Mass Spectrometric Determination of Association Constants of Bovine Serum Albumin (BSA) with *para*-Sulphonato-Calix[*n*]arene Derivatives

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Abstract

Electrospray Ionization Mass Spectrometry (ESI/MS) has been used to determine the association constants (K_{As}) and binding stoichiometries for parent *para*-Sulphonato-calix[*n*]arenes and their derivatives with bovine serum albumin (BSA). K_A values were determined by titration experiments using a constant concentration of protein. K_A measurements were carried out in a methanol–formic acid solution. 5,11,17,23–tetra-Sulphonato-calix[4]arene (**1a**) and 25-mono-(2-aminoethoxy)-5,11,17,23-tetra-Sulphonato-calix[4]arene (**1d**) interact strongly with BSA showing 3 non-equivalent binding sites with $K_{A1} = 7.69 \times 10^5$ M^{-1}, $K_{A2} = 3.85 \times 10^5$ M^{-1}, $K_{A3} = 0.33 \times 10^5$ M^{-1} and $K_{A1} = 1.69 \times 10^5$ M^{-1}, $K_{A2} = 2.94 \times 10^5$ M^{-1}, $K_{A3} = 0.60 \times 10^5$ M^{-1}, respectively. The strength of the interactions between the calixarene and BSA is inversely proportional to the size of macrocyclic ring: $n = 4 > n = 6 >> n = 8$.

Introduction

The biological activity of calix[*n*]arene derivatives was first reported by Cornforth in 1957 [1], however, the total insolubility of the simple calix[*n*]arene derivatives in aqueous solution effectively blocked research in the field. With the appearance of the *para*-Sulphonato-calix[*n*]arenes in 1984 [2], highly water soluble molecules became readily available and Atwood, in 1996, demonstrated the activity of various *para*-Sulphonato-calix[*n*]arene derivatives as calcium-dependent chloride ion channel blockers [3]. Subsequently the biological activity of the *para*-Sulphonato-calix[*n*]arenes has been extended to include anti-thrombotic behaviour, antiviral activity, inhibition of Lysyl Oxidase activity [4]. Recently, the interaction between the *para*-Sulphonato-calix[*n*]arenes and the prion protein has been shown to amplify the detection limits of this protein in Western Blot immunodetection [5]. Other water soluble calix[*n*]arenes have also been demonstrated to possess biological activity, for example protein surface receptors based on calix[4]arene with peptide loops attached [6]. Carboxylic acid calix[*n*]arene derivatives have been used to solubilize, with retention of catalytic activity, the cationic protein Cytochrome *c* in chloroform [7].

Considerable research has been undertaken on the complexing of amino-acids and small cationic peptides by the *para*-Sulphonato-calix[*n*]arenes, by NMR titration, micro-calorimetry and RP-HPLC. As expected complexing is generally strongest with the basic amino-acids Lysine (Lys) and Arginine (Arg) with association constants in the range of 200–3000 M^{-1} [8]. For di- and tri-peptides of Lys and Arg much stronger binding has been reported with *para*-Sulphonato-calix[6]arene [9].

The serum albumins are the most abundant circulatory proteins, with serum concentrations of up to 40 g/l. They are globular proteins, of around 67 kDa mass, and have an ovoid shape of $4 \times 4 \times 14$ nm. In solution, the serum albumins are present as homo-dimers [10]. The biological functions of the serum albumins include fatty acid transport, with three known binding sites at Arg 116, Lys 350, Lys 474, cation and anion transport, as well as acting as transporters for certain included drugs. The protein is covered by patches of both hydrophilic and hydrophobic zones allowing it to strongly adhere to surfaces where it then serves as an anchoring layer for the adhesion of other proteins.

The use of mass spectrometry for the study of the complexing of small molecules with the serum albumins has been demonstrated [11]. We recently reported the use of Electrospray Mass Spectrometry for the observation of the formation of complexes between the *para*-Sulphonato-calix[*n*]arenes and bovine serum albumin (BSA) [12], in which we demonstrated that while non-specific binding of up to 50 molecules of *para*-Sulphonato-calix[4]arene with BSA occurs in solution, under ES/MS conditions specific binding occurs. Both, the high molecular mass of BSA, 67 kDa, and the very low

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solubility of certain of the resulting complexes rule out the study of serum albumin-calix[*n*]arene complexes by other methods. Given the high concentration of the serum albumins in physiological fluids, for future either *in vivo* or for diagnostic applications in physiological fluids use of the *para*-Sulphonato-calix[*n*]arenes, it is of considerable importance to obtain detailed knowledge of this complexing. The complexing of the calix[*n*]arenes by serum albumins may allow them to circulate freely and with suitable association constants, the complexes may act as a reservoir for the controlled release of bio-active calix[*n*]arene derivatives. However, very high association constants could effectively lead to the removal of the calix[*n*]arenes and inhibit their activity.

Soft Ionisation mass spectrometric methods including MALDI and Electropsray Ionisation (ESI) are particularly well suited to the study of non-covalent complexes of biomolecules [13] and macrocycle molecule [14]. Three different strategies exist for the determination of association constants by ESI [15]; first, the use of 'melting curves' involving raising the temperature of the analyze solution and determination of the percentage of intact complex present, for example the analysis of complementary DNA strands [16]. In the second approach competition experiments, third, direct titration of a biomolecule with a ligand, or vice versa may be used [17]. It has been clearly demonstrated by a wide body of work that the association constants obtained by the third method are an accurate reflection of those observed in solution. However, care must be taken with regard to two problems: first that the observed complexes do not result from gas-phase cluster formation or non-specific solution interactions and second that the ionization efficiency of the biomolecule and complex remain constant through the whole concentration range of the titration experiment. Ensuring that integration of the total peak intensity remains constant over the range studied best solves this latter problem.

In this paper we report on the use of ESI titration experiments to study the complexing of a series of 12 *para*-Sulphonato-calix[*n*]arene derivatives, the parent macrocycles where *n* = 4, 6 or 8 and their 2-carboxy methoxy, 2-amido methoxy and 2-amino ethoxy derivatives with BSA. For the *para*-Sulphonato-calix[4]arene derivatives three binding sites are observed, while for the *para*-Sulphonato-calix[6]arene and *para*-Sulphonato-calix[8]arene derivatives only one or two binding sites are present. The association constants observed are in the range from $7.46 \times 10^3 \ M^{-1}$ to $10^6 \ M^{-1}$. For three calix[*n*]arene derivatives, K_{A1} is smaller than K_{A2}.

Experimental

Sample preparation

BSA (fatty acid free) was purchased from Sigma and used without further purification. The parent *para*-Sulphonato-calix[*n*]arenes and their derivatives were synthesized according to the literature method [18, 19] and were purified by RP-HPLC prior to use. The mass spectra of solutions containing the *para*-Sulphonato-calix[*n*]arene derivatives and BSA were carried out in CH_3OH-H_2O mixture (50:50; v/v) with 0.1% of formic acid. In the titration experiments, the final concentration of the BSA was retained constant at 5 pmol/μl. To 10 μl of the BSA solution was added increasing volumes of the relevant calix[*n*]arene derivative solution from 0 to 24 pmol/μl, the final volume was adjusted to 0.1 ml. A mass spectrum of each sample was recorded. A minimum of 15 points was required to obtain the best fit for calculating the dissociation constant value.

ESI/MS mass spectra

All experiments were performed using a Sciex API 165 quadrupole mass spectrometer, associated with an Electrospray Ionisation (ESI) source operating in the positive ion mode. Compounds were introduced by direct infusion of solutions at 5 μl/min flow rate in a CH_3OH/H_2O (50/50, v/v) mixture containing 0.1% of HCOOH. Mass spectra were acquired at 35 V orifice voltage value. The scan range was set at m/z 900–2300. Multi-charged ion spectra were deconvolved in the 64- to 74-kDa mass range with a step-size of 1 Da and 20 iterations were summed to improve the signal to noise ratio. Deconvolution of the raw mass spectra allows observation and subsequent integration of peaks arising from BSA at 66.5 kDa and peaks arising from the complexes. The area obtained was calculated using the digitising software UN-SCAN-IT (Silk Scientific. Inc) and is assumed to represent the quantity of associated calix[*n*]arene/protein species formed during the titration experiment. It is assumed that the ionization response factors of BSA and the BSA–calix[*n*]arene complexes are the same.

Calculation of K_A

For the calculation of the K_A values are assumed that there are *n* binding sites that are not necessarily equivalent, the association constants are derived as $K_A = 1/K_D$, with K_D = dissociation constant :

$$K_{D1} = [P][C]/[PC] \qquad [1]$$

$$K_{D2} = [PC][C]/[PC_2] \qquad [2]$$

$$K_{Dn} = [PC_{n-1}][C]/[PC_n] \qquad [3]$$

where P is the free BSA, C is the free calix[*n*]arene, PC is the 1:1 Protein–Calix[*n*]arene complex, PC_2 is the 1:2 Protein–Calix[*n*]arene complex and the equilibrium constants can also describe *n* non-equivalent binding sites:

$$K_1 = [P][C]/[PC] \qquad [4]$$

$$K_2 = [P][C]^2/[PC_2] \qquad [5]$$

where $K_1 = K_{D1}$ and $K_2 = K_{D1} \times K_{D2}$. Following the derivation in van Holde's Physical Biochemistry [19], the relationship below is derived :

$$([P]+[PC]+[PC_2]+\cdots[PC_n])/[P]$$
$$=[C]^n/(K_{D1}K_{D2}...K_{Dn})+\cdots[C]^2/(K_{D1}K_{D2})+[C]/K_{D1}+1$$
$$[6]$$

The intercept will be 1 for a calix[n]arene concentration of 0. A plot of measured $([P] + [PC] + [PC2] + \cdots [PCn]) / [P]$ versus added $[C]$ has been fit to a n order polynomial function [20].

For calculating the association constant, it was assumed that the total signal response for each individual species was proportional to the concentration of that species in the gas phase and in solution.

Results and discussion

Among the amino-acid sequence of BSA, a total of 99 basic amino-acids (59 Lys, 23 Arg and 17 His), that might non-specifically bind to the *para*-Sulphonato-calix[n]arenes by electrostatic interactions are present. The *para*-Sulphonato-calix[n]arene derivatives used in this study are given in Figure 1. We have previously shown by ^{1}H-NMR titrations that they all bind strongly (K_{ass} 1000–2000 M^{-1}) to Lys and Arg, slightly less strongly to His (K_{ass} 200–500 M^{-1} for **1a–d**, K_{ass} 500–2000 M^{-1} for **2a–d** and K_{ass} 700–2800 M^{-1} for **3a–d**), and that the carboxylic acid derivatives **1b**, **2b** and **3b** also bind strongly to Asp [17]. Observed association constants with regard to other aminoacids are generally much lower.

In Figure 2 are given the raw mass spectra for (a) BSA alone and (b) BSA in presence of **1b** at a 1:3 ratio. Under the positive mode ionization constants used ions with charge ranging from 30^+ to 68^+ are observed for BSA alone, an expansion for the range 42^+ to 47^+ is given in 2a$_1$. In the presence of **1b** ions in the range 30^+-68^+ are observed, in Figure 2b$_1$ an expanded view of the range 45^+-47^+ is given. Species corresponding to both

the 1:1 BSA−**1b** and the 1:2 BSA-**1b** complexes are clearly seen.

Deconvolution of the raw mass spectra, Figure 3a, b, allows observation and subsequent integration of peaks arising from BSA at 66.5 kDa, the 1:1 complex at 67.3 kDa, the 1:2 complex at 68.1 kDa and the1:3 complex at 68.9 kDa.

After integration of the total peak intensities the values were summed over the total range of *para*-Sulphonato-calix[n]arene derivative concentrations used in the titration experiments. A small decrease in the sum of intensities from 1 for BSA alone to 0.95 was observed for all the derivatives even at the lowest *para*-Sulphonato-calix[n]arene concentration. However, the sum then remains constant at 0.95 throughout the range of concentrations. This small decrease in intensity is associated with the disappearance of a small shoulder at 66.5 kDa in the BSA peak. Given this, we consider that the condition of an equal ionization probability between BSA and the BSA:*para*-Sulphonato-calix[n]arene complexes is satisfied.

A typical peak intensity titration curve (BSA-**1b**) is given in Figure 4. The intensity of not complexed BSA diminishes rapidly and effectively disappears into the experimental background noise at BSA:**1b** ratio above 1:5. The peak for the 1:1 complex increases up to a ratio of 1:3 BSA:**1b**, is almost constant up to a ratio of 1:5. For comparison of the binding as a factor of macrocycle size for **1a**, **2a** and **3a** are given in Figure 5.

The effective association constants were determined using following equations: $K_A = 1/K_D$ and equation (6):

$$([P] + [PC] + [PC_2] + \cdots [PCn])/[P]$$
$$= [C]^n/(K_{D1}K_{D2}K_{Dn}) + \cdots [C]^2/(K_{D1}K_{D2})$$
$$+ [C]/K_{D1} + 1$$

where the polynomial order was taken from the number of complexes, and hence the number of binding sites observed, Figure 6. The observed association constants, number of binding sites, the polynomial order and correlation constant are given in Table 1. The maximum number of binding sites is three, for **1a** and **1d**, two for **1b**, **1c**, **2c** and **2d** and one for **2a**, **2b** and **3a–d**.
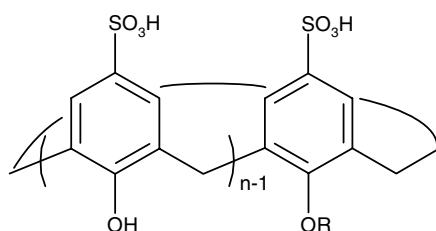
1a-d (n=4), **2a-d** (n=6), **3a-d** (n=8)
R= -H (a); -OCH$_2$COOH (b); -OCH$_2$CONH$_2$ (c); -OCH$_2$CH$_2$NH$_2$ (d)

Figure 1. Molecular structure of *para*-Sulphonato-calix[n]arene derivatives.
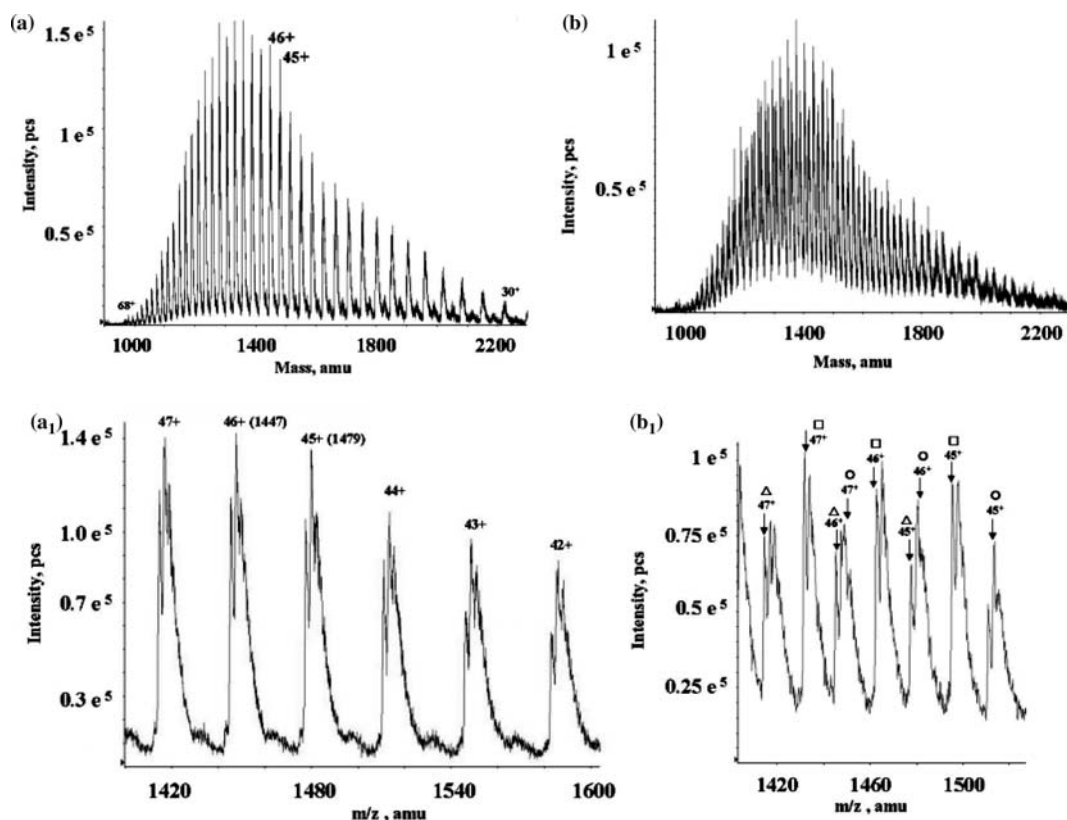
Figure 2. View of mass spectra of BSA alone (a) and the complexing of BSA with **1b** (b) at molar ratio 1:3 BSA/Calixarene before deconvolution. View of mass spectra of BSA alone (a₁) in the range from 42^+ to 47^+ and the complexing of BSA with 1b (b₁) in the range from 45^+ to 47^+ at molar ratio 1:3 BSA/Calixarene after deconvolution. At this molar ratio, BSA is present as the uncomplexed form (Δ) and the complexed forms with one molecule of 1b (□) and with two molecules of **1b** (○).

It is thus clear that binding of the *para*-Sulphonato-calix[*n*]arenes to BSA occurs via specific interactions at particular binding sites and not through non-specific electrostatic interactions with the 99 basic amino-acids present on the exterior of BSA.

From Figure 5, it is apparent that the total binding of the *para*-Sulphonato-calix[*n*]arenes to BSA decreases with increasing size of the macrocyclic ring, $4 > 6 \gg 8$. This is reflected in the association constants K_{A1} 7.69×10^5 M^{-1}, K_{A2} 3.85×10^5 M^{-1} and K_{A3}
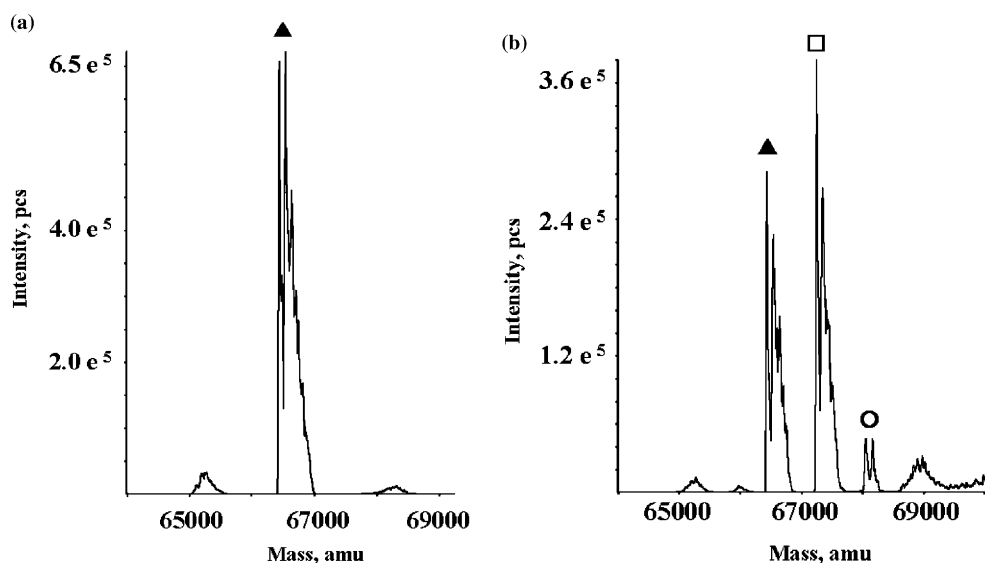

Figure 3. View of mass spectra of BSA alone (a) and the complexing of BSA with **1b** (b) at molar ratio 1:3 BSA/Calixarene after deconvolution. At this molar ratio, BSA is present as the uncomplexed form (▲) and the complexed forms with one molecule of **1b** (□) and with two molecules of 1b (○).
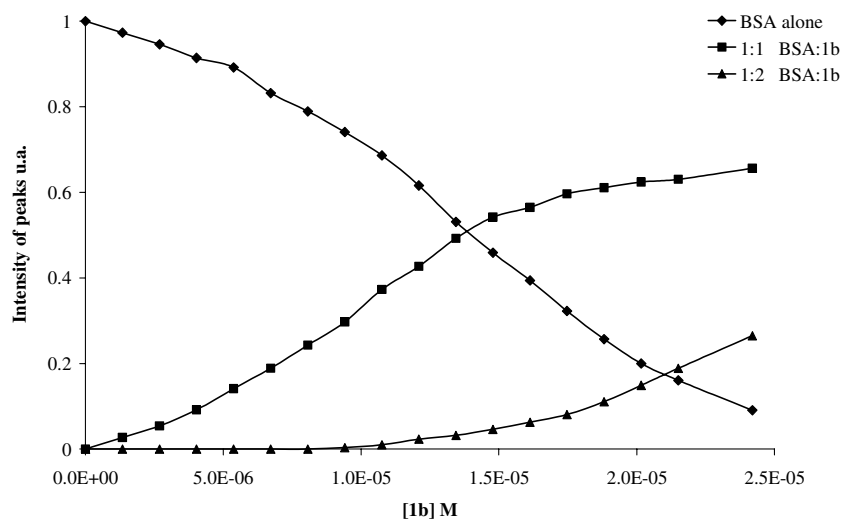
Figure 4. Plot of intensity of peaks of BSA versus the increasing concentration of **1b.**
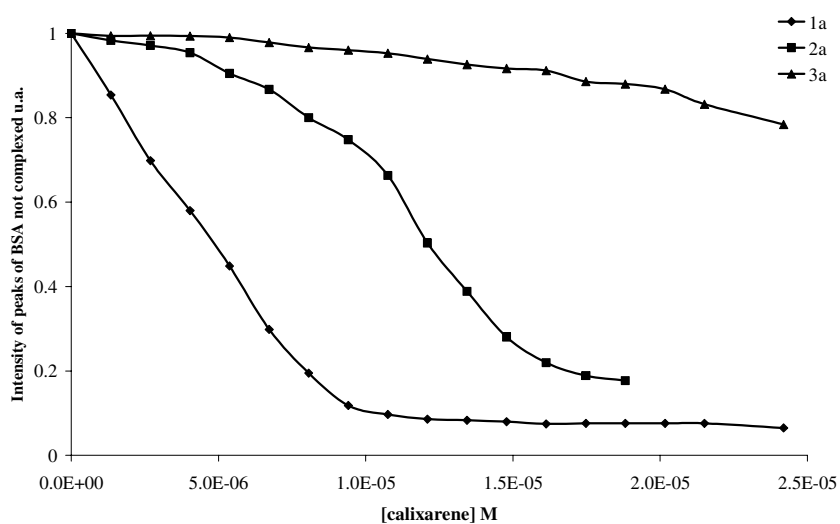
Figure 5. Comparison of uncomplexed BSA in presence of increasing concentration of **1a**, **2a** and **3a.**

$$y = 3E+15x^3 + 5E+10x^2 - 169352x + 1$$
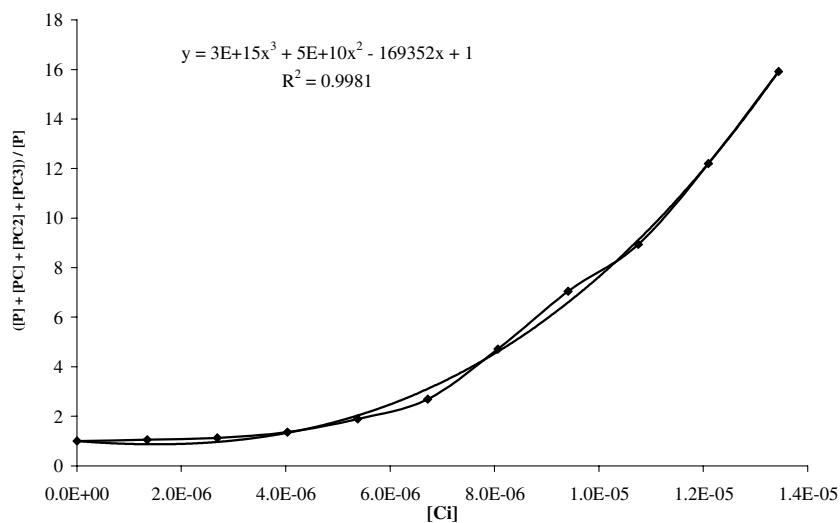$$R^2 = 0.9981$$

Figure 6. Representation of a trinomial fit of complexing of BSA with **1d**. $K_{Ds} = 1/K_{As}$ are determinated with a correlation coefficient of 0.998.

Table 1. Association constants values derived from ES/MS data for the binding of parent *para*-Sulphonato-calix[*n*]arenes and its derivatives to BSA

	K_{A1} ($\times 10^5$ M^{-1})	K_{A2} ($\times 10^5$ M^{-1})	K_{A3} ($\times 10^5$ M^{-1})	R^2
1a	7.69	3.85	0.33	0.987
1b	1.15	0.87	–	0.973
1c	1.72	2.33	–	0.998
1d	1.69	2.94	0.60	0.998
2a	0.40	–	–	0.965
2b	0.29	–	–	0.972
2c	0.48	2.04	–	0.994
2d	2.08	0.48	–	0.940
3a	0.07	–	–	0.945
3b	0.26	–	–	0.985
3c	0.14	–	–	0.970
3d	0.19	–	–	0.980

0.33×10^5 M^{-1} for **1a**, K_{A1} 0.40×10^5 M^{-1} for **2a** and K_{A1} 0.07×10^5 M^{-1} for **3a**. With regard to the *para*-Sulphonato-calix[4]arene derivatives, both the parent molecule **1a** and the 2-amino ethoxy derivative **1d** bind to three sites on BSA. The first two interactions are strong; for **1a** $K_{A1} = 7.69 \times 10^5$ M^{-1} and $K_{A2} = 3.85 \times 10^5$ M^{-1}, for **1d** $K_{A1} = 1.69 \times 10^5$ M^{-1} and $K_{A2} = 2.94 \times 10^5$ M^{-1}. The third interaction is much weaker; $K_{A3} = 0.33 \times 10^5$ M^{-1} for **1a** and $K_{A3} = 0.60 \times 10^5$ M^{-1} for **1d**. The 2-carboxy methoxy, derivative **1d** and the 2-amido methoxy **1c** binding to only two sites, here: $K_{A1} = 1.15 \times 10^5$ M^{-1} and $K_{A2} = 0.87 \times 10^5$ M^{-1} for **1b**, $K_{A1} = 1.72 \times 10^5$ M^{-1} and $K_{A2} = 2.33 \times 10^5$ M^{-1} for **1c**. While for **1a** and **1b** the second binding constant is lower than the first, for **1c** and **1d** the second binding interaction is stronger than the first.

From the titration curves, see for example Figures 4 and 5, the different binding events are sequential in nature. First, binding of one calix[*n*]arene molecule occurs, when there is almost saturation of the binding of the first molecule, interaction of the second occurs. This suggests that for **1c** and **1d**, either cooperative binding at the same site occurs or that a second stronger binding site is made available by binding at the first site.

For **1b**, **1c** and **1d**, the K_{A1} values are essentially the same; this suggests that binding probably occurs at the same site. Also, K_{A2} values are similar for all four Sulphonato-calix[4]arene derivatives, suggesting that binding again occurs at the same site.

With respect to the *para*-Sulphonato-calix[6]arene derivatives **2a** and **2b** only bind to one site with K_{A1} values of 0.40×10^5 M^{-1} and 0.29×10^5 M^{-1}, respectively. Now, **2c** and **2d** bind to two sites: for **2c** $K_{A1} = 0.48 \times 10^5$ M^{-1} and $K_{A2} = 2.04 \times 10^5$ M^{-1}, while for *2d* $K_{A1} = 2.08 \times 10^5$ M^{-1} and $K_{A2} = 0.48 \times 10^5$ M^{-1}. Again, from the titration curves, the binding process is sequential.

The K_{A1} values for **2a**, **2b** and **2c** are all very close, while the K_{A2} value of **2d** is essentially the same and

closely similar to the K_{A3} values and close to K_{A1} or K_{A2} values observed for **1a** and **1d**. Similarly, the K_{A2} value of **2c** and the K_{A1} value of **2d** are essentially the same. This would appear to imply that the *para*-Sulphonato-calix[6]arene derivatives bind to the third site of the para-Sulphonato-calix[4]arene derivatives. For **2c** and 2d, either the first or second binding site of the *para*-Sulphonato-calix[4]arene derivatives is available.

For the *para*-Sulphonato-calix[8]arene derivatives, much weaker binding occurs at only one site with K_{A1} values of respectively 0.07×10^5 M^{-1}, 0.26×10^5 M^{-1}, 0.14×10^5 M^{-1} and 0.19×10^5 M^{-1} for **3a**, **3b**, **3c** and **3d**.

It has been established that BSA has three sites for anionic molecules and in particular for long chain fatty acids, salicylate, and some sulfonamides [23]. Binding of these anions involves, as expected, Arginine or Lysine residues with Lys-474 being implicated in the primary binding site, Lys-350 in the secondary site and Lys-116 in the weakest site. The three binding sites of BSA are present in domains I, II and III in order of increasing affinity [24]. The results obtained for the *para*-Sulphonato-calix[*n*]arenes in this study are hence in agreement with previous biochemical studies of anion binding to BSA [23].

Conclusion

In conclusion, we have demonstrated the use of ESI mass spectrometry for the study of the binding of *para*-Sulphonato-calix[*n*]arene derivatives to the BSA. The observed association constants are in the range 10^4–10^6 M^{-1}. As has been previously noted for other organic anions, three binding sites may be present. The strength of binding varies inversely with the size of the *para*-Sulphonato-calix[*n*]arenes.

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