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A First Approach to the Study of Calixarene Solid Lipid Nanoparticle (SLN) Toxicity

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Abstract

The haemolytic effects of a series of amphiphilic calix-arene derived Solid Lipid Nanoparticles (SLNs) have been measured on human erythrocytes. The effects of both the hydrophobic chain length and the nature of the polar head-groups on the amphiphilic calix[4]arene have been investigated. For all systems, no haemolytic effects are observed.

Abbreviations: BSA, Bovine Serum Albumin; O.D., Optical Density; PCS, Photon Correlation Spectroscopy; PBS, Phosphate Buffered Saline; SLN, Solid Lipid Nanoparticle

Introduction

Calix[n]arenes [1] are, with cyclodextrins [2] and crown ethers [3], one of the major class of supramolecular hosts. Widely used in material science and solid-state chemistry, their biological interest is much less well developed. However, previous work has shown their activity against tuberculosis [4], in the inhibition of enzyme activity [5] (lysyl-oxidase), as chloride ion channel blockers [6], and as anti-coagulant [7]. Some modified calixarenes have been shown to act as antibody mimic in surface protein recognitio [8] and as ion channel for the transport of ions across phospholipid bilayer [9, 10].

More recently we have shown that amphiphilic calixarenes are able to form highly stable monodisperse nanoparticles in water [11, 12] opening new prospects in their use for drug delivery. The calix-arene derived nanoparticles, or more correctly Solid Lipid Nanoparticles (SLNs) show excellent stability at physiological ionic strength (154 mM). Similar nanoparticles based on the cyclodextrins have been extensively studied for drug transport [13].

It is evident that for medical application of calix-arene or other supramolecular SLNs, such systems should not provoke amongst others immunogenic responses, opsonisation reactions by serum albumin surface aggregation and haemolytic effects on erythrocytes.

In the case of cyclodextrins, while serum albumin surface aggregation has not yet been studied, both immune response by the formation of cyclodextrin-specific antigens and the strong haemolytic effects [14], arising from cholesterol [15] and phospholipid [16, 17] complexation, and mitigate strongly against their intravenous use. The haemolytic effects may be overcome by certain synthetic modifications [18, 19].

In the case of calix-arene based carrier systems, no immune response has been found for the parent tert-butyl-calix[n]arenes [20] and work in progress has shown no neutrophil activation by the SLNs [21]. We have recently shown that while a single or double layer of serum albumin molecules adheres to the SLNs no aggregation or large accumulation occurs even at the physiological concentration of 40 g/L [22]. Given the much lower size of the molecular cavity of calix[4]arene as compared to β-cyclodextrin it is unlikely that inclusion with cholesterol will occur. Indeed Langmuir compression isotherm studies confirm this hypothesis, with no molecular complexation observed [23].

In this work, we present the first study on the haemolytic effects of calix-arene based Solid Lipid Nanoparticles (SLNs) prepared from different amphiphilic calixarenes (Figure 1). The effects of both the hydrophobic chain length and the nature of the polar head-groups on the amphiphilic calix[4]arene are reported, and we demonstrate the lack of any haemolytic properties of these systems.

Experimental

General

Amphiphilic calixarenes have been synthesized as previously described [24]. All chemicals were purchased from Acros Organics, Phosphate buffer saline (pH 7.4) was prepared by dissolving 1.9 mmol of NaH_2PO_4, 8 mmol of Na_2HPO_4, 7.493 g of NaCl in 1 L of de-ionised water. Drabkin reagent (sodium carbonate, potassium ferrocyanide and potassium cyanide) was purchased from Sigma. (Warning: Drabkin reagent is highly toxic and must be manipulated

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with plastic gloves, contacts with eyes, skin, ingestion and inhalation must be avoided.)

SLN preparation

Nanoparticles are prepared by the solvent displacement method [25]. The relevant amphiphilic calixarene was dissolved in tetrahydrofuran (5 mg/mL) and 3 mL of this solution was added to 50 mL of phosphate buffer saline (PBS) pH 7.4 and stirred during 1 minute at 500 rpm. The tetrahydrofuran was eliminated by evaporation under reduced pressure and the volume adjusted to 100 mL with water. The pH of all solutions was verified to be equal to 7.4.

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 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 relevant SLN suspensions, 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, centrifuged at 1200 g during 5 min. 20 μL of the supernatant was added in 2 mL of Drabkin reagent. The quantity of hemoglobin 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 respectively water (total haemolysis due to hypotonic stress) and PBS. 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.

Results and discussion

The haemolytic effect of SLNs based on calix-arene bearing acyl chains of various length (C_6:C_{12}; **1**, **2**, **3**, and **4**) at the upper rim functionalised and in the case of **4**, 1,3-disubsituted by diethyl-phosphonate (**5**) and phosphonate functions (**6**), scheme 1.

All compounds form stable Solid Lipid Nanoparticle (SLN) colloidal suspensions in water, and in phosphate buffered saline (PBS) solutions. In PBS, the hydrodynamic diameters as measured by Photon Correlation Spectroscopy (PCS) are given in Table 1.

The measurements of the haemolytic properties of the calix-arene derived SLNs **1**–**6** were carried out on freshly washed erythrocytes, using the standard procedure. Drabkin's reagent was used to stabilize the hemoglobin released from lysed erythrocytes, and the optical density (O.D.) at a wavelength of 540 nm was measured. A positive control using pure water to totally lyse the cells was carried, and the results given in Table 2 express the O.D. measured for **1**–**6** as ratios of the O.D. obtained from water lysed cells.

Table 1. Hydrodynamic diameter of calix-arene based Solid Lipid Nanoparticles prepared in PBS measured by Photon Correlation Spectroscopy

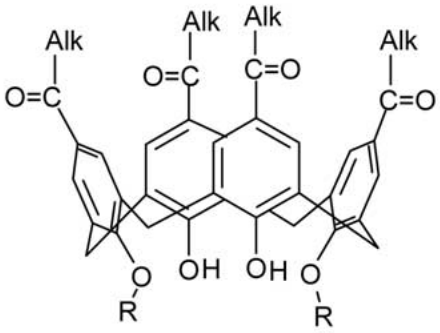
Compound	**1**	**2**	**3**	**4**	**5**	**6**
Average hydrodynamic diameter (nm)	340	353	350	350	325	260

Table 2. Results of the haemolysis experiments (expressed as a percentage of total haemolysis caused by hypotonic stress) at calix-arene SLN concentrations varying from 50 to 150 mg/L, values are derived from the O.D. measured at 540 nm

Concentration (mg/L)	**1**	**2**	**3**	**4**	**5**	**6**
50	0.0	0.0	0.5	0.0	0.0	1.8
75	0.0	3.2	0.0	0.0	0.0	0.0
100	0.0	0.5	0.0	0.0	0.25	0.0
125	0.0	0.0	0.0	0.0	0.62	0.0
150	0.0	0.0	0.0	0.0	0.0	0.0

It is evident that for all concentrations of **1**–**6** up to 150 mg/L cause zero haemolysis of the erythrocytes. Previous work on the interfacial interactions of **4** and **6** with various phospholipids have shown that while **6** is fully miscible with di-palmitoyl-phosphatidic acid (DPPA) and its derivative bearing choline (DPPC), serine (DPPS) and ethanolamine (DPPE) head-groups, **4** is immiscible with DPPA, DPPE and DPPS and only partially miscible with DPPC [26]. With regard to cholesterol **4**, **5** and **6** show total immiscibility, but more importantly do not form inclusion complexes [23]. The known haemolytic effects of the cyclodextrins arise from the formation of inclusion complexes with various lipids and particularly cholesterol, removing these components from the cell membrane and thus provoking the destruction of the integrity of the cell envelope. Apparently the lack of haemolytic properties of **1**–**6** may be due to a lack of inclusion of various membrane components and hence no perturbation of the cell membrane. This can be compared to the haemolytic effects of various cyclodextrin derivatives for which the onset of haemolytic effects are in the range of 1–2 mM. In the case of β-cyclodextrin derivatives for which acyl functions are coupled at either the primary or secondary face such haemolytic effects have been reported to be reduced [19].

Natural polyphenols have also been observed to produce zero haemolytic effects. It appears that even for the purely synthetic calix-arene systems, the presence of polyphenolic functions is concurrent with a lack of haemolytic properties. The above *in-vitro* results may open the way to possible medical applications of calix-arene SLNs as transport systems in intravenous use.



Alk= $CH_3(CH_2)_n$

	n	R
1	4	H
2	6	H
3	8	H
4	10	H
5	10	$PO(OEt)_2$
6	10	$PO(OH)_2$

Scheme 1. General formulae of the amphiphilic calix-arenes used in the haemolysis experiment.

Conclusion

The measurement of the haemolytic effects of a series of amphiphilic calix-arene derived Solid Lipid Nanoparticles shows that neither variations in chain length of the hydrophobic moiety nor variation in the nature of the polar head group induce haemolysis in human erythrocytes.

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