Enhanced detection of the pathogenic prion protein by its supramolecular association with *para*-sulfonato-calix[*n*]arene derivatives†

Anthony W. Coleman,*[a] Florent Perret,[a] Sébastien Cecillon,[a] Aly Moussa,[b] Ambroise Martin,[c] Maryline Dupin[d] and Hervé Perron[d]

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The supramolecular interaction between the pathogenic form of the prion protein and derivatives of the *para*-sulfonato-calix[*n*]arenes has been demonstrated and two putative binding sites determined; this interaction leads to an amplification of the Western Blot immunological detection of the prion protein by the SAF84 antibody.

Introduction

The disease class of transmissible spongiform encephalopathies covers a series of transmissible neurodegenerative diseases, including in sheep, scrapie; in cattle Bovine Spongiform Encephalopathy (Mad Cow Diseases); in deer, Chronic Wasting Disease and in humans, Kuru, Gerstmann–Straüssler–Scheinker disease, Fatal Familial Insomnia, Fatal Sporadic Insomnia, and the various form of Creutzfeldt–Jakob Disease.[1] It has been determined that the infectious agent associated with all the diseases is the prion protein. The prion protein (PrP^c), present as non-glycosylated, mono-glycosylated and bi-glycosylated glycoforms, is mainly expressed in neuronal and lymphoid tissues and the gene coding for PrP^c is present in mammals as well as in non-vertebrates.[2]

While the peptide sequence is widely conserved, both glycosylation and the GPI lipid structure have proved to be widely variable in nature.[3] The change from the normal forms of the prion protein PrP^c to the pathogenic forms PrP^{sc} is associated with conformational changes in the peptide backbone from a high degree α-helical structure to a conformer in which a much higher degree of β-sheet structures are present.[4] This shift in protein conformation is accompanied by a change in detergent solubilisation, and induces resistance to proteolytic degradation along with a tendency to aggregation. The conformal change related to genetic factors, induces polymorphisms including that at codon 129 which causes Creutzfeldt-Jakobs Disease.[5] Also, an auto-catalytic shift due to contact with xeno-proteic materials arriving from external sources can initiate TSE infections such as BSE and v-CJD.[6]

The prion protein structure contains a multi-histidine peptide sequence capable of binding a wide range of metallic cations,[7] but which is easily proteolytically cleaved. This protein structure also contains a spherical core sequence with a generally highly negatively charged surface.[8] However, at least two positively charged pocket, rich in lysine or arginine residues, are always present, see Fig. 1.

The presence of this positively charged binding pocket is related to the capacity of PrP to interact with the heparan sulfate glycofragment present at the cell surface[9] and the 37-kDa/67-kDa laminin receptor (LRP).[10]

There are two types of interaction between LRP and PrP. The first involves the heparan sulfate chains of an Heparan sulfate proteoglycan as intermediary. Putative binding sites for the heparan sulfate glycosylaminoglycan chains may be

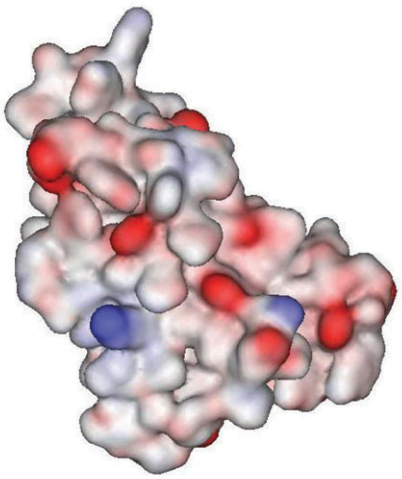

Fig. 1 (a) Charge surface of the fragment 125–231 of the PrP. In red, negatively charged surface. In blue positively charged surface. (b) Amino acids sequence of the bovine PrP protein, SAF84 epitope in red, putative binding sites for *para*-sulfonato-calix[*n*]arene derivatives underlined.

[a] *Institut de Biologie et Chimie des Protéines (IBCP UMR 5086, CNRS, University Lyon 1, 7 passage du Vercors, F69367 Lyon, France. E-mail: aw.coleman@ibcp.fr; Fax: 33 472722690; Tel: 33 472722640*
[b] *Agence Française de Sécurité Sanitaire des Aliments, F69364 Site de Lyon, France*
[c] *Université Claude Bernard, 8 avenue Rockefeller, 69373 Lyon Cedex 08, France*
[d] *BioMérieux Advanced Technology Unit, R&D Neurological diseases, Chemin de L'Orme, F69280 Marcy L'Etoile, France*
† Dedicated to Professor George Gokel on the occasion of his 60th birthday.

identified at positions 99–113 and 148–161 in the protein sequence. Both peptide sequences are rich in lysine and arginine residues, corresponding to known glycosylaminoglycan binding sequences.[11] The second mechanism proceeds *via* a direct interaction between PrP and LRP involving the domain PrPLrpbd1 of PrP. This interaction allows the anchorage of the PrP at the surface *via* its GPI anchor and stabilises it.[3]

The calix[*n*]arenes are a class of organic molecular host molecules widely used in supramolecular chemistry.[12] They are phenolic macrocyclic rings composed usually of four, six or eight phenolic rings. Although the first report of their bioactivity was published by Conforth *et al*. in 1955,[13] it is only within the last ten years that there has been a remarkable resurgence in the interest of the biological and pharmaceutical application of the calix[*n*]arenes.[14] Direct bioactivity of these synthetic host molecules, has been shown for antithrombotic activity,[15] anti-viral activity,[16] ion-channel blocking[17] and enzyme inhibition,[18] as well as the reappraisal of their antitubercular activity.[13]

While the complexation of amino-acids by *para*-sulfonato-calix[*n*]arene is well documented, their interactions with proteins[20] and their toxicity is less well studied.[21] It has been clearly demonstrated that the strongest interactions, as would be expected, are with the basic amino acids lysine (K) and arginine (R),[19] with association constants of for *para*-sulfonato-calix[4]arene with arginine, K_A = 1546 M^{-1}; and with lysine, K_A = 1356 M^{-1}; **2a**–arginine, K_A = 3090 M^{-1}; **2a**–lysine, K_A = 2200 M^{-1}; **3a**–arginine, K_A = 10 083 M^{-1}; **3a**–lysine, K_A = 4288 M^{-1}. Studies on the binding of di- and tri-peptides of lysine and arginine show much higher binding constants, for example, **1a**–KK = 3800 M^{-1} , **1a**–KR = 3700 M^{-1}, **1a**–RK = 4300 M^{-1} **1a**–RR = 7700 M^{-1}, **1a**–KKK = 30 000 M^{-1}, **1a**-RRR = 35 000.[22] The increase in the values of the association constants suggests that there is not simple additivity in the interactions between the *para*-sulfonato-calix[*n*]arenes and peptides containing positively charged amino acids. Mono-substitution at the lower rim of the *para*-sulfonato-calix[*n*]arenes induces selective changes in the binding of amino-acids that are dependent both on the nature of the substituent and on the size of the macrocyclic ring.[19] The effects of mono-substitution of the *para*-sulfonato-calix[*n*]arenes on their binding to proteins have been demonstrated for the anti-coagulant properties of these molecules,[23] and also with respect to binding to Bovine Serum Albumin.[20]

We have recently demonstrated that a number of substances including streptomycin allow enhancement of the detection of TSE infections *in vitro*.[24] In this paper we describe the amplification of Western-Blot immunodetection of the prion protein based on the interaction of the *para*-sulfonato-calix[*n*]arenes, and certain of their mono-substituted derivatives, with the prion protein. This supramolecular association results in amplification of antibody–prion interaction and thus leads to increased sensitivity in the detection of the PrP^{res} pathogenic protein.

Results and discussion

The molecular structures of the *para*-sulfonato-calix[*n*]arene derivatives used in this study are shown in Fig. 2. They were

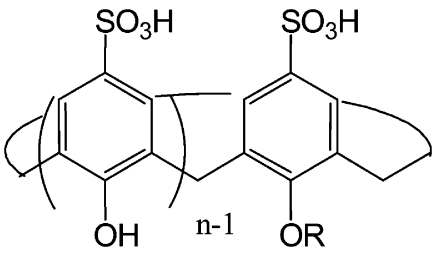

Fig. 2 Structure of *para*-sulfonato-calix[*n*]arene and their derivatives. **1**: n = 4, *para*-sulfonato-calix[4]arene; **2**: n = 6, *para*-sulfonato-calix[6]arene; **3**: n = 8, *para*-sulfonato-calix[8]arene; (a) R = H, *para*-sulfonato-calix[*n*]arene; (b) R = (CH_2)COOH, *para*-sulfonato-calix[*n*]arene-mono-*O*-methoxycarboxylic acid; (c) R = $(CH_2)_2NH_2$, *para*-sulfonato-calix[*n*]arene-mono-*O*-ethoxyamine.

synthesised according to the literature methods[19] and purified to diagnostic standard using RP-HPLC, with a water–acetonitrile gradient of 40%.

For Western Blot detection in the absence of *para*-sulfonato-calix[*n*]arenes, PrP^{sc} was extracted from confirmed-positive BSE cattle brain tissue and of Scrappie-positive, sheep and goat brains then treated with Proteinase K to proteolyse all proteins other than the resistant form (PrP^{res}) of the Prion.[1]

For Western Blot detection in the presence of *para*-sulfonato-calix[*n*]arenes in PBS was carried out by adding 0.1 μmol calixarenes to 100 μl of the 10% brain extract, incubated at 37 °C for 1 h prior to digestion with Proteinase K for another hour.

All other procedures used were identical between the two methods except when otherwise mentioned. Thus after this proteolysis the samples were taken up into the denaturing Lamaeli buffer, heated to 100 °C, centrifuged and the supernatants were loaded onto the gel. After one-dimensional electrophoresis in a sodium dodecyl sulfate–15% polyacrylamide gel (SDS-PAGE) as described by Laemmli,[25] the proteins were then electrophoretically transferred onto nitrocellulose membranes. The immune-detection of PrP^{res} was carried out using the SAF84 antibody,[26] for which the known epitope lies between amino acids 126–160 of the human numbered sequence of PrP.

Successive dilutions of extracted PrP^{res} extract were carried out prior to Western Blot detection in order to determine the maximum dilution at which PrP^{res} could be observed.

In preliminary experiments the interaction parameters between *para*-sulfonato-calix[*n*]arenes and the prion protein, before or after digestion with Proteinase K, were explored (Fig. 3(a)). The obtained results show that, for addition of calixarene, **2b** before digestion with 10 μl PK at 300 μg ml^{-1}, the PrP^{res} signals were amplified in presence of 1 or 2 μl of 0.1 M calixarene, **2b**. At increased volumes of the *para*-sulfonato-calix[*n*]arene solution, from 4 μl and above added before PK digestion, interference with the PK activity was observed. On the contrary, lowered PrP^{res} signal detectability was recorded when the *para*-sulfonato-calix[*n*]arene was added after digestion with either of two PK concentrations. After repeated experiments the optimum reaction parameters to be used in routine testing of field samples were determined; addition of 1 μl of *para*-sulfonato-calix[*n*]arene at 0.1 M to 100 μl 10%

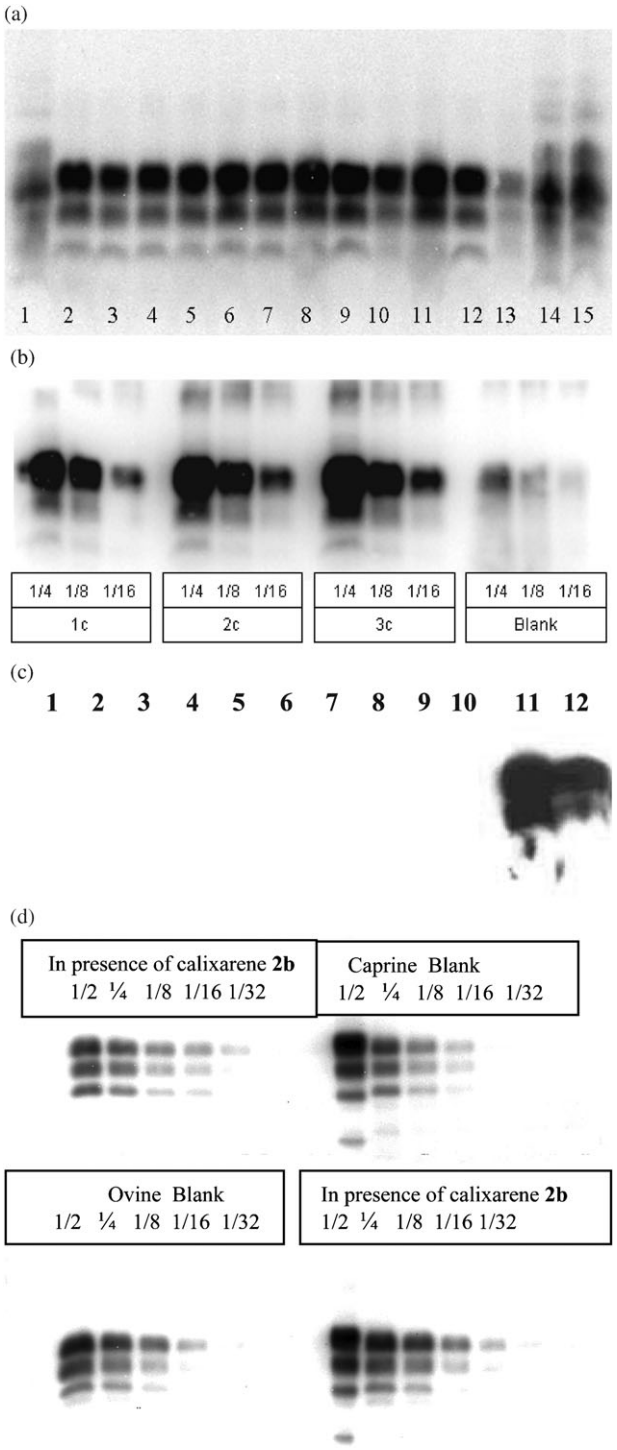

Fig. 3 (a) Western Blot detection of PrP^{res} prepared from BSE confirmed positive cow brain, culled at a rendering plant, in the presence of 0 µl (tubes 1–3), 1 µl (tubes 4–6), 2 µl (tubes 7–9), 4 µl (tubes 10–12) or 8 µl (tubes 13–15) of 0.1 M calixarene **2b**; which were added before PK treatment (tubes 5, 6, 8, 9, 11, 12, 14 and 15) or after digestion with 10 µl PK at 200 µg ml^{-1} (tubes 4, 7, 10 and 13). In tube 1 no PK was added, 10 µl of 200 µg ml^{-1} PK was added to tubes 2, 5, 8, 11 and 14 and 10 µl PK at 300 µg ml^{-1} was added to tubes 3, 6, 9, 12 and 15. (b) Western Blot detection of bovine PrP^{res} prepared in the presence of *para*-sulfonato-calix[*n*]arene derivatives **1c**, **2c** and **3c**. The PrP^{res} were diluted at 1/4, 1/8 and 1/16. The blank was carried in the absence of calixarenes. (c) Western blot detection of PrP^{res} present in brains of calves of 6 months age (lanes 1–10) and from confirmed positive BSE cattle brains (lanes 11 and 12) in the presence of 1 µl of a 0.1 M solution of *para*-sulfonato-calix[*n*]arene derivative **2b**. (d) Western Blot detection of ovine PrPres (left) and caprine PrP^{res} (right) diluted at 1/2, 1/4, 1/8, 1/16 and 1/32 with addition of *para*-sulfonato-calix[*n*]arene derivative **2b**. The blank was carried out in absence of calixarene.

brain suspensions, incubation for an hour at 37 °C then digestion with 10 µl PK at 300 µg ml^{-1} for another hour.

The blank lanes in Fig. 3(b) show that the maximal dilution for immune-detection of PrP^{res} by SAF84 in the absence of calix[*n*]arene derivatives is at the dilution of 1/8. However, in presence of calixarenes **1c**, **2c** and **3c**, increased intensities of PrP^{res} Western Blot bands was recorded until the dilution 1/16. Two to eight times higher PrP^{res} titres were observed in the presence of various *para*-sulfonato-calix[*n*]arene derivatives.

The supramolecular interaction of calix[*n*]arene **2b** with PrP^{res} allows the extension of the detection limit of the pathogenic protein by at least a factor of 8. It is clearly evident that this extension of the detection limit of the PrP^{res} should not be accompanied by the appearance of the detection of false positive samples of the protein as is shown in Fig. 3(c). Thus we selected *para*-sulfonato-calix[*n*]arene derivative **2b** for further experiments.

In order to probe effects of calixarenes on the detectability of PrP^{res} present in brain extracts obtained from a known positive scrapie infected sheep and a goat, SAF84 Western Blot immunodetection of PrP^{res} obtained results showed again amplification of the obtained PrP^{res} band signals (Fig. 3(d)). The observed molecular mass of the non-glycosylated band of the goat PrP^{res} in presence and absence of calixarenes was lower at the dilution 1/2 and increased to the normal size at the following dilutions.

It has been noted above that both the size of the calix[*n*]arene macrocycle and mono-substitution at the phenolic face may lead to significant modification of the interactions between the *para*-sulfonato-calix[*n*]arenes and amino-acids[19] or proteins.[21] In order to probe the differences arising from the size of the macrocycle and of the nature of mono-substitution at the phenolic face in the interaction between PrP^{res} and *para*-sulfonato-calix[*n*]arenes, SAF84 Western Blot immunodetection of bovine PrP^{res} was carried out in the presence of nine calix[*n*]arene derivatives using brain extracts from a known positive case. Three dilutions of the extracted PrP^{res} were used pure, diluted at 1/4 and diluted at 1/16. The results are shown in Fig. 4.

Optical scanning of Western Blot electrophoregrams for 1 h allowed the determination of the optical density (counts mm^{-2}) of the three bands, non-glycosylated, mono-glycosylated and di-glycosylated, corresponding to the three glycoforms of PrP^{res}. Given that the experiment was carried out on a single sample we consider it reasonable that the optical densities reflect the differences in binding of the *para*-sulfonato-calix[*n*]arenes to the PrP^{res} protein.

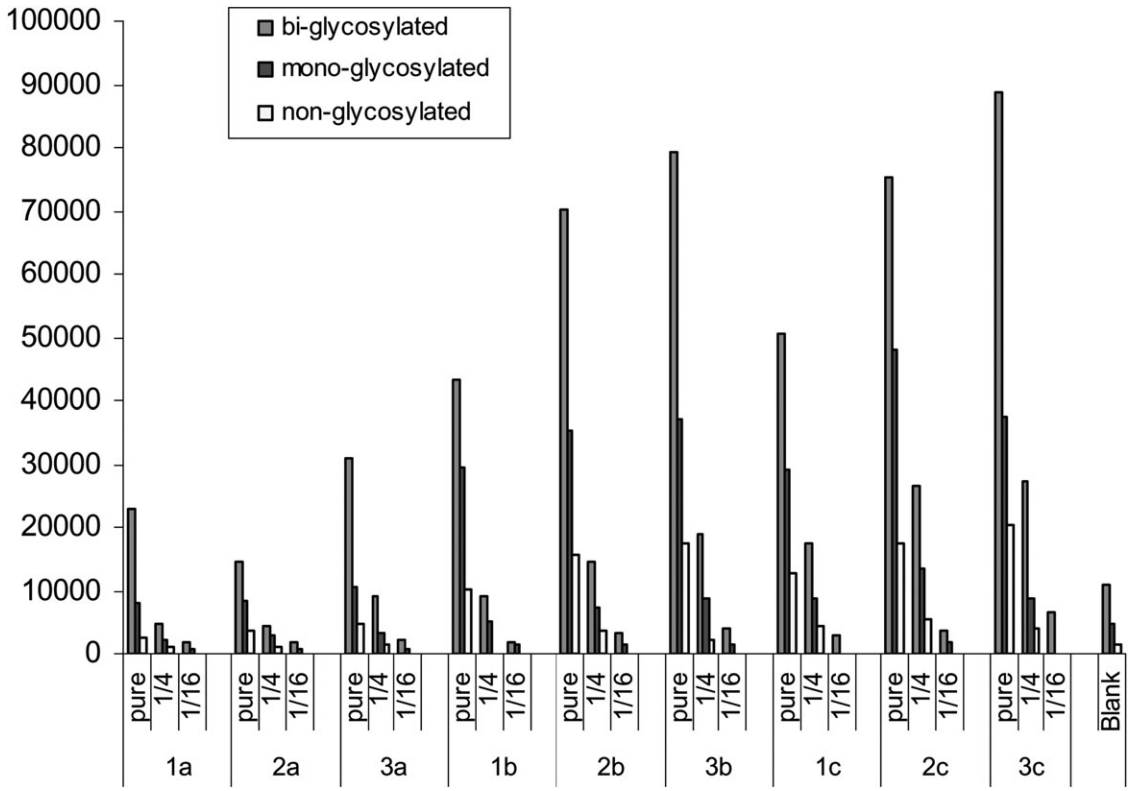

Fig. 4 Optical densities of scanned Western Blots showing the interaction of various calixarenes and brain extracts from a known positive sample. Counts mm^{-2} of the Western Blot bands obtained from PrP^{res} pure and diluted to 1/4 and 1/16.

The optical density for the undiluted blank, *i.e.*, in the absence of the *para*-sulfonato-calix[*n*]arene derivatives, is 11 000 counts mm^{-2} as shown in the histogram.

For the unsubstituted *para*-sulfonato-calix[*n*]arenes, **1a** (23 000 counts mm^{-2}), **2a** (15 000 counts mm^{-2}), **3a** (31 000 counts mm^{-2}), there exists a small amplification in the optical densities with **3a**. Here the effect of the size of the macrocycle is $8 > 4 > 6$.

However much stronger amplification of the signal is observed for both the monomethoxycarboxy and monoethoxyammonium derivatives. Here, there is an effect arising from the macrocycle size (n), with $n = 4 < n = 6 < n = 8$ for both types of mono-substitution (43 000, 70 000 and 79 000 counts mm^{-2} for **1b**, **2b** and **3b**, respectively; and 51 000, 75 000 and 89 000 counts mm^{-2} for **1c**, **2c** and **3c**, respectively), the strongest amplifications are observed for **3b** and **3c** showing 7-fold and 8-fold amplification of the signal, respectively. The difference in Western Blot detection between the two types of substitution are however relatively small. From the above the choice of **2b** as the *para*-sulfonato-calix[*n*]arene derivative of choice may at first sight appear to be non-optimal, however as the main aim of this work is to produce a working industrial scale diagnostic test for the various prion associated diseases the much lower yields associated with mono-substitution of calix[8]arene as compared to those for calix[6]arene,[19] rule out the use of *para*-sulfonato-calix[8]arenes in such a diagnostic test.

It is clear that the supramolecular interactions between certain *para*-sulfonato-calix[*n*]arenes, in particular *para*-sulfonato-calix[8]arene monoethoxyamine and the pathogenic prion PrP^{res} lead to an amplification of the detection of this protein by Western Blot immunodetection using the SAF84 antibody. This amplification may be postulated to arise from a more favourable presentation of the epitope for SAF84, arising from complexation between the calix[*n*]arene derivative and PrP^{res}.

Two putative binding sites for the *para*-sulfonato-calix[*n*]arenes are present in PrP^{sc}. These occur between Trp99-Ala113 and Arg148-Gln161 in the peptide sequence of the protein, shown below in Fig. 5. Both these peptide sequences are rich in positively charged amino-acids, 99–113: WNKPSKPKTNMKHVA, and 148–161: RYYRENMHRYPNQ, respectively, in the PrP human numbered sequence. Similar sequences are present in bovine and ovine PrP. The SAF84 epitope is situated at 126–164 in the peptide sequence and thus is positioned between the two probable binding sites.

Binding of the *para*-sulfonato-calix[*n*]arenes to the two peptides was demonstrated by ESI mass spectrometry (Fig. 6).[20] Analyses of the relative peak intensities of the observed complexes suggest that there is little differentiation in the binding of the *para*-sulfonato-calix[*n*]arenes. However, in the current study the complexation of the *para*-sulfonato-calix[*n*]arenes with the peptides lead to precipitation and no quantitative data could be obtained.

Pathogenicity of the prion protein is associated with conformational changes in the structure of the protein with presence of beta-sheets increasing. However, ^{1}H NMR studies by Prusiner showed that while the sequence 124–231 is

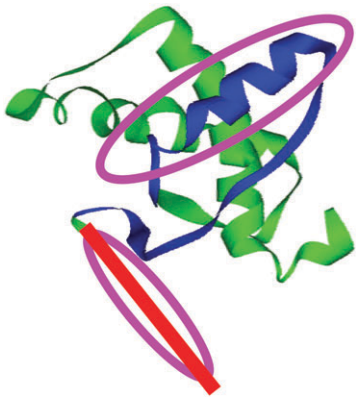

Fig. 5 Structure of the PrP. Blue line: epitope for SAF84. Red line: according to Prusiner is non-structured.[27] Pink ovoids show the putative binding sites for *para*-sulfonato-calix[*n*]arenes.

structured as an alpha-helix, the sequence 90–123 is unstructured.[27] The presence of mobile peptide segments in the amyloid associated HET-s prion, also containing lysine and arginine amino-acids has also recently been reported by Meier and co-workers.[28]

We propose that interaction between the *para*-sulfonato-calix[*n*]arenes and the binding site or sites on PrP^{res} may cause a structuring effect on certain peptide sequences and thus leads to a change in the three dimensional presentation of the epitope for SAF84, leading to the observed amplification of the detection of PrP^{res}.

The established parameters for PrP^{res} detection in presence of *para*-sulfonato-calix[*n*]arenes were tested in parallel with the official technique,[29] and the results obtained were, in general, in concordance with those obtained for this method. However, a small but significant number of BSE suspected cattle brains were detected weak but positive in presence of *para*-sulfonato-calix[*n*]arenes but were officially declared negative. Careful repeated testing with the official WB method confirmed these weak positive results, Fig. 7. The positive controls (lane 15 in both in the presence and absence of **2b**, and lane 11 only in the presence of **2b**), and three other field samples (lanes 12–14), were positive in the presence and absence of calixarenes.

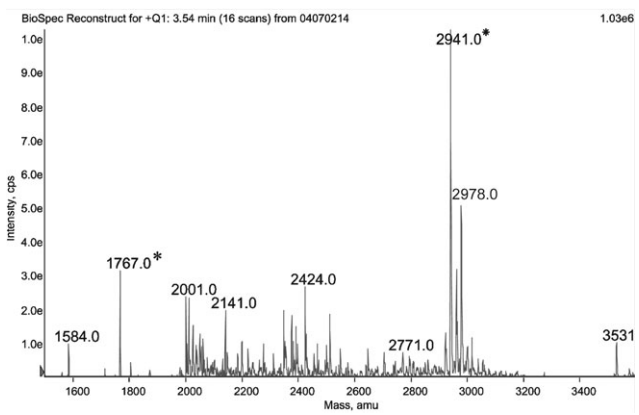

Fig. 6 ES/MS spectra of complexation of calixarene **2b** with peptide 99–113. The peak at 1767 UMA corresponds to peptide, the peak at 2941 UMA corresponds to the complex.

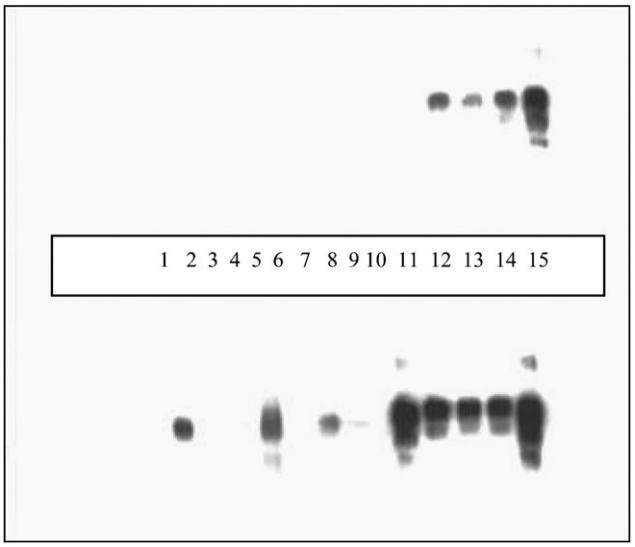

Fig. 7 Western Blot detection of 13 BSE suspected field samples in absence (upper) and in presence (lower) of 1 μl of 0.1 M *para*-sulfonato-calix[*n*]arene **2b**. Two confirmed BSE positive controls were loaded onto lane 11 (lower well) and in lane 15 (upper and lower wells). Buffer was loaded in lane 11 (upper well).

Higher PrP^{res} band signals were recorded in the presence of calixarenes. Three samples loaded on lane 2, 6 and 8 were negative in absence of calixarene **2b** but were weakly positive in its presence. The other seven animals were found negative for PrP^{res} even in presence of **2b** and were officially confirmed negative.

Finally, by the use of *para*-sulfonato-calix[*n*]arenes and streptomycin together for PrPres extraction from BSE brain suspension, highly amplified PrPres bands were obtained showing that higher detection sensitivity is achieved (Fig. 8).

Conclusion

In conclusion we have shown that the supramolecular interaction between *para*-sulfonato-calix[*n*]arene derivatives and, in particular, the *para*-sulfonato-calix[8]arene-monoethoxyamine and the pathogenic prion protein PrP^{res} yields an amplification in the Western Blot detection by the SAF84 antibody. Higher amplification of PrP^{res} bands were observed by the use of both calixarenes and streptomycin. This yields to a considerable extension of the lower detection limit of this protein which is

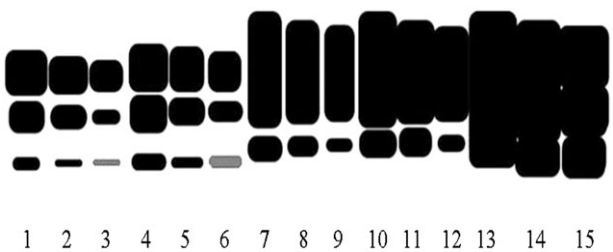

Fig. 8 PrPres detection of diluted (1/5, 1/10 and 1/20) BSE brain in the absence (lanes 1–3) and in the presence of 1 μl of 0.1 M calixarenes (lanes 4–6) and after their precipitation of the latter with 5% (lanes 7–9), 10% (lanes 10–12) and 15% (lanes 13–15) streptomycin 0.7 M.

responsible for Bovine Spongiform Encephalopathy and a new variant of Creutzfeldt–Jakob Disease. The utility of this new method is underscored by its ability to detect positive samples, hitherto undetectable by standard tests, and hence substantially improve current efforts to prevent contact of the human population with infectious, prion containing, biomaterials. The method has proved to be transposable from animal PrP^{res} to human PrP^{res},[30] and the results of this will be reported in a subsequent paper.

Experimental

General

All chemicals were purchased from Acros Organic other than Proteinase K purchased from Boehringer and used without further purification. All chemical reactions were carried out under nitrogen.

^{1}H and ^{13}C NMR spectra were recorded at 25 °C on a Varian NMR instrument operating at 500 and 121.5 MHz.

ESI/MS experiments were performed on a Perkin Elmer Sciex API 165; MALDI-TOF on a Voyager-DE PRO (Applied Biosystems).

Synthesis of the *para*-sulfonato-calix[*n*]arene derivatives

All the calix[*n*]arene derivatives used in this study were synthesised by the literature methods and the physical data were in full accord with those previously published.[19]

Preparation of the PrP samples in the absence of *para*-sulfonato-calix[*n*]arene

0.5 g of the confirmed TSE positive brain tissue was ground in 4.5 ml of 5% glucose solution to obtain a 10% W/V suspension. Then 10 µL of Proteinase K at 200 µg ml^{-1} were added to 100 µL of the 10% brain homogenate. The mixture is vortexed and incubated at 37 °C for 1 h, 100 µl of denaturing Lamaeli buffer were added, heated to 100 °C, centrifuged and the supernatants are loaded onto SDS-PAGE gel.

Preparation of the PrP samples in the presence of *para*-sulfonato-calix[*n*]arene

Before PK digestion. To 100 µl of the above 10% brain suspension was added 1 µl of 0.1 M the relevant *para*-sulfonato-calix[*n*]arene. After 1 h at 37 °C, 10 µl of Proteinase K at 300 µg ml^{-1} was added and the mixture vortexed and incubated at 37 °C for another hour. After adding 100 µl Laemmli denaturing buffer,[26] 5 min heating at 100 °C, centrifugation at 12 000*g* for 5 min, supernatants were recovered for run on SDS-PAGE.

After PK digestion. To 100 µl of the above 10% brain suspension was added 10 µl of Proteinase K at either 200 or 300 µg ml^{-1} and the mixture vortexed and incubated at 37 °C for 1 h then 1 µl of 0.1 M calixarene was added, vortexing and the tube held for 1 h at 37 °C. After adding 100 µl Laemmli denaturing buffer, 5 min heating at 100 °C, centrifugation at 12 000*g* for 5 min, supernatants were recovered for run on SDS-PAGE.

Preparation of PrP samples using *para*-sulfonato-calix[*n*]arene derivatives and streptomycin

To 100 µl of the above 10% brain suspension was added 1 µl of 0.1 M calixarene **2b**. After 1 h at 37 °C, 10 µl of Proteinase K at 300 µg ml^{-1} was added and the mixture vortexed and incubated at 37 °C for another hour. Then 5, 10 or 20 µl of streptomycin 0.7 M were added and the mixture was subjected to vortex stirring then re-incubated at 37 °C for 1 h. After adding 100 µl Laemmli (10) denaturing buffer, 5 min heating at 100 °C, centrifugation at 12 000*g* for 5 min, supernatant liquids were rejected. To each pellet 100 µl of 50% v/v 8-M urea and Laemmli denaturing buffer was added. After vigorous vortex stirring, 5 min heating at 100 °C, and 5 min centrifugation at 12 000*g*, the second supernatant liquids were collected and run on SDS-PAGE.

Preparation of the samples for Western Blotting

The samples were loaded either non-diluted or diluted 1 : 2 with Laemmli denaturing buffer to the desired dilution, heating for 5 min at 100 °C, centrifugation at 12 000*g* for 5 min, supernatants were recovered for runs on SDS-PAGE.

Immunodetection of PrP^{res}

After running on a one-dimensional electrophoresis in a sodium dodecyl sulfate–15% polyacrylamide gel (SDS-PAGE) the proteins were then electrophoretically transferred onto nitrocellulose membranes and immunoblotted at room temperature for 60 min with a monoclonal antibody directed to the 126–160 amino-acids epitope. The secondary antibody (1/5000) was a horseradish peroxidase-conjugated goat anti-mouse IgG (H + L). Blots were finally washed and signals were detected by chemiluminescence either with an ECL kit (Amersham) onto films (Biomex light, Kodak) or with super Signal Ultra (Pierce) and visualization in Fluor S Multimager (BioRad).

Peptide synthesis

Peptides were synthesized on a Milligen 9050 apparatus with Fmoc-OH/DIC/Hobt chemistry. The peptides were also cleaved by a TFA solution with classical scavengers and precipitated in diethyl ether. The precipitate was centrifuged. The centrifugation pellet was solubilized in water and lyophilised. The crude peptides were dissolved in water and purified on a Vydac column (C18, 5 µm, 250 × 10 mm) with an appropriate gradient of B (70% acetonitrile, 0.09% TFA solution in water).

Purified peptides were characterized by Electrospray Mass Spectrometry at 1766 UMA for peptide 99–113, and 1825 UMA for peptide 148–161, and by HPLC apparatus HP 1100 on analytical column Vydac (C18, 5 µm, 250 × 4.6 mm) in a gradient of 30 min from 10% to 90% of B.

Electrospray mass spectrometry

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^{-1} flow rate in a CH_3OH–H_2O (50 : 50, v/v) mixture containing 0.1% of HCOOH. To improve the signal-to-noise

ratio, 20–30 scans were accumulated, with ion spray and orifice potential set to 5000 and 35 V. The scan range was set at m/z 1000–4000.

The samples were introduced as 20 mM of the relevant calixarene solution with 1 equivalent of the relevant peptide, in a 0.02 M ammonium acetate buffer (pH = 7).

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