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Haemolytic properties of some water-soluble *para*-sulphonato-calix-[*n*]-arenes

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Abstract

In this paper, we describe the haemolytic effect of parent *para*-sulphonato-calix-[*n*]-arenes and their derivatives bearing one pendant group at the lower rim of calix-arene towards human erythrocytes. A maximum of 30% of haemolysis has been observed for *para*-sulphonato-calix-[8]-arene for a concentration of 200 mM representing 300 g of calix-arene per liter of human blood, *para*-sulphonato-calix-[4]-arene and *para*-sulphonato-calix-[6]-arene show much lower haemolytic effects, 0.5 and 8%, respectively at 200 mM concentration. Coupling of a methoxy-carboxylate function at the phenolic group reduces haemolytic effects in all cases. The presence of an ethoxy-amine function increases the haemolytic behaviour for the calix-[4]-arene and calix-[6]-arene systems, but reduces the effect for the calix-[8]-arene derivatives.
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1. Introduction

The possible use of water-soluble supramolecular complexes as transport systems for bio-active compounds or as biologically active molecules has provoked strong interest for a number of years, in particular with respect to the cyclodextrins (Szetjli, 1988; Huekama, 2002). In the case of the calix-[*n*]-arenes, although their use in biological and medical applications was held back by a lack of readily available water-soluble derivatives with the emergence of the highly water-soluble *para*-sulphonato-calix-[*n*]-arenes, it has became evident that the calix-[*n*]-arenes show a wide range of interesting biological activities. Interestingly the initial reports on the biological activity of the calix-arenes date back to 1955 when Cornforth demonstrated the anti-tubercular properties of certain compounds (Cornforth et al., 1955). Of the available water-soluble calix-arenes derivatives, the *para*-sulphonato-calix-[*n*]-arenes are the most widely studied for their pharmacological properties. The chloride ion channel blocking properties of the *para*-sulphonato-calix-[*n*]-arenes were patented by Atwood et al. (1996), subsequently patents on the anti-thrombotic (Hwang et al., 1995) and anti-viral properties of various calix-arenes were obtained by Harris (1995), and on the inhibition of the activity of the enzyme, lysyl-oxydase by Aubert-Foucher et al. (1998). Reinhoudt has reported the lack of immunogenic properties of calix-[4]-arene coupled to BSA (Gansey et al., 1999). The use of peptido-calix-[4]-

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arenes, in which four peptide loops are arrayed around a central calix-arene core, as receptors for protein surface recognition has been reported by Hamilton (Lin et al., 1998; Park et al., 1999). Recently, the formation of ion channel both in vitro and in vivo by the calix-[4]-arene tetrabutylamide has been demonstrated by Sidorov et al. (2002). Both Ungaro and ourselves have published a number of studies on the complexation of amino acids by the *para*-sulphonato-calix-[*n*]-arenes (Douteau-Guével et al., 1998, 1999; Arena et al., 1999; Sansone et al., 1999; Kalchenko et al., 2001, 2002; Da Silva and Coleman, 2003).

Apart from the work of Reinhoudt, all these studies have been carried out in vitro on the isolated biological molecules. In order to proceed to full bio-medical applications, it is necessary to obtain information on the interactions, first at the cellular level and then in vivo, between molecules and biological entities. For medical applications, the toxicity of molecules is evidently a key factor, and the measure of the haemolytic properties represents a useful starting point. Indeed, many of the medical applications of the cyclodextrins have been held up by the haemolytic activity of β-cyclodextrin and many of its derivatives, which effectively precludes their use as injectable drug-carrier systems (Leray et al., 1995; Bost et al., 1997; Nagase et al., 2002; Memisoglu et al., 2003).

In this paper, we wish to report on the haemolytic properties of a series of *para*-sulphonato-calix-[*n*]-arene derivatives. In a previous study, we have shown that the complexation of the *para*-sulphonato-calix-[*n*]-arenes by bovine serum albumin is strongly dependant on the size of the macrocycle. Complexes of 1:1, 1:2 and 1:3 BSA/calix-arene stoichiometry were observed in the case of *para*-sulphonato-calix-[4]-arene. For *para*-sulphonato-calix-[6]-arene 1:1 and 1:2 complexes are observed while with *para*-sulphonato-calix-[8]-arene only a 1:1 complex is observed. With regard to amino acid complexation in general the association constant increases with increasing size of the macrocycle. However, we have observed that both the strength of complexation and the selectivity of complexation are strongly modified by the presence of pendant group (2-carboxy methoxy group, 2-amido methoxy group and 2-amino ethoxy group) functions at the lower rim of the *para*-sulphonato-calix-[*n*]-arenes. In particular both the carboxylic acid and amine functions induce strong complexation of aspartic acid. As a consequence, it is necessary to study, both, the effects of macrocycle size and the effects due to the presence of pendant lower rim groups on the haemolytic properties of the *para*-sulphonato-calix-[*n*]-arenes.

2. Experimental

All calix-[*n*]-arene derivatives were synthesised according to the literature methods, and their physical properties are in full agreement with previously published data (Da Silva et al., 2001; Da Silva and Coleman, 2003).

Chemicals were purchased from Acros Organics, phosphate buffer saline (PBS) pH 7.4 was prepared by dissolving NaH_2PO_4 (1.9 mmol, 228 mg), Na_2HPO_4 (8 mmol, 1.14 g), NaCl (128 mmol, 7.4 g) in 1 l of de-ionised water. Drabkin reagent (Na_2CO_3 80%, $K_4Fe(CN)_6$ 16%, KCN 4% w/w) was purchased from Sigma (Drabkin and Austin, 1935). (Warning: Drabkin reagent is highly toxic and must be manipulated with non-permeable gloves, contact with eyes, skin, ingestion and inhalation must be avoided).

2.1. Haemolysis tests

Haemolysis tests were carried out on washed erythrocytes: 50 ml of freshly drawn human blood was centrifuged in a polypropylene vial with 50 ml of physiological serum (NaCl 0.9%) during 5 min at $1200 \times g$. The supernatant was removed and this operation was repeated twice. The volume of the washed erythrocytes was adjusted to 50 ml with PBS.

To 500 μl of the PBS buffered solution of water-soluble calix-arene derivatives, were added 500 μl of the suspension of erythrocytes. After moderate manual stirring, the mixture was incubated during 30 min in a thermostated bath at 37 °C and centrifuged at $1200 \times g$ during 5 min. A 20 μl aliquot of the supernatant was added to 2 ml of Drabkin reagent. The quantity of haemoglobin was assayed spectrophotometrically at 540 nm. To ensure results reproducibility all assays were repeated twice. Positive and negative controls have been realised by replacing the sample with water (total haemolysis due to hypotonic stress) and PBS, respectively. The percentage of haemolysis is expressed as the ratio between the absorbance of the

sample (corrected using the value obtained for PBS) and the absorbance of the positive control.

3. Results and discussion

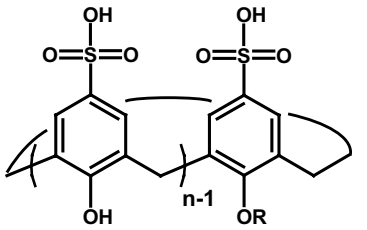
The structures of the various calix-[*n*]-arene derivatives tested for haemolytic properties are given in Scheme 1.

The study of the haemolytic effect of water-soluble *para*-sulphonato-calix-[*n*]-arenes was carried out on freshly washed erythrocytes. Drabkin's reagent was used to stabilize the haemoglobin released from lysed red cells and the complex absorbed at 540 nm (Moore et al., 1981). The percentage of haemolytic effects is described as follows:

$$\text{Haemolysis (\%)} = \frac{\text{Optical density of sample with calixarene}}{\text{Optical density of sample with distilled water}} \times 100$$

It can be clearly seen that the haemolytic effects increase with the ring, with a maximum value of 30% at a 200 mM concentration of **3a**. For **1a**, there is effectively no haemolytic effect, while a small effect of 8% is observed in the case of **2a**. It should be noted that a 200 mM concentration represents 150 g/l for **1a**, 220 g/l for **2a** and 300 g/l for **3a**. At 50 mM concentrations, all three parent compounds show less than 5% haemolytic effects.

No haemolytic effects (<5%) were observed for any of the water-soluble calix-arene derivatives at a concentration of 50 mM (average 40 g/l) (Figs. 1–3).



1 n=4, **2** n=6, **3** n=8

1a, 2a, 3a R= -H
1b, 2b, 3b R= -CH_2COOH
1c, 2c, 3c R= -CH_2CONH_2
1d, 2d, 3d R= -$CH_2CH_2NH_2$

Scheme 1. Schematic representation of different water-soluble *para*-sulphonato-calix-[*n*]-arenes.

The presence of pendant group at the lower rim of calix-arene does not alter the behaviour of the macrocyclic compounds towards to red blood cells except for **2d** and **3d** for which the haemolytic effect attains the highest values. Thus, the presence of a pendant amine group may close the way to possible medical applications of such calix-arene derivatives. In contrast for a concentration of 100 mM in calix-[*n*]-arene, the presence of a methoxy-carboxylate function reduces the haemolytic effect for the calix-[4]-arene derivative from 0.5 to 0.1%, for the calix-[6]-arene derivative from 5 to 2% whereas calix-[8]-arene derivative the haemolytic effects are effectively the same 9% in both cases. For *para*-sulphonato-calix-[4]-arene the

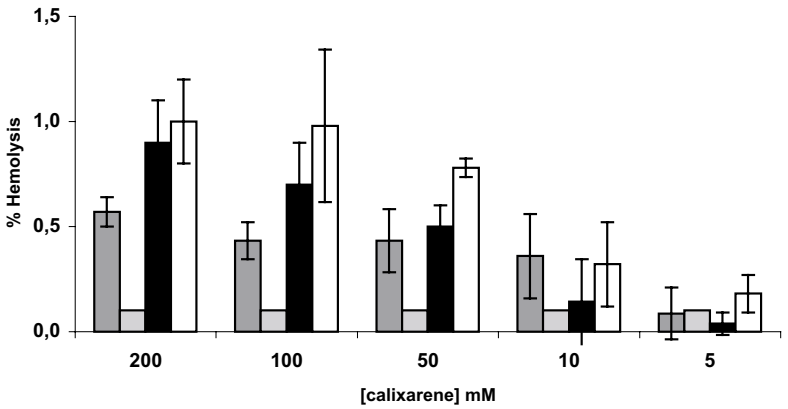


Fig. 1. Comparison of haemolysis percentage between *para*-sulphonato-calix-[4]-arene **1a** (dark grey) and its derivatives **1b** (clear grey), **1c** (black) and **1d** (white).

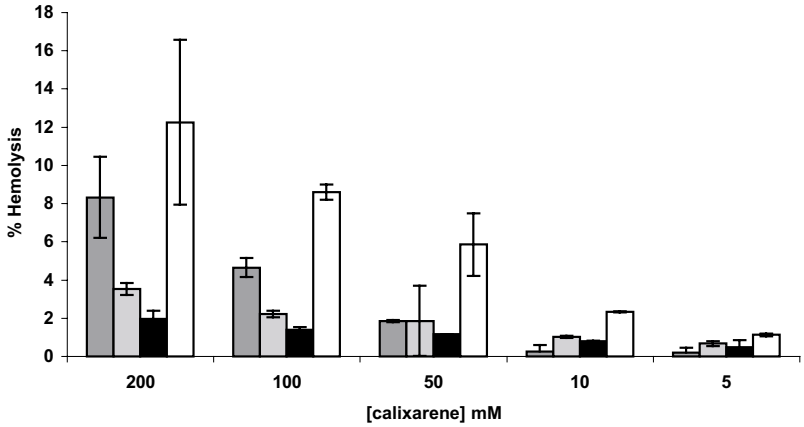


Fig. 2. Comparison of haemolysis percentage between *para*-sulphonato-calix-[6]-arene **2a** (dark grey) and its derivatives **2b** (clear grey), **2c** (black) and **2d** (white).

ethoxy amide function increases the haemolytic effect at concentrations of 50 mM or greater, whereas for the *para*-sulphonato-calix-[6]-arene and *para*-sulphonato-calix-[8]-arene the haemolytic effect is decreased with respect to the parent macrocycle at all concentrations.

The above results may be compared to those of the cyclodextrins where for the native compounds, haemolytic effects are observed in the concentration range of 2 mM (Bost et al., 1997). The work of Shiotani et al. shows that unmodified β-cyclodextrin provokes 100% haemolytic effect at a concentration of 3 mM and that the presence of sulphates group on β-cyclodextrin decreases the haemolytic effect, with 100% heamolysis now observed at a concentration of 20 mM (Shiotani et al., 1995). Similar effects were observed by Macarak et al. (1991). Similarly, zero haemolytic effects have been reported for the pentosan polysulfate (Bertoux et al., 1977). A recent paper of Shahgaldian et al. reported the non-toxicity of solid lipid nanoparticles based on amphiphilic calix-[4]-arenes against human red blood cells at concentration of 150 mg/l (Shahgaldian et al., 2003).

Natural tea polyphenols (TPP) have been observed to reduce the haemolytic effect on rabbit red blood cells, by free radical species (O_2^- and H_2O_2

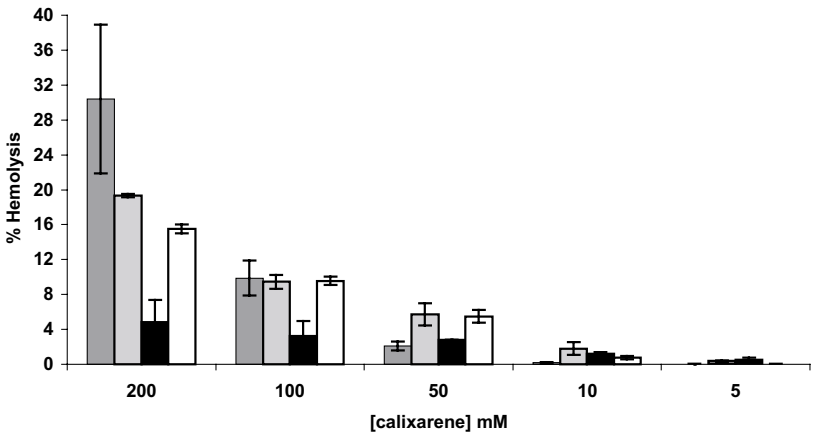


Fig. 3. Comparison of haemolysis percentage between *para*-sulphonato-calix-[8]-arene **3a** (dark grey) and its derivatives **3b** (clear grey), **3c** (black) and **3d** (white).

species) induced by 8-aminoquinoline, from 35 to 5% at 10 μg/ml of TPP (Grinberg et al., 1997). The haemolytic effect (100%) induced by hypotonic phosphate buffer on human erythrocytes is reduced to 22% by the polyphenol flavonoid quercetin (Pawlikowska-Pawlega et al., 2003).

From the above, the presence of a polyphenolic skeleton and sulphonate groups, in the series of compounds studied in this article, are both major factors in the low to zero haemolytic effects observed.

4. Conclusion

In this study, we have shown that *para*-sulphonato-calix-[4]-arene and three of its lower rim mono-substituted derivatives have effectively no haemolytic effects for concentrations up to 200 mM. For the *para*-sulphonato-calix-[6]-arene derivatives a maximum of 12% haemolysis occurs for the ones bearing an amine function at concentration of 200 mM. Haemolysis of 29% is observed for *para*-sulphonato-calix-[8]-arene at 200 mM. However, for all derivatives, haemolysis of less than 5% are observed for concentrations below of 50 mM. The haemolytic effect increases with macrocycle ring size.

For the *para*-sulphonato-calix-[4]-arene and *para*-sulphonato-calix-[6]-arene derivatives, the presence of a single ethoxy-amine function on the lower rim increases the haemolytic effect at all concentrations. In all cases, the presence of a carboxy-ethoxy function leads to a significant reduction of the haemolytic effects at concentrations of 50 mM and above. It may be deduced that the *para*-sulphonato-calix-[*n*]-arenes and their derivatives will be innocuous with regard to haemolytic effects at doses of less than 50 mM.

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