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Monolayers and Langmuir–Blodgett films of amphiphilic tetramethylsulphonatocalix[4] resorcinarene and their interactions with polyethyleneimine and caeruloplasmin

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The paper deals with the surface films of amphiphilic tetramethylsulphonatocalixresorcinarene ($R = C_{11}H_{23}$) **1** insoluble in water but forms rigid stable non-collapsing films at the water–air interface. Compression isotherms were used to investigate the interactions of the macrocycle films with two polymers fed to the aqueous subphases: synthetic – polyethyleneimine (PEI) and natural – an oxidase enzyme caeruloplasmin (CP). The interactions of the surface films of **1** with these substrates are predominantly dictated by the nature of the macromolecules and not by macrocycle interactions with their individual fragments. CP having retained its globular structure was extracted in the layer of **1** and became the dominating component of the film. The synthetic macromolecule of PEI, six times lower in its weight than CP, did not affect the morphology of the interfacial film, involved in the interactions only with its hydrophilic part directed inside the water.

Keywords: tetramethylsulphonatocalix[4]resorcinarene; polyethyleneimine; caeruloplasmin; Langmuir–Blodgett films; monolayers

Introduction

In extensive studies of the complexation properties of the aromatic macrocycles, predominantly represented by calix[n]arenes and resorcin[n]arenes, three main groups with regard to their macrocycle–substrate-binding location can be distinguished in the solution, in the solid state and at the interface. For direct structural studies in the solid state, X-ray is the most accepted method (*1*); in solution the range of available tools varies significantly – from different optical spectroscopy methods (*2*) to potentiometry (*3*) and NMR spectroscopy (*4*). Interactions at the phase boundary are probed by tensiometry (*5*), by chromatography and separation techniques, by immobilisation in the form of SAMs (*6*) and in polymeric matrices (*7*). The phase boundary location is unique due to the pre-orientation of the molecules forming the interfacial film and interacting with the components present in the bulk solution. Interactions with clusters of molecules arranged at the interface are more preferable than the individual ligand–substrate binding (*8*).

Introduction of spatially separated hydrophilic (hydroxy-, bipyridil-, sulphonato-groups etc.) and hydrophobic groups enables orientation of molecules at the interface (Figure 1). For calix[n]arene and calix[n]resorcinarene films deposited at the water–air interface orientation of the upper rim of the cavity towards aqueous phase favours cation–π interactions. They can be detected tensiometrically (*5*), but usually they are not strong enough to affect the morphology of the film. On the other hand, molecules of the interfacial film can participate in at least two other kinds of interactions. First, electrostatic Coulomb interactions in which molecules of the subphase form an ordered structure along the interfacial film and, hence, perturb the surface layer packing. The second are hydrophobic interactions resulting in the extraction/transfer of molecules of the subphase into the interfacial film (*9*).

Polyethyleneimine (PEI, 50 kDa) used in the present study is a highly branched macromolecule; the ratio of its amino-groups is 1:2:1 for primary:secondary:tertiary (Figure 2). Besides the properties mentioned above, it is known as an artificial membrane carrier used for gene delivery due to its ability to form complexes with DNA and to play the role of a proton-sponge in physiological conditions (*10*).

Caeruloplasmin (CP, 132 kDa) is a multi-copper oxidase, containing most of the copper present in the plasma and exhibiting catalytic activity towards the oxidation of iron. As has been recently demonstrated, it comprises a single polypeptide chain of 1046 amino-acid residues forming six domains arranged in a triangular array (Figure 2). Six copper ions located in the depth of the protein folds provide its catalytic activity towards Fe^{2+} oxidation. Three Na^{+} ions coordinated near the base of each

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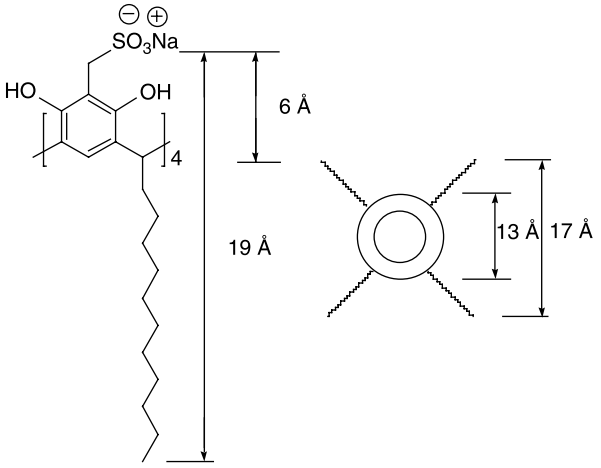

Figure 1. Structure and dimensions of calix[4]resorcinarene **1**.

of the three protuberances formed by glycan moieties (upper part of the triangular pyramid) contribute to the rigidity of the protein structure, whereas the Ca^{2+}-binding site characteristic of domain 1 presumably mediates the conformation of at least one of the glycan moieties and thus is of importance in the surface binding of CP (*11*).

Complexation of tetramethylsulphonatocalix[4]resorcin arenes with shorter aliphatic substituents is well documented (*3*, *12–14*). The common trait shared by these compounds

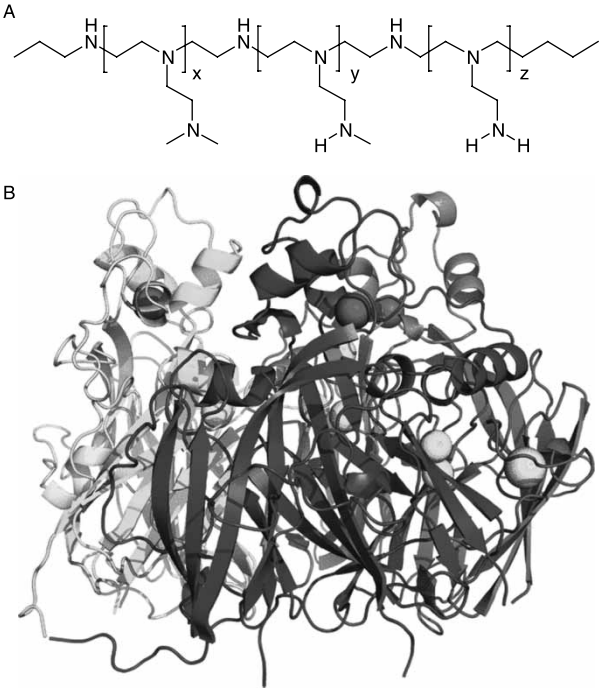

Figure 2. Structures of (A) polyethyleneimine (PEI) and (B) caeruloplasmin (CP) (reproduced from Ref. (*11*) with the permission of IUCr). The overall organisation of the CP molecule, showing the six cupredoxin domains and the locations of the metal-binding sites: Cu^{2+}, Ca^{2+} and Na^{+} ions (*11*).

is their amphiphilic nature (*5*). While in solution, depending on the concentration, two patterns of their complexation behaviour towards large polymeric molecules can be distinguished. Below critical micelle concentration (CMC), interactions of calixresorcinarenes are aimed at individual functional groups of the macromolecules. For example, in aqueous solution the analogue of **1** with amyl lower rim substituents forms inclusion complexes with PEI based on interactions with its amino-groups. These interactions are clearly manifested as up-field shift in respective ^{1}H NMR spectra and in diffusion NMR experiments. At concentrations above CMC, the presence of amphiphilic calixresorcinarenes in the same solution with macromolecules results in the formation of nano-sized hydrophobic domains due to multiple hydrophobic interactions. Recently, we have demonstrated that micromolar concentrations of PEI, La(III), and tetramethylsulphonatocalix[4]resorcinarene present in aqueous solutions catalyse the hydrolysis of *O*-ethyl-*O*-*n*-nitrophelylchoromethylphosphonate. The catalytic effect was attributed to the formation of aggregates, playing the role of nano-reactors and creating the concentration gradient between the aggregate and the bulk of solution (*15*).

Numerous publications cover the binding properties of calix[n]arenes monolayers (*16*). To our knowledge, this is the first study dealing with the binding properties of a calix[4]resorcinarene interfacial film. **1** bearing four methylsulphonato-groups on the upper rim and four undecyl chains on the lower rim demonstrates amphiphilic properties and, unlike its methyl- and pentyl-substituted analogues (*17*), is nearly insoluble in water. Here, we report on Langmuir films of **1** studied both tensiometrically (compression isotherms) and by AFM. The examined films were formed on subphases of pure water, universal buffer solutions (pH range of 1.68–12), and aqueous solutions of PEI and CP. Under these conditions, it is possible to consider the interactions exclusively with the aromatic cavity and the upper rim substituents and to eliminate the effects of the lower rim aliphatic substituents leading to micelle formation.

Materials and methods

Synthesis of tetramethylsulphonatocalix[4]resorcinarene **1**. Initial resorcinarene with four $C_{11}H_{23}$-aliphatic substituents on the lower rim and no substituents on the upper rim was prepared as described elsewhere (*18*). Four methylsulphonato-groups were introduced by the reaction of 5.52 g (5 mmol) of this resorcinarene with 2.05 g (2.5 mmol) of 37% aqueous formaldehyde and 3.15 g (2.5 mmol) of sodium sulphate in 50 ml of water. The reaction mixture was kept at 70°C for 10 h, cooled and neutralised by diluted HCl to pH 6.5. The product was separated by the addition of 50 ml of acetone; the precipitate was centrifuged and dried for 30 h in vacuum at 70°C. The dry precipitate was boiled

in methanol (3 × 50 ml) to remove sodium chloride, filtered and dried. Obtained product (6.8 g, yield 88.6%) was an orange crystalline powder melting above 360°C. Found, %: C 58.28, H 7.24, S 7.90, $C_{76}H_{116}Na_4O_{20}S_4$ (1569.98). Calculated, %: C 58.14, H 7.45, S 8.17. ^{1}H NMR spectra, δ, ppm (DMSO-d_6): H_a 4.21 t, H_b 7.25 s, $CH_2SO_3^-$ 3.85 s, $(CH_2)_{10}$ 1.23 m, CH_3 0.83 t.

PEI of branched structure and the average molecular mass of 50 kDa was purchased from Aldrich as a 50% aqueous solution. CP KF 1.16.3.1 (*19*) was obtained from ImBio (N.Novgorod, Russia) as dry lyophilised samples. Its freshly prepared aqueous solutions were diluted to prepare respective subphases. The quality of solutions was controlled by the intensity of their absorbance spectra; the ratio $A_{610nm}/A_{280nm} = 0.0048$ ensured its ≥89% purity. Arachidic acid (>99%) was purchased from Merck and used without further purification. Buffer solutions were prepared from the mixture of 0.04 M phosphoric, acetic and boric acids. About 100 ml of the acidic mixture were adjusted to the desired pH by adding a certain amount of 0.2 M NaOH (*20*). Buffer solutions were freshly prepared and their pH was controlled with pH metre pH-320 SET (Wissenschaftlich Technische Werkstäten GmbH, Weilheim, Germany).

Isotherm investigations were carried out at 298 K on the automated set-up described in Ref. (*21*). The size of the Teflon® trough was 200 × 137 × 2 mm. Langmuir balance was equipped with IR-sensor providing the precision of ±0.05 mN/m of the surface pressure, Π, measurements. The standard deviation of the effective molecular area determination relative to arachidic acid standard was 2–5%. Chloroform solution of **1** was spread dropwise with a chromatographic syringe on the surface of the respective aqueous subphase. The first 10 μl portion of the chloroform solution of **1** was allowed to equilibrate on the aqueous surface for 10–15 min before the deposition of the next 5 μl. Addition of the following 5 μl was performed 2–3 min after the complete spread of the previous portion to achieve the macrocycle surface concentration of ~0.03 nM/cm^2. Deposition of the chloroform solution of **1** and the solvent evaporation took ~40 min. Compression of the films was performed at the rate of 60 cm^2/min. Spreading time and compression speed were chosen to provide as low hysteresis of the films as possible. Each isotherm was recorded for at least 10 times on freshly prepared solutions of both **1** and a subphase.

AFM experiments were carried out for the films deposited on 1 mm thick glass supports and were prepared either by horizontal transfer of the monolayers according to the Langmuir–Blodgett technique or by the direct deposition of chloroform solutions of **1** on the glass support. The latter were dried in vacuum for 8 h and then exposed to the aqueous solution of the respective subphase for 40 min. The measurements were performed on a scanning probe microscope Solver Bio (NT-MDT Co., Zelenograd, Russia) using a non-contact mode for vacuum-dried films.

Results and discussion

Two experimental techniques applied to the interfacial films of amphiphilic calixresorcinarene **1** were used in the present study: compression isotherm measurements and AFM. The results were interpreted in terms of the macrocycle interactions with the components of the subphase.

Compression isotherms of **1** on water and buffer solutions (pH 1.65–12) are given in Figure 3. The shift of the isotherms recorded with buffer solutions relative to those obtained on pure water subphase indicates a higher stability of the interfacial layer in the presence of a buffer. Corresponding values of A_0 and compression coefficient, β, are given in Table 1.

The isotherms of **1** recorded on PEI subphases are presented in Figure 4. Corresponding effective area of **1** gets larger with the increase of PEI concentration though the correlation is not linear. A double increase of PEI concentration results in the 17.85% growth of A_0, while the four times higher PEI concentration gives a 60% increase of it. The monolayer remains stable on 1–4 μM PEI solutions and does not collapse reaching the plateau at Π of 0 mN/m. Depending on the polymer concentration, aqueous solutions of PEI have a pH value of 8.7–9.7. At pH 10, less than 0.5% of PEI amino-groups are protonated (*22*). pK_a values of the methyl-substituted analogue of **1** reported earlier (*5*, *23*) are 9.0 ± 0.08,

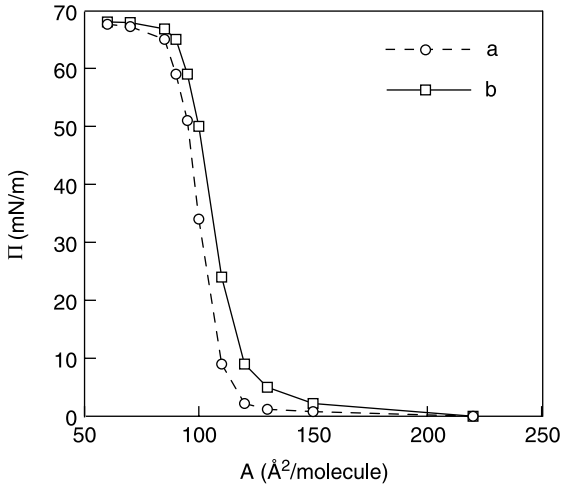

Figure 3. Surface pressure – area isotherms of the layer of **1**: (a) on water and (b) on aqueous buffer solution subphases; the isotherms were identical at the range of pH 1.65–12.

Table 1. Effective area of the macrocycle molecule (A_0), compression coefficient (β) and pH of the subphase for the monolayers of **1** deposited on the interfaces of water, buffer, PEI and CP solutions.

Concentration, μM	A_0, Å^2/mol	β, $\times 10^{-17}$ N/m^3	pH
Water and buffer solution subphases			
0	115 ± 2	2.2 ± 0.1	7[a]
	125 ± 2	1.7 ± 0.1	1.68–12
PEI subphases			
1	140 ± 2	1.5 ± 0.1	8.7
2	185 ± 2	1.1 ± 0.1	9.3
4	200 ± 2	0.9 ± 0.1	9.7
CP subphases%			
0.023	135 ± 2	2.48 ± 0.1	6.0
0.047	140 ± 2	2.40 ± 0.1	6.0
0.095	155 ± 2	1.37 ± 0.1	6.0
0.19	185 ± 2	0.90 ± 0.1	6.0
0.38	205 ± 2	0.80 ± 0.1	6.0
0.76	240 ± 2	0.60 ± 0.1	6.0
1.52	300 ± 2	0.40 ± 0.1	6.0
3.04	350 ± 2	0.29 ± 0.1	6.0

[a] pH is defined by **1** present in solution.

9.3 ± 0.1, 10.8 ± 0.3 and 10.6 ± 0.1, respectively. According to Ref. (*14*), its analogues with longer lower rim substituents have a lower pK_{a1}. For a water-soluble macrocycle with four CH-groups, it is 7.83 ± 0.04 (pK_{a2-4} values are 9.49 ± 0.05, 10.6 ± 0.20, 10.3 ± 0.1 and 4.32 ± 0.08, respectively). This suggests that **1** in the monolayer deposited on PEI-containing subphase undergoes at least partial deprotonation forming penta- or hexaanions.

The greatest change of compression isotherms was observed for the monolayer of **1** on CP subphase. It was clearly pronounced even on submicromolar solutions of CP (Figure 5 and Table 1). The A_0 value grows with the increase of CP concentration. Compression coefficient, β decreased eight times with a 132-fold increase of concentration. This indicates a change of the interfacial layer morphology, from rigid to a loose one. CP adsorption on the surface layer was quantified by molecular adsorption, N^S_{CP}, calculated as: $N^S_{CP} = (1/A_0)_{water} - (1/A_0)_{CP}$, where $(1/A_0)_{water} = 88$ mol/Å and $(1/A_0)_{CP}$ is a surface concentration of **1** on the CP-containing subphase (Figure 6). The change in morphology in the presence of a protein is usually caused by adsorption of its globules at the interfacial films. To avoid formation of mixed monolayers, the following experiments were conducted on 0.023 μM solutions of CP.

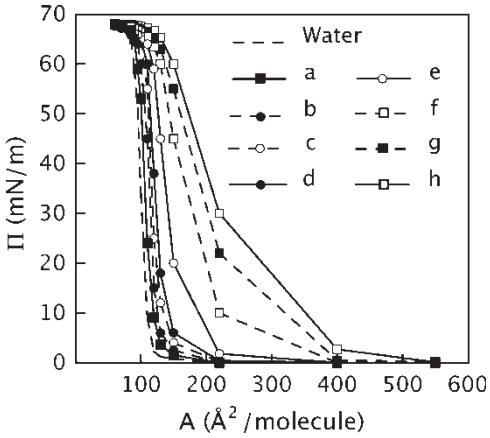

Figure 5. Surface pressure – area isotherms of the layer of **1** on the aqueous subphases containing: (a) 0.023, (b) 0.047, (c) 0.095, (d) 0.19, (e) 0.38, (f) 0.76, (g) 1.52 and (h) 3.04 μM of CP.

Morphology of the films of **1** formed on the three considered subphases was examined by the non-contact mode AFM. It was performed with the films transferred on glass substrates according to the Langmuir–Blodgett technique. They were compared with the films deposited by direct spray and spread of a respective chloroform solution on a glass substrate. Molecules forming interfacial films are capable of a spontaneous

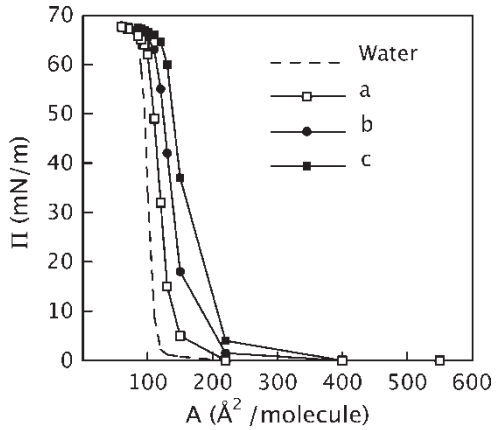

Figure 4. Surface pressure – area isotherms of the layer of **1** on the aqueous subphases containing: (a) 1, (b) 2 and (c) 4 μM PEI.

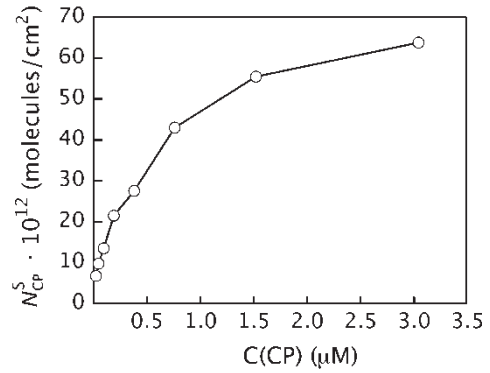

Figure 6. Molecular adsorption of CP, N^S_{CP}, on the film of **1** as a function of CP concentration in the aqueous subphase.

re-orientation in the course of the film preparation due to the tendency to minimise the surface Gibbs energy at the film–air interface. A thin glass support suitable for AFM measurements provides a surface polarity similar to that of the aqueous solutions, making the two conditions of film preparation rather similar. The AFM images of the films obtained by different methods were very much alike (Figure 7). Importantly, the height of the films does not correspond to the real thickness of the macrocycle monolayer. A fraction of the aqueous subphase is inevitably carried to the glass support during the film transfer. The quantity of water dragged over upon the film transfer strongly depends on the hydration of the interfacial layer.

The films obtained on pure water and PEI subphases had clearly visible craters, while the one obtained on CP solution was smooth and pit free. The thickness of the films was the greatest for PEI subphase (203.94 nm) and the smallest for the one containing CP (8.38 nm), whereas the film obtained on pure water had a crater depth of 131.84 nm. Scanning of the macrocycle film from CP subphase in the non-contact mode amplified the signal. It appeared as white spots on AFM images (Figure 7(C)). In the case of proteins, this is typically attributed to formation of flattened globular spheres on the examined surface.

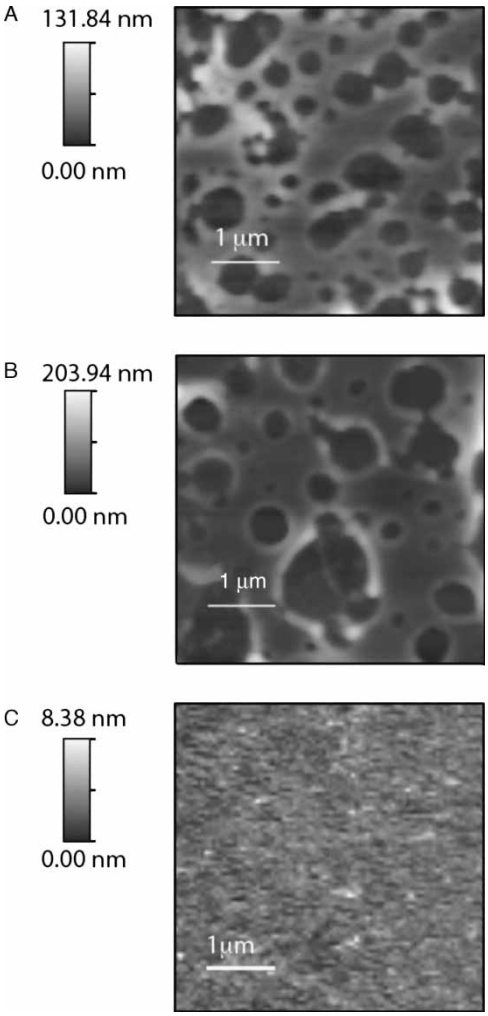

Figure 7. AFM images of the thin films of **1** transferred on glass support from (A) water and aqueous subphases containing (B) PEI (1 μM) and (C) CP (0.095 μM).

Similar to amphiphilic calix[n]arenes, **1** due to its four undecyl substituents forms rigid films at the water–air interface. Its hydrophilic part is oriented towards the aqueous subphase and the hydrophobic one towards the air. The former is responsible for the orientation of the individual molecules at the interface and the latter provides an overall film stability owing to the multiple hydrophobic interactions of undecyl chains, similar to those in lipid bilayers.

As it was demonstrated in Ref. (*9*), variation of the effective molecular area, A_0, obtained from compression isotherms of the films is largely affected by the conformational behaviour of the macrocycle. It depends on the size and the nature of the lower rim substituents and on the macrocycle interactions with the components of the subphase. The latter often interfere with the intermolecular interactions of the molecules forming the film, i.e. electrostatic attraction/repulsion, hydrogen bonding and ion–dipole interactions ($-SO_3^- \cdots HO-$) as well as deprotonation of the upper rim OH-groups upon the variation of pH of the aqueous subphase.

As its less lipophilic analogues, in contact with the aqueous media, **1** exists in the form of a tetraanion adopting cone conformation of C_{4V} symmetry stabilised by the intermolecular hydrogen bonding of the OH-groups of the neighbour resorcinol rings. Its surface area of 114 $Å^2$ on pure water is similar to that of tetraalkylphenylazocalix[4]arene (122–126 $Å^2$) (*9*). 10 $Å^2$ increase of the mean molecular area is observed for buffer solutions in the pH range of 1.68–12 (Figure 2 and Table 1). Identical compression isotherms observed for the whole pH range correspond to buffer/electrolyte effects.

Depending on the polymer concentration, PEI subphase has a pH of 8.7–9.7 (Table 1). The observed increase of the mean molecular area of **1** is not linear. It tends to reach a plateau with the increase of the polymer concentration. As mentioned above, the variation of the subphase pH such as is insufficient for the observed change of A_0. On the other hand, AFM images of the films of **1** formed on water and PEI subphases are quite alike (Figure 7). In spite of the change in A_0, the presence of PEI in the subphase does not disturb the packing of the macrocycles' aliphatic substituents directed towards the air. The thickness of the film transferred from PEI subphase is about 1.5 times greater than that in the case of pure water. Such a change is too big to be ascribed only to a different amount of water carried with the film on the glass support. PEI, as a branched polymer molecule,

is most likely to be oriented along the hydrophilic part of the interfacial film of **1** facing water. The interactions are strong enough to affect the thickness of the interfacial film. They neither cause a loosening of the film nor affect the packing of its lipophilic part exposed to air. It is worth mentioning that, in solution, in the presence of amyl-substituted analogue of **1**, PEI forms inclusion complexes that appear as a strong up-field shift of amino-group protons in ^{1}H NMR (*15*). Nevertheless, due to the difference in the experimental conditions, a strict interpolation of these results to the present data obtained with PEI is restricted.

Drastic changes of compression isotherms and AFM images were observed for films of **1** on CP subphases. The mean molecular area increased up to 420 Å^2 (more than thrice) in the presence of CP in the subphase (3.04 μM). The respective AFM images showed the formation of a smooth crater-free interface indicating a dramatic change in the film morphology. Its mean height decreased to 8.38 nm. This is, however, not an unambiguous quantitative characteristic of the film thickness since, as mentioned above, in the course of film transfer a fraction of the aqueous subphase is inevitably carried to the glass substrate. Formation of the flat globular structures observed on AFM images clearly point to protein intercalation into interfacial films. Loosening of the film character is in a good agreement with the decrease of β-coefficient: for the film of **1** on 3.04 μM CP solution it is eight times lower than that on water (Table 1). Finally, for the subphase of 0.095 μM CP, protein concentration in the interfacial layer was 6.15×10^{15} mol/cm^2 (Figure 7(B)), while the concentration of **1** in all of the reported experiments was 1.8×10^{13} mol/cm^2. Apparently, this excess of CP by 100 times in the film is responsible for the observed change in its morphology. It is the protein, and not the macrocycle, that defines the film structure and compression coefficient. Increase of the mean molecular area, A_0, for these films also correlates with the high number of CP molecules present in the film. The extreme stability of the obtained **1** and CP mixed films suggests that **1** plays the role of 'glue', stabilising the films that predominantly comprise CP, which by itself does not form any distinct interfacial films. CP transfer into the films of calixresorcinarene can be considered as an extraction driven and stabilised by multipoint weak interactions of CP globules with amphiphilic macrocycle **1** providing a lipophilic environment of the interfacial layer (Table 2).

Table 2. Surface concentration (number of mol/cm^2 and number of mol/bath) of CP in the surface films of **1**, in the aqueous subphases, and the fraction of CP intercalated into the interfacial film of **1**.

C, μM	$N^S_{CP} \times 10^{12}$ mol/cm^2	$N^S_{CP} \times 10^{15}$ mol/bath	$N^{total}_{CP} \times 10^{15}$ mol/bath	N^S_{CP}/N^{total}_{CP}
0.023	12.88	3.53	7.59	46.50
0.047	15.53	4.25	15.5	27.43
0.095	22.44	6.15	31.4	19.61
0.19	32.90	9.02	62.7	14.38
0.38	38.18	10.5	125	8.34
0.76	45.29	12.4	251	4.95
1.52	53.62	14.7	502	2.93
3.04	58.39	16.0	1000	1.59

Summary

Amphiphilic water-insoluble tetrasulphonatomethyl-calix[4]resorcinarene **1** was synthesised and studied tensiometrically. Due to the presence of four sulphonato-methyl-groups at the upper rim, it is able to form stable non-collapsing films at the water–air interface. Compression isotherms of the films deposited from chloroform solutions were recorded for the aqueous subphases of different composition. Namely, changes of compression isotherms were considered upon variation of the subphase buffer pH and in the presence of macromolecules, PEI and CP.

It was found that the compression isotherm of the film of **1** on the aqueous buffer subphase remains stable and identical across the pH range of 1.68–12. Unlike the earlier reported interfacial films formed by neutral calixarenes, packing of the film comprising tetraanionic species of **1** is unaffected by deprotonation of the macrocycle OH-groups. The slight difference between compression isotherms recorded on buffer and water subphases is, presumably, caused by electrolyte/ionic strength effects.

Interaction of **1** with PEI and CP follows different scenarios. CP retaining its globular structure even in diluted solutions is extracted from water into the lipophilic film of calixresorcinarene, which changes its morphology. The change is clearly seen in AFM images and in the series of the obtained compression isotherms. Highly branched PEI, as seen from respective compression isotherms, undoubtedly interacts with the film of **1**. The high degree of its hydration and the absence of compact macromolecule folding do not permit its extraction into the interfacial film of **1** as in the case of CP.

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