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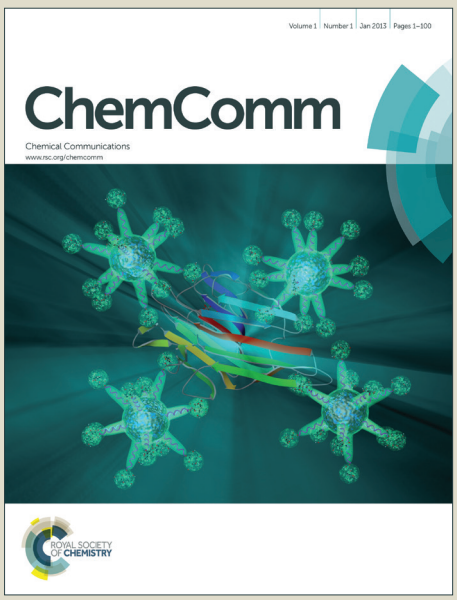


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# Structural requirements for anti-oxidant activity of calix[n]arenes and their associated anti-bacterial activity

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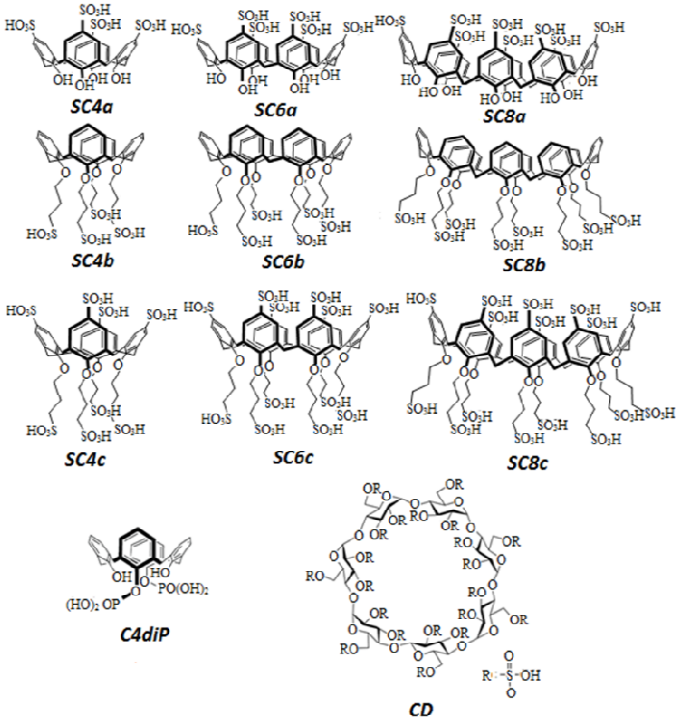
**Treatment of neural cells with calix[n]arenes featuring sulphonate moieties and linked to Ag nanoparticles results in reduced reactive species. For gram+ bacteria there is an inverse correlation between anti-bacterial activity and ROS reduction whereas for gram- bacteria only calix[6]arenes bearing O-alkyl sulphonate functions act as ROS inhibitors and anti-bacterial agents.**

Injury to the central nervous system is characterized by altered $Ca^{2+}$ flux and associated increases in reactive species, thought to be triggered by excess glutamate release [1, 2]. If reactive species are not controlled by endogenous anti-oxidants, oxidative stress ensues, and this is a feature of traumatic brain and spinal cord injury as well as secondary degeneration [3-5]. Anti-oxidants have been used in pre-clinical studies to treat injury to the central nervous system, but new agents are needed in order to generate complementary combinations of anti-oxidant therapies to control multiple facets of oxidative stress[3]. Furthermore, antioxidants often suffer from lack of solubility and resultant poor bioavailability [6, 7], limiting their clinical usefulness.

Calix[4]arenes have been used as therapeutic delivery systems for anti-oxidants and have also been shown to have inherent anti-oxidant properties [8]. As such they have the potential to provide a combinatorial anti-oxidant strategy for neurotrauma. The sulphonated calix[n]arene derivatives all have solubilities above 100mM in water making them attractive as therapeutic agents. The *para*-sulphonato-calix[n]arenes are well documented for their biological activities, [9] while the silver nanoparticles capped with *para*- and O-sulphonated derivatives have shown interesting anti-bacterial activities with selective action against gram+ or gram- bacteria. [10]

However the structural requirements for anti-oxidant and anti-bacterial activity are unknown, making elucidation of the mechanisms of action problematic. Here we assess the effects of a range of calix[n]arene structure on viability and production of reactive oxygen species (ROS) by neuronal cells stressed with an excitotoxic concentration of glutamate. We also have studied possible association between effects on ROS and anti-bacterial activity, as the presence of radicals and hence ROS is one of the proposed mechanisms for the anti-bacterial action of silver nanoparticles [11].

Calix[n]arenes were synthesized by literature methods, see SI and the sulphated cyclodextrin is commercially available. (Scheme 1). Calix[n]arenes were linked to Ag nanoparticles (Ag_NP) by direct interaction during the reduction of $Ag^+$ to $Ag^0$ (Scheme S1, SI). The linking of calix[n]arenes to Ag nanoparticles may reduce the strongly negative charge of the calixarene tail groups, thereby aiding in membrane penetration and perhaps increasing antioxidant efficacy, compared to calix[n]arenes not linked to Ag nanoparticles.




**Scheme 1** SC(n)a, SC(n)b and SC(n)c correspond, respectively, to para-sulphonatocalix[n]arene, O-propyl sulphonate calix[n]arene and O-propyl para-sulphonato-calix[n]arene; with (n) corresponding to the number of phenolic units in the macrocycle. C4diP corresponds to calix[4]arene dihydroxyphosphonic acid and CD corresponds to sulphated β-cyclodextrin.

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Toxicity of nanoparticle preparations in biological systems remains an ongoing concern. We therefore assessed the effects of the calix[n]arene and cyclodextrin preparations on viability of neuronal cell that had been stressed with an excitotoxic concentration of glutamate, using Pheochromocytoma (PC12) neuronal-like cells. Calcein dye was used to selectively stain viable cells. Images were taken at 2 locations in triplicate culture wells at each concentration, using a Nikon Inverted Fluorescence microscope. Viable cells (green) were counted, expressed relative to unit area and mean ± S.E.M calculated. Data were assessed using ANOVA with a significance value of $p \leq 0.05$, using SPSS statistical software (IBM). There were no significant differences in PC12 cell viability following glutamate stress and treatment with any of the calix[n]arene or cyclodextrin preparations at 100 μg $mL^{-1}$, compared to untreated, glutamate stressed control cells ($F=1.42$, $p=0.12$). Choice of calix[n]arene and cyclodextrin concentration was in line with our previously reported studies and higher than concentrations previously shown to exhibit antioxidant activity [8]. Representative images of cells treated with selected preparations demonstrate that treated PC12 cells displayed normal morphology, extending small neurites (Figure 1).

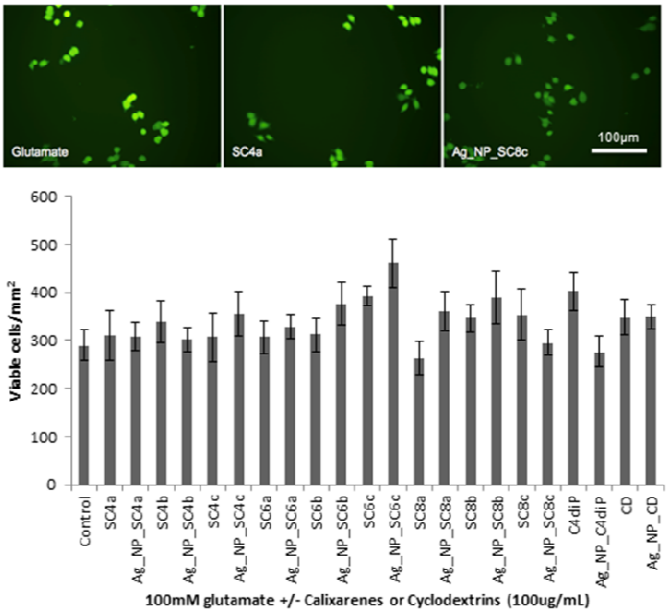


**Fig. 1** Viability of PC12 cells that have been stressed with 100mM glutamate and treated with 100ug/mL of the various calix[n]arene preparations for 1 hour. Viability was assessed using calcein dye to label live cells and representative images of glutamate stressed untreated cells and glutamate stressed cells treated with SC4a or AG_NP_SC8c are shown. Quantification of viability data are expressed as mean ± S.E.M live cells/ area assessed.

Having established that the calix[n]arene and cyclodextrin preparations were not toxic to neural cells at a concentration of 100μg $mL^{-1}$, we assessed the effects on the preparations at 1 – 100 μg $mL^{-1}$, on intracellular generation of ROS. Fluorescence of the reactive dye chloromethyl dichlorodihydro-fluorescein diacetate (CM-$H_2$DCFDA) was used to indicate intracellular reactive species. Cellular fluorescence was measured on an Enspire multimode plate reader using an excitation wavelength of 480 nm and emission wavelength of 530 nm (Software version 4.1). Results for each experiment were expressed as mean ± S.E.M. arbitrary units of fluorescence intensity data for each group of calix[n]arene preparations (e.g. SC4 calixarene preparations), and were assessed using ANOVA and Dunnett's post hoc tests with a significance value of $p \leq 0.05$, using SPSS statistical software.

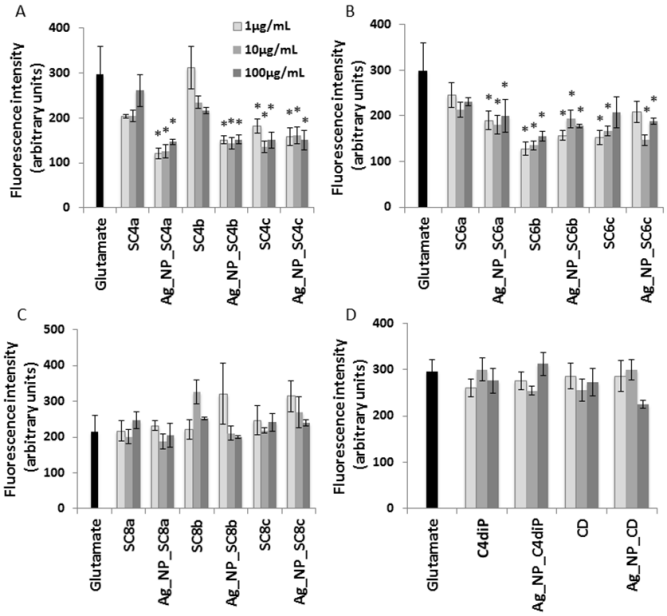


**Fig. 2** Effects of calix[n]arene and cyclodextrin preparations on ROS production by glutamate stressed PC12 cells. Cells were stressed with 100mM glutamate and treated with 1, 10 or 100 μg $mL^{-1}$ calixarene or cyclodextrin preparations. ROS production was quantified using DCFH-DA and results expressed as mean ± S.E.M fluorescence intensity (arbitrary units): * $p<0.05$, significantly different from glutamate only control. Experiments were conducted 2-3 times and representative results displayed.

Treatment with each concentration of Ag_NP_SC4a resulted in significantly reduced ROS production by PC12 cells ($F=5.87$, $p\leq0.0001$: note p value for least effective concentration provided), whereas treatments with SC4a not linked to Ag_NP were not effective at reducing ROS ($p=0.06$, Figure 2A). Efficacy of Ag_NP_SC4b was similarly dependent on linkage to Ag_NP ($p=0.001$): SC4b alone had no significant effect on ROS ($p=0.16$). In contrast, Ag_NP_SC4c and SC4c were both effective at reducing ROS at the tested concentrations ($p=0.001$, $p=0.01$ respectively). Treatment with the SC6 preparations followed a similar pattern (Figure 2B). Reduction of ROS with SC6a required linkage to Ag_NPs ($F=3.11$, $p=0.05$). However, SC6b and Ag_NP_SC6b were both effective at reducing ROS ($p=0.001$, $p=0.001$ respectively), as were SC6c and Ag_NP_SC6c ($p=0.002$, $p=0.02$ respectively). Note that there were some concentration dependent effects following treatment with the SC6c preparations but differences were minor and unlikely to be biologically meaningful. No reductions in ROS were detected following treatment with the SC8 calixarene preparations regardless of linkage to Ag_NP ($F=1.53$, $p=0.13$), or with the C4diP or CD preparations ($F=0.84$, $p=0.61$).

Our data demonstrate that the length and orientation of the sulphated tail groups on calix[n]arenes, as well as their interaction with Ag_NP, impact upon their intrinsic anti-oxidant activity, and that cyclodextrins lack anti-oxidant activity at the tested concentrations. The highly anionic groups of the calix[n]arenes may reduce interactions with the negative exterior of the plasma membrane [9]. Nanoparticle surface charge is a determining factor of cellular uptake [10, 11] and those with cations present on their surface are more easily internalised due to electrostatic interactions with the negatively charged cell surface. Attachment of calix[4a,4b,6a]arenes to silver-capped nanoparticles resulted in reduced intracellular ROS, perhaps *via* Ag cations reducing the negative charge of the calix[n]arenes and increasing internalisation within cells. Alternatively, the functional groups of the calix[n]arenes may be sequestered within the Ag_NP surface (Scheme S1, SI). Differential

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efficacy was also observed when comparing calix[n]arene preparations of the same ring size, but with different tail structures present. In the cases of both 4- and 6-moeity ring sizes, the most effective structure was that of SC(n)c, which corresponds to sulphonic acid groups present above and below the plane of the macrocycle. Calix[n]arenes have been shown to selectively associate with, [12, 13] and transport [14] various cations present in biological systems, readily forming anionic derivatives, although the electrostatic interactions are not well understood and are thought to vary largely depending on the degree of charge localisation within the ions [15]. While the antioxidant mechanism of the SC(n)c is not known, complex intracellular anionic interactions are likely to be involved, as supported by our previous demonstration of antioxidant activity of calix[4]arenes that also featured anionic groups above and below the ring [8]. The observation that the calix[8]arenes failed to reduce ROS production, regardless of concentration or linkage to Ag_NP, suggests that there is a size limit, as well as structural requirements, to calix[n]arene efficacy, perhaps due to size exclusion of the calix[n]arene cup structure or protein binding incompatibility. Note that Ag_NP without calixarene capping degrade rapidly, limiting any likely biological effects if dissociation occurs[16]. Antioxidant efficacy is often associated with antibacterial activity[17], but the mechanism and structural requirements of the association are as yet unclear. We had previously assessed efficacy of calix[n]arene and cyclodextrin capped Ag nanoparticles at inhibiting growth of gram + bacteria and gram – bacteria, by measuring cell growth as a percentage of growth in untreated cultures (Table 1).[10] There exists an inverse relation between the antioxidant efficacy of Ag-NPs capped by *para*-sulphonato-calix[n]arenes and Gram+ antibacterial activities. Gram – antibacterial activity was significant in the cases of SC6b and SC6c and in both cases, associated with the reduction of ROS species. Molecules are active with regard to ROS reduction as the isolated molecule and also as the capped NPs (Table 1).

**Table 1 Summary of antioxidant activity of calix[n]arenes and antioxidant and antibacterial activities of calix[n]arene capped silver nanoparticles. ROS levels are expressed as a % of values from control. Similarly, antibacterial levels are expressed as a % of growth compared to an untreated control. The silver nanoparticles showing an inverse dependence between their Gram + anti-bacterial and ROS activities are highlighted in red. The silver nanoparticles showing a direct association between their Gram - anti-bacterial and ROS activities have been highlighted in blue.**

| Molecule | ROS level (%) | Molecule capped on silver nanoparticles | ROS level (%) | Gram + Bacterial growth (%) | Gram - Bacterial growth (%) |
|---|---|---|---|---|---|
| **SC4a** | 68 | **SC4a**_Ag_NP | 42 | 81 | 100 |
| **SC6a** | 72 | **SC6a**_Ag_NP | 61 | 55 | 100 |
| **SC8a** | 93 | **SC8a**_Ag_NP | 87 | 33 | 99 |
| **SC4b** | 73 | **SC4b**_Ag_NP | 48 | 97 | 99 |
| **SC6b** | 43 | **SC6b**_Ag_NP | 53 | 98 | 46 |
| **SC8b** | 102 | **SC8b**_Ag_NP | 93 | 81 | 89 |
| **SC4c** | 51 | **SC4c**_Ag_NP | 51 | 99 | 84 |
| **SC6c** | 51 | **SC6c**_Ag_NP | 49 | 98 | 62 |
| **SC8c** | 101 | **SC8c**_Ag_NP | 111 | ND* | ND* |
| **C4diP** | 88 | **C4diP**_Ag_NP | 86 | ND* | ND* |
| **CD** | 86 | **CD**_Ag_NP | 76 | ND* | ND* |

ND*: Not Determined

From the above it is possible to postulate that while the action of calix[n]arene capped nanoparticles with regard to gram+ positive bacteria involves radical species and is inhibited by ROS reducing systems, for gram – bacteria the action is highly specific, involving only calix[6]arene derivatives carrying O-alkyl sulphonate groups at the phenolic face and is not related to ROS reducing activity.

## Conclusion

Calix[n]arenes featuring sulphonate groups above and/or below the plane of the macrocycle have intrinsic anti-oxidant capacity: less sulphonated calix[n]arenes require linkage to Ag_NPs to achieve similar efficacy. Calix[n]arenes may be used to carry additional therapeutic agents to provide a combinatorial anti-oxidant strategy for treatment of neurotrauma and other diseases. Associated antibacterial activity may enhance therapeutic potential in certain clinical scenarios featuring infection. The mechanism of the anti-bacterial action remains a quandary for gram- bacteria.

## Notes and references

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