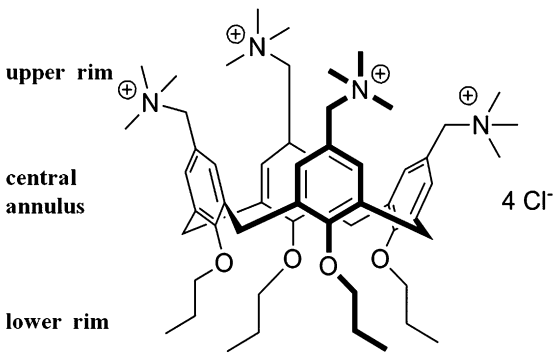
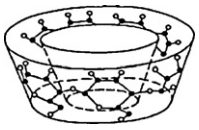
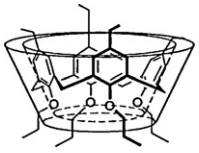
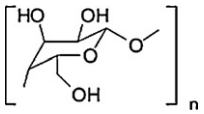
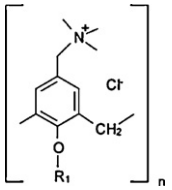


Fig. 1. The structure of studied aminoalix.

Table 2
Comparison of structural and physicochemical properties of studied aminocalix with natural cyclodextrins.

Property	Cyclodextrins			Aminocalix
	α	β	γ	
Shape				
Unit				
Number of units	6	7	8	4
Molecular formula	$C_{36}H_{60}O_{30}$	$C_{42}H_{70}O_{35}$	$C_{48}H_{80}O_{40}$	$C_{56}H_{88}Cl_4N_4O_4$
Formula weight	972.9	1135.0	1297.1	1023.1
Inner cavity diameter, Å	5.2[a]	6.6[a]	8.4[a]	3.0[b]
Aqueous solubility, M	0.12	0.016	0.17	>0.55

[a] According to Connors (1997).
[b] Mean size of cavity, which is formed by repeating phenolic units (Yang and de Villiers, 2005).

where S_0 is the intrinsic solubility of the drug, and Slope is the slope of a linear phase-solubility diagram. A linear phase-solubility diagram is often thought to indicate formation of 1:1 D:C complex but a positive deviation from linearity is thought to indicate formation of 1:2 D:C complex. Due to the structural similarities of CAs and CDs it is valid to suggest the described mechanism of solubilization can also take place in the case of CAs. However, as mentioned previously, the studied CA possesses surfactant properties and can solubilize drugs by another mechanism, i.e. through non-covalent interaction of a drug with the CA aggregates formed. Moreover,

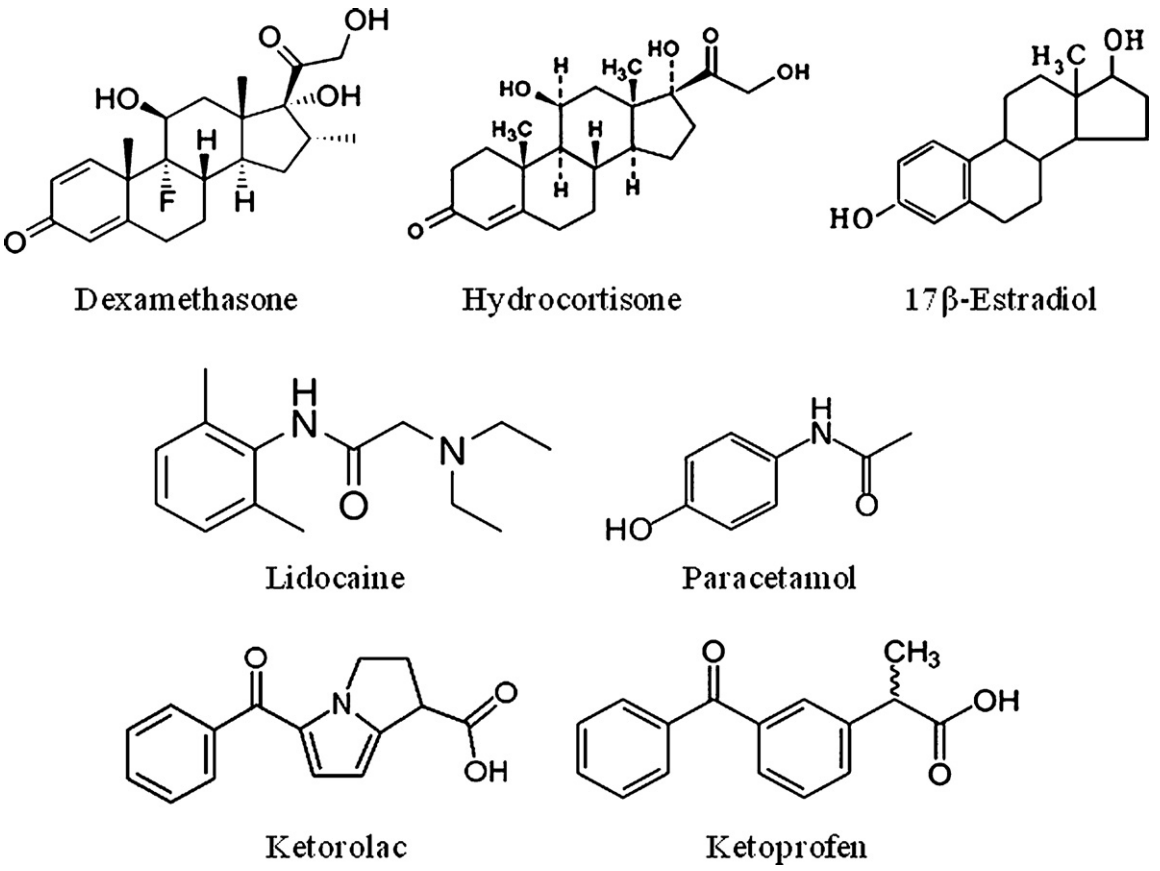


Fig. 2. The chemical structure of the drugs used.

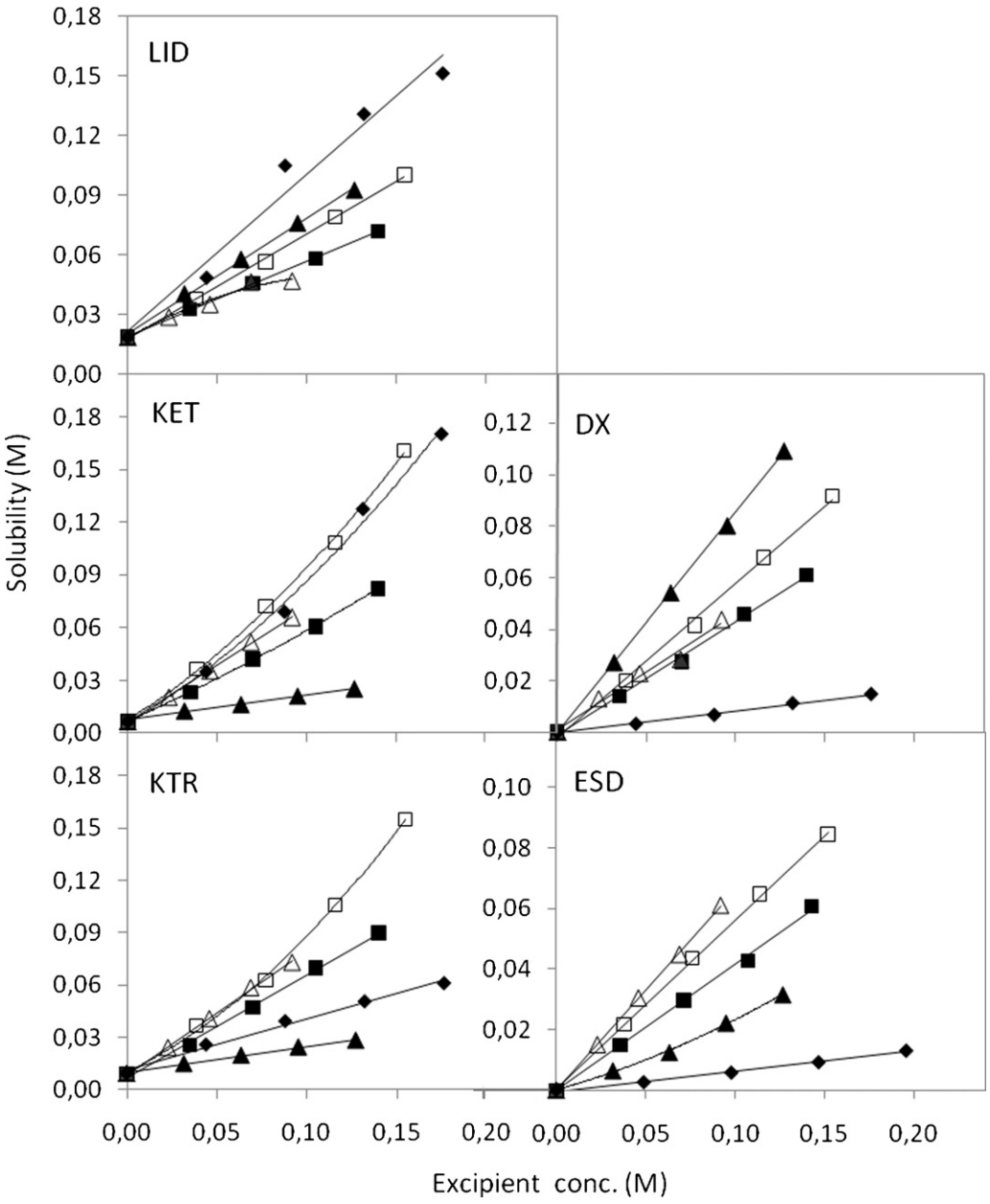


Fig. 3. Phase-solubility diagrams of drugs in aqueous buffer solution in the presence of various solubilizers at ambient temperature and pH 7.4: lidocaine (LID); ketoprofen (KET); dexamethasone (DX); ketorolac (KTR); 17β-estradiol (ESD); HPβCD (■); HPγCD (▲); RMβCD (□); SBEβCD (△); aminocalix (♦).

both mechanisms can coexist and even interact, where the inclusion complex formation promotes micelle or other aggregate type formation which, in turn, increases the solubilizing effect (Kurkov et al., 2010). In such cases the total solubility of a drug is defined as:

$$S_{tot} = S_0 + [D/C] + S_A \quad (5)$$

where [D/C] is the 1:1 inclusion complex concentration and S_A is the drug concentration solubilized by the aggregates (Loftsson et al., 2002). In the case of simultaneous 1:2 inclusion complexes formation Eq. (5) takes a more complex form. This equation can be used for both the CDs and the CAs. In this case the main difference between the aminocalix and CDs chosen is that for aminocalix the term [D/C] in the equation is likely to be small in comparison to the S_A term, whereas for CDs the contrary is true since the CDs tendency to form aggregates (especially at concentrations lower than 10% (w/v)) is weaker (Messner et al., 2010).

The solubility experiments gave the following results shown in Fig. 3 and collected in Table 3. The observed phase-solubility diagrams demonstrate that the aminocalix possesses evident solubilizing ability which is comparable to that of the CDs considered.

It was important to evaluate the solubilizing effect of aminocalix more thoroughly. For this purpose, the solubilities of the drugs in PBS containing 5% (w/v) of the excipient were estimated from the phase-solubility diagrams. The results are displayed in Fig. 4. Aminocalix improves the solubility to a greater extent than reference CDs in the case of lidocaine, paracetamol and ketorpofen. The steroid drugs are better solubilized by all the CDs considered, than by aminocalix, whilst ketorolac is solubilized by aminocalix similarly to SBEβCD but not as effectively as by non-ionized βCD derivatives.

The solubilizing effects observed in Fig. 4 can only be applied for comparison of excipients to each other with regard to a certain drug since drug hydration peculiarities do not influence such analyses.

Table 3
The parameters of complex formation for aminocalix and some cyclodextrins with selected drugs based on phase-solubility experiments in phosphate buffer solution pH 7.4 at ambient temperature. S_0 (exp) and S_{int} are the experimental and intersept intrinsic solubilities of drugs, respectively; "slope" is the phase-solubility diagrams slope; R^2 is the pair correlation coefficient.

Excipient	S_0 (exp) (M)	Type of solubility profiles	Slope	$S_{int.}$ (M)	R^2
Dexamethasone					
HPβCD	$(1.8 \pm 0.2) \times 10^{-4}$	A_L	0.439	−0.001	0.997
HPγCD		A_L	0.853	−0.00003	1.000
SBEβCD		A_L	0.447	0.0012	0.981
RMβCD		A_L	0.597	−0.0018	0.997
Aminocalix		A_L	0.085	−0.0001	0.997
Hydrocortisone					
HPβCD	$(7.2 \pm 0.3) \times 10^{-4}$	A_L	0.863	0.0015	0.999
HPγCD		A_L	0.826	0.0006	1.000
SBEβCD		A_L	0.814	0.0011	1.000
RMβCD		A_L	0.785	0.0015	0.998
Aminocalix		A_L	0.217	0.0008	1.000
17β-estradiol					
HPβCD	$(1.2 \pm 0.6) \times 10^{-5}$	A_L	0.418	−0.0002	0.998
HPγCD		A_P	0.164 (0.675)	0.0001	0.999
SBEβCD		A_L	0.660	−0.00007	1.000
RMβCD		A_L	0.558	0.0005	1.000
Aminocalix		A_L	0.066	−0.0004	0.996
Lidocaine					
HPβCD	$(1.9 \pm 0.1) \times 10^{-2}$	A_L	0.377	0.019	1.000
HPγCD		A_L	0.581	0.0204	0.997
SBEβCD		A_N	0.391	0.0187	0.990
RMβCD		A_L	0.529	0.0175	0.999
Aminocalix		A_L	0.792	0.0213	0.970
Paracetamol					
HPβCD	$(9.8 \pm 0.1) \times 10^{-2}$	A_L	1.435	0.1009	0.999
HPγCD		A_L	1.594	0.0988	0.999
SBEβCD		A_L	1.172	0.0977	1.000
RMβCD		A_L	1.656	0.0981	1.000
Aminocalix		A_L	1.543	0.1239	0.963
Ketorolac					
HPβCD	$(9.0 \pm 0.4) \times 10^{-3}$	A_L	0.587	0.0071	0.998
HPγCD		A_L	0.151	0.0096	0.995
SBEβCD		A_L	0.707	0.0085	0.999
RMβCD		A_P	0.492 (2.864)	0.01	0.999
Aminocalix		A_L	0.291	0.01	0.991
Ketoprofen					
HPβCD	$(6.7 \pm 0.3) \times 10^{-3}$	A_L	0.538	0.0054	0.998
HPγCD		A_L	0.145	0.0074	0.994
SBEβCD		A_L	0.652	0.0062	0.999
RMβCD		A_P	0.642 (2.204)	0.0075	0.999
Aminocalix		A_P	0.612 (1.953)	0.0055	0.995

The solubility enhancement of the drugs was estimated at aminocalix concentration of 176 mM (or 18% (w/v)). For this purpose the following parameter was introduced:

$$\delta = \frac{S - S_0}{S_0} \times 100\% \quad (6)$$

where δ is the solubility enhancement factor, S and S_0 are the drug solubilities in the presence and absence of aminocalix, respectively. The solubility enhancement factor is equal to 100% when the solubility is doubled due to the aminocalix presence, i.e. when $S = 2S_0$. Fig. 5 illustrates the relative solubilizing effect of aminocalix exerted upon the considered drugs.

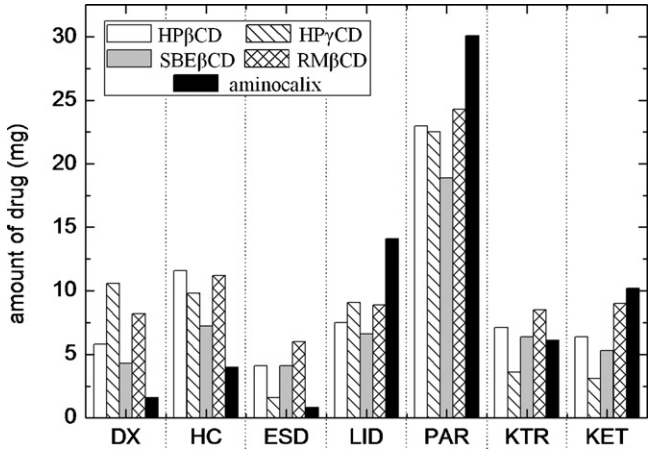


Fig. 4. The amount of dissolved drug in 1 ml of buffer solution containing 5% (w/v) of corresponding excipient.

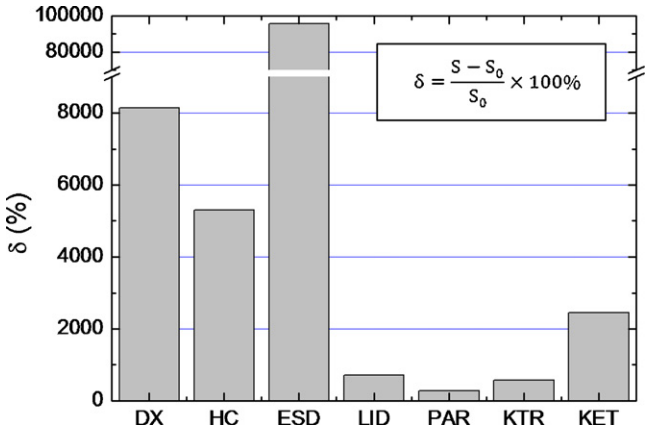


Fig. 5. Solubilizing effect of 18% (w/v) aminocalix on different drugs (δ is the solubility enhancement factor, S and S_0 are the solubility in the presence and absence of complexing agent, respectively).

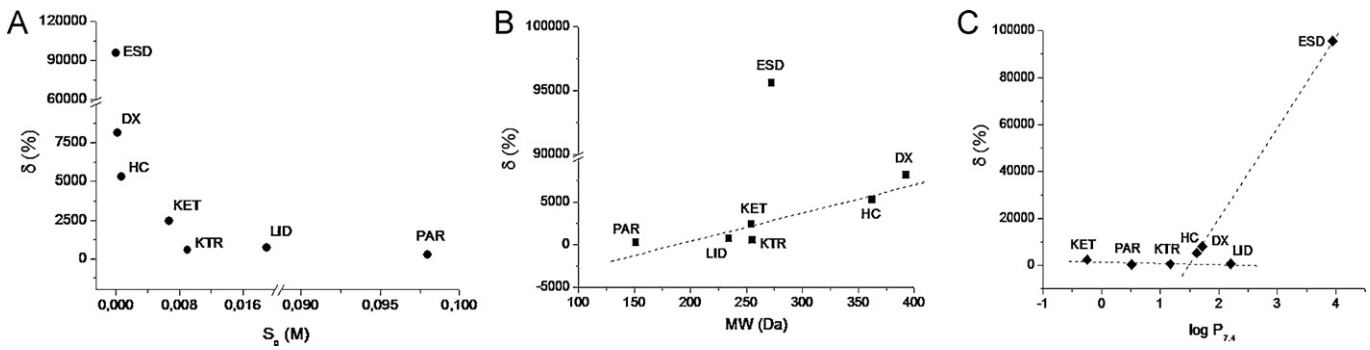


Fig. 6. Relationships found between the solubility enhancement factor, δ, and (A) intrinsic solubility, S_0, (B) molecular weight, MW, and (C) partitioning coefficient, $\log P_{7.4}$, of considered drugs.

The increase in solubilization is ranked as follows: ESD > DX > HC > KET > LID > KTR > PAR. Aminocalix shows the greatest relative solubilization of steroids (especially with 17β-estradiol) in comparison to the rest of the drugs. This is most probably due to the pronounced hydrophobicity of steroids and as a consequence of their very low intrinsic aqueous solubility (S_0). Fig. 6A shows that there is an exponential dependence between the δ-factor and S_0: a very small intrinsic solubility (i.e. of 10^{-4}–10^{-5} order of magnitude in M units) leads to accelerating increase of relative solubilization. It should be mentioned that in such cases the error in δ value estimation is at its maximum.

In an attempt to understand the nature of the aminocalix solubilizing phenomena a search of correlations between δ and some drug properties was undertaken. Molecular weight can serve as a rough measure of molecular size, which is one of the critical factors for inclusion complexation. The better a molecule fits in the cavity and the deeper it is inserted into it, the stronger the inclusion complex is which leads to solubilization promotion. Thus, the solubility enhancement factor was plotted versus drug molecular weight which resulted in the relationship shown in Fig. 6B. A linear relationship is observed for all the drugs except for 17β-estradiol the solubilisation of which is significantly higher than the general trend. Such a relationship supports the suggestion that inclusion complexation plays a minor role in the solubilizing ability of aminocalix. Larger molecules (e.g. cyclosporine A (Kurkov et al., 2010)) are usually poorly solubilized by CDs whose action is primarily based on an inclusion mechanism since their size and shape hampers drug insertion into the cavity. As can be seen in Fig. 6B in the case of aminocalix the larger drug molecular weight favors the solubilization extent. Comparison of low molecular weight drugs to high molecular weight ones within those studied leads to the conclusion that the mass increment is caused by hydrocarbon skeleton growth which is known to be hydrophobic. Thus, it was interesting to search for relationships between discussed parameters and partitioning coefficient at pH 7.4, $\log P_{7.4}$, of the drugs. However, no correlation was found between the molecular weight and the partitioning coefficient for this drug series. On the contrary, δ-factor showed a relationship with $\log P_{7.4}$ (Fig. 6C). It can be interpreted as follows: solubilization for relatively hydrophilic drugs, i.e. acids and bases containing polar groups, does not depend on their lipophilicity and remains equally small within a rather wide range of $\log P_{7.4}$ from −0.25 to 2.2. Again, this observation shows that inclusion complexation is unfavorable: the aminocalix molecule possesses positive charges located at the wider rim, which would attract the negatively charged acidic drugs promoting complex formation and solubilization. However, the relationship between δ-factor and $\log P_{7.4}$ as well as the fact that the strongest solubilizing effect being observed for neutral lipophilic steroids, is only explicable if it is considered that their solubilization is based on non-specific interaction with a lipophilic fraction of aggregate (for instance, a micelle core or interior of a vesicle wall).

Thus, correlation analysis supports the supposition that the solubilizing effect of aminocalix is based mostly on interaction of the drug molecules with aggregates formed by aminocalix rather than by 1:1 inclusion complexation. From this viewpoint it is worthwhile to study in detail the micellization ability of aminocalix within the studied concentration range.

3.2. Properties of aminocalix and its behavior in aqueous solutions

The freshly prepared aqueous solutions of aminocalix are turbid and foamy, in other words aminocalix behaves as a typical surfactant. Therefore, a study was undertaken to analyze the aminocalix surface-active properties and to determine the CAC value.

The aqueous solubility of aminocalix was estimated using a UV-spectrophotometer (λ = 272 nm, extinction coefficient ε = 2398) and found to be greater than 570 mg/ml or 57% (w/v) at room temperature (22–23 °C).

Three different techniques – UV-spectrophotometry, vapour pressure osmometry and tensiometry – were employed to measure the CAC value of aminocalix. All these measurements yielded consistent results which indicated an inflection of obtained lines of concentration dependences at aminocalix concentration of about 1% (w/v) (Fig. 7).

A simple explanation of such inflections would be the beginning of the aggregation process, thus the concentration of 1% (w/v) was concluded to be a CAC value for aminocalix. However, the critical micelle concentration for aminocalix in water at 17 and 30 °C was reported earlier (Arimori et al., 1993, 1995) to be significantly smaller with a value of 0.001% (w/v). Such large differences prompted us to investigate solutions over a range of aminocalix concentrations between 0.1 and 1.2% (w/v) more thoroughly using DLS and TEM as particle evaluation methods.

3.3. Aggregate formation

Initially, the aqueous solutions of aminocalix were investigated by means of DLS. The results, namely peak intensity area, mass distribution and hydrodynamic diameter values are collected in Table 4. Due to the method limitations the slowest (micro-scale) peaks were interpreted as artifacts and were excluded from further consideration.

The mass distribution of aminocalix with respect to particle size distribution was estimated applying a method described elsewhere (Gonzalez-Gaitano et al., 2002) and assuming formation of spherical particles, like micelles or vesicles. Exclusion of the micro-scale intensity peak from the calculation did not influence the mass distribution estimation results since the intensity of this peak is low

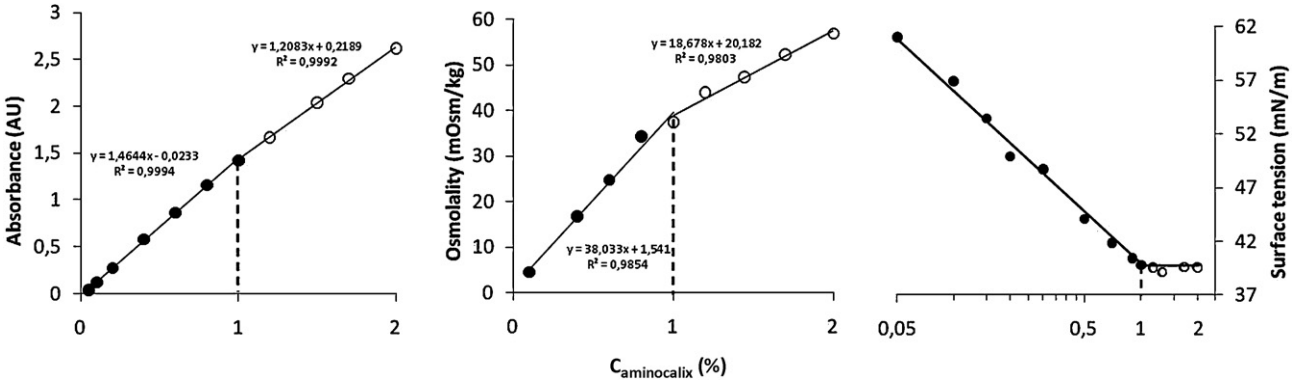


Fig. 7. Absorbance (λ = 330 nm), osmolality and surface tension plotted versus concentration of aminocalix.

Table 4
The DLS results for aminocalix freshly prepared aqueous solutions of different concentrations. $C_{aminocalix}$ is the concentration, *A* is the peak intensity area, *M* is the mass distribution, *d* is the hydrodynamic diameter.

$C_{aminocalix}$ (mg/ml)	*A* (%)			*d* (nm)			*M* (%)	
	1[a]	2[a]	3[a]	1	2	3	1	2
1	48	48.2	2	205	661	5430	97.1	2.9
5	94.7	–	5.3	377.2	–	5243	100	–
8	23.4	71	5.6	131.3	538.2	4452	95.8	4.2
10	59.4	37.9	2.8	255	782	5360	97.8	2.2
12	22.6	76.1	1.3	133	455	5510	92.2	7.8
12[b]	3.8	95.6	0.6	19.9	177	4570	96.5	3.5

[a] 1 is small, 2 is middle, and 3 is large (micro-scale) size population, respectively.
[b] Filtrated through 0.45 μm filter.

and the R_3-value is so large that their mass contribution according to Eq. (2) would be tending to zero.

The analysis of scattered light intensities showed that in the unfiltered freshly prepared aminocalix solutions generally two polydisperse size populations exist with hydrodynamic diameter varying approximately from 100 to 800 nm (Fig. 8A).

The dimensions of the aminocalix molecule (the long axis is 1.5 nm and short axis is about 1 nm (Arimori et al., 1995)) cannot be related to the observed peaks indicating that virtually all dissolved aminocalix molecules participate in aggregate formation. In addition, the 1.2% (w/v) aminocalix solution was analyzed after filtration through 0.45 μm membrane filter. Comparison of the intensity diagrams of filtered and unfiltered solutions show that the largest particles comparable with filter pore size (i.e. those with hydrodynamic diameter around 455 nm) are removed from the solution. Instead, a new small population of smaller aggregates with diameter around 20 nm appears. Moreover, structural changes are detected at concentrations above 10 mg/ml or 1% (w/v): the balance between the two size populations shifts towards larger aggregates (Fig. 8B). This phenomenon can explain the UV-absorbance, osmolality and surface tension concentration dependences (Fig. 7), which show abrupt inflections at concentration of aqueous aminocalix about 1% (w/v). Thus, our results do not disagree with the ones from Arimori et al. (1993, 1995), but supplement them: the 0.001% (w/v) point corresponds to monomers → micelles transition, whilst the 1% (w/v) point corresponds to a micelles → vesicles or vesicles → vesicles' transition, i.e. the shift of equilibrium between two different vesicle types. The reason for such a spontaneous transition is not obvious, but it could be driven by repulsive forces between charges during solute-solute interactions when the distance between nanoparicles becomes critically short. The larger aggregate size would result in a less dense

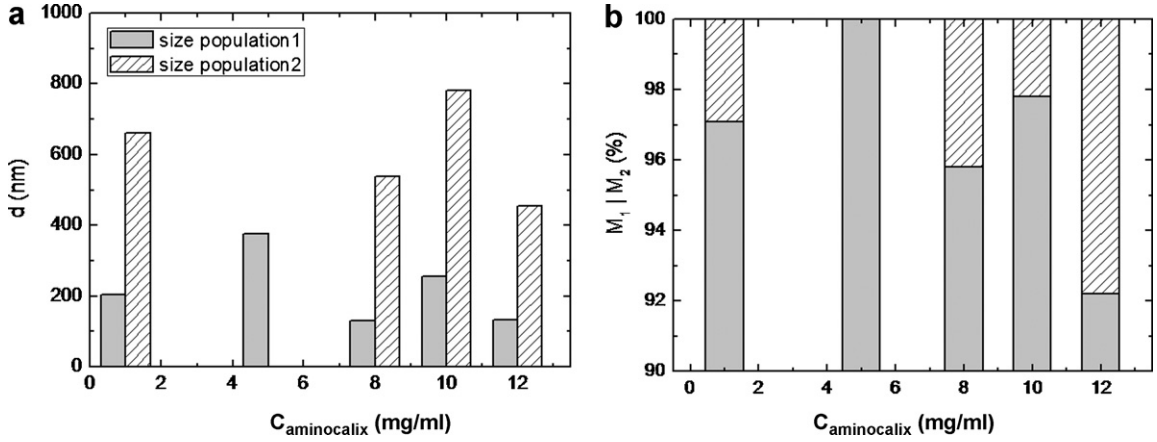


Fig. 8. Particle size (A) and mass (B) distribution of aminocalix among aggregates in freshly prepared aqueous solutions of different concentrations (size classification corresponds to Table 4).

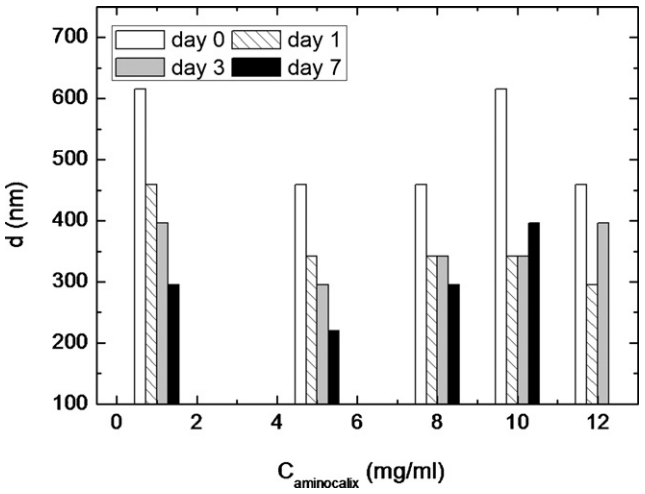


Fig. 9. Kinetic changes in maximum particle size at different concentrations of aminocalix.

solute arrangement and consequently diminish repulsive forces.

The DLS data which were collected during the 7 days storage of solutions show a kinetic effect on size distribution. A slight evolution towards a smaller size distribution in most cases can be observed (Fig. 9). These results differ from the DLS results previously reported (Arimori et al., 1993, 1995) where in aqueous 1.2% (w/v) aminocalix solution aggregates with a diameter of 2–4 nm were detected at 30 °C. However, the absence of experimental conditions in this report does not enable an investigation of these discrepancies. The large vesicles (aggregates) appear to be metastabile and slowly disintegrate to form more stable smaller vesicles.

The aminocalix particles were also viewed by TEM. The TEM images of samples prepared from aminocalix 1.2% aqueous solution are shown in Fig. 10. The figure clearly shows that the sample represents a mixture of spherical objects (presumably vesicles) of diverse size. The diameter of aggregates varies from relatively small – particles about 80 nm (small black dots in Fig. 10A) to about 1.3 μm – the diameter of the biggest particles. In Fig. 10B particles with diameter of about 130, 250, 400 nm as well as particles with larger diameter of about 2 μm are clearly discernible. Fig. 10C shows large formations, which are 1–2 μm wide. In general, the results of TEM study agree with DLS data showing spherical aggregates of diverse sizes are present in concentrated aminocalix solution.

Thus, aminocalix was shown to have profound ability to aggregate where virtually all molecules participate in aggregate formation even at low concentrations. This property represents a key distinction in the solution behavior of aminocalix in comparison to the considered CDs.

The phase-solubility results show that aminocalix is an efficient solubilizer for acidic, basic and neutral drugs of different chemical structure. Lidocaine, paracetamol and ketoprofen are solubilized by aminocalix more efficiently than by the CDs. The comparison of solubilization effects caused by aminocalix in relation to the intrinsic solublities of the chosen drugs revealed that the steroid representatives are solubilized to a greater extent than polar drugs, which could be explained by their low intrinsic solubility, extreme hydrophobicity and molecular size. All these observations are consistent with intricate mechanism of drug-aminocalix interaction. Independent methods showed that aminocalix in contrast to the CDs has a pronounced ability to self-aggregate forming spherical nanoparticles in relatively dilute aqueous solution. The main difference in solubilization nature of aminocalix and CDs lies in the balance between two mechanisms: inclusion complexation and drug-aggregate interaction. Although a small number of publications concerning solubilization of drugs by CAs (Millership, 2001; Yang and de Villiers, 2004a,b, 2005, 2006; Yang et al., 2008) have been repotrted, the action mechanism of ionic CAs is still not well understood. The "drug/CA" systems are usually considered in terms of "guest–host" interactions, resulting in formation of inclusion complexes with 1:1 stoichiometry (Millership, 2001; Yang and de Villiers, 2004a). Increase in drug solubility in such cases must be due to complex formation between the drug and the CA afforded by weak forces including hydrogen bonding, π–π interactions, electrostatic interactions and/or dipole–dipole moments (Yang and de Villiers, 2004a,b). The inner cavity diameter (diameter of central annulus, which consists from repeating phenolic units) of calix[4]arenes is relatively small or about 3 Å (Yang and de Villiers, 2005) but the distance between opposite and neighboring N^+ groups in the aminocalix is 12 Å and 9 Å, respectively (Shi and Schneider, 1999). Thus, there is a possibility for relatively small guest molecules or for some moieties of bigger molecules to be included in the cavity of the aminocalix. In view of the fact that aminocalix is bearing ionic groups on the upper rim of the molecule, the interaction between this excipient and some drugs may be expected to be of electrostatic origin, similarly to the cases described by Shi and Schneider (Shi and Schneider, 1999). Apparently, the solubilization mechanism of ionized ketoprofen and ketorolac in PBS pH 7.4 (pK_a 4.45 and pK_a 3.54, respectively) by aminocalix is somehow related to their electrostatic interaction. Besides, it has been described that during inclusion of some aromatic guests into CA cavity π–π interaction takes place (Rehm et al., 2009). The authors reported that positively charged CA can also interact as host molecules with non-polar aromatic guests via additional cation-π mechanism promoting host-guest association.

However, it is known that amphiphilic macrocyclic molecules, like CAs, behave in aqueous solutions as typical surfactants that aggregate to form micelles (Arimori et al., 1995; Mchedlov-Petrossyan et al., 2006; Rehm et al., 2009). Taking into account self-aggregation of the studied aminocalix (Arimori et al., 1995), it is likely that drug solubilization by aminocalix is based on micellar effects. The interaction between CAs with a similar ionic struc-

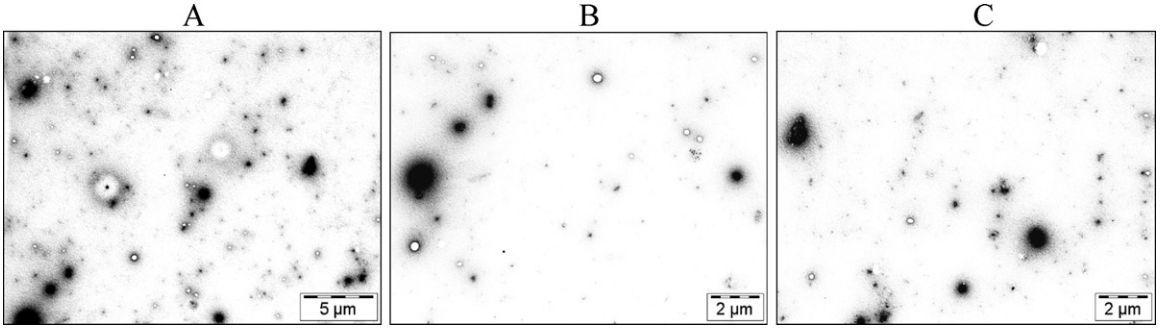


Fig. 10. (A–C) Three different TEM images of the same sample of aminocalix in 1.2% aqueous solution.

ture and dyes (Mchedlov-Petrossyan et al., 2006, 2009) has been studies and it has been proposed that the possible mechanism of interactions is due to the micellar effect rather than docking of the indicator dyes into calixarene cavities. Thus, relation of aminocalix's solubilizing ability to drug interaction with lipophilic vesicle regions should not be excluded. Equally with other interaction types, hydrophobic bonding can significantly contribute to increased solubility of such non-polar hydrophobic molecules as dexamethasone, hydrocortisone and 17β-estradiol. Another fact is that, in comparison to the CDs, the positively charged aminocalix solubilization of positively charged lidocaine is greater than for the negatively charged ketoprofen and ketorolac, supports a suggestion about a significant role of drug inclusion into a micellar core. For better understanding of aminocalix as a solubilizer it would be worthwhile to analyze the terms of aminocalix mechanism of solubilizing action separately. This will be a matter of future studies.

4. Conclusions

The studied cationic aminocalix possesses pronounced solubilizing properties. In general, aminocalix improved the aqueous solubility of lidocaine, paracetamol and ketoprofen more efficiently than the CDs tested. However, maximum solubility enhancement was observed for steroidal drugs. DLS and TEM experiments showed that in the studied concentration range aminocalix exists in aggregates (presumably vesicles) of two different size populations. Correlation analysis of the relationship between the physicochemical properties of the drugs and the solubilizing ability of aminocalix supports the supposition that the mechanism of solubilization is based on formation of aminocalix aggregates rather than by formation of drug/aminocalix inclusion complexes.

Acknowledgments

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