Improving Spin Probe Methodologies to Investigate Supramolecular Assemblies

Improving Spin Probe Methodologies to Investigate Supramolecular Assemblies

Marco Lucarini*[a]

Dedicated to Prof. Gian Franco Pedulli on the occasion of his 80th birthday

Abstract: In this report, our work describing the use of spin probes in the field of supramolecular chemistry and how electron spin resonance (EPR) has been used for detecting and identifying supramolecular assemblies is shortly reviewed. Selected examples are reported, including paramagnetic host-guest complexes, self-assembled systems doped with spin probes, spin-labelled macrocycles and open shell mechanical interlocked structures (MIMs) such as rotaxanes, in which the dumbbell, the wheel or both are tagged with nitroxide **1.**

1. Introduction

Since the first report of stable and persistent free radical,[1] organic radical chemistry has played an important role in many aspects of chemical science over more than a century. Radicals are key reactive intermediates in many relevant chemical processes like combustion, polymerization, oxidative damage atmospheric chemistry, synthetic chemistry etc.[2] Although organic radicals are generally transient species, they can become very persistent when delocalization and steric protection appropriately cooperate like in 2,2,6,6-tetramethylpiperidinyloxyl (TEMPO) radical where the unpaired electron is stabilized by delocalization between oxygen and nitrogen atoms and the four methyl groups prevent its disappearance by disproportionation reaction.[3] The existence of persistent radicals makes electron paramagnetic resonance (EPR)[4] manageable as a supplementary spectroscopically tool to investigate the properties of chemical systems. Compared to popular NMR techniques, the main advantages of EPR are the sensitivity of the method (micromolar concentrations of radical can be detected); the possibility of obtaining kinetic information in the submicrosecond time range; the possibility of measuring distances between radical centers in the range 0-100 Å.[4] Because of these favourable features, spin probing and spin labelling methodologies have largely expanded beyond the classical context of biology and structural biochemistry to other fields. In the last two decades, our group (and many others) systematically explored the use of organic radicals as spin probes and spin labels in supramolecular chemistry. This topic has been reviewed regularly,[5] and only selected examples, mainly reported from our group in Bologna, will be shortly described here. Because of these favourable features, spin probing and spin labelling methodologies have largely expanded beyond the classical context of biology and structural biochemistry to other fields. In the last two decades, our group (and many others) systematically explored the use of organic radicals as spin probes and spin labels in supramolecular chemistry. This topic has been reviewed regularly,[5] and only selected examples, mainly reported from our group in Bologna, will be shortly described here.

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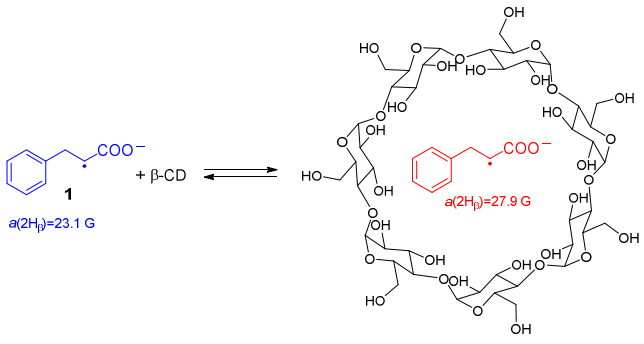
2. Host-guest systems

2.1. Benzyl *tert*-butylnitroxide as spin probe

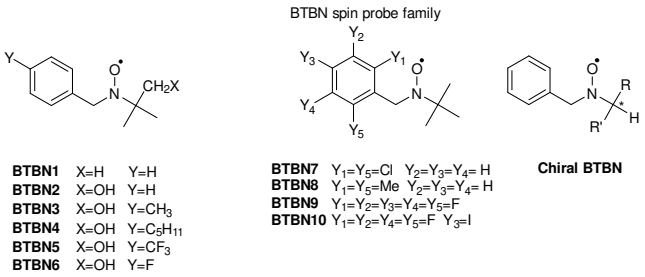
Our interest in the use of EPR for studying supramolecular chemistry started in 1996 in the context of a collaborative project with Prof. B. P. Roberts at University college of London. We succeed in including inside the hydrophobic cavity of a β–cyclodextrin (β-CD) the very short lived α-carboxyalkyl radical **1** (Scheme 1).[6] The inclusion leads to a change in the conformation adopted by the radical upon inclusion as indicated by the variation of the hyperfine splitting of the two β-hydrogen atoms, $a(2H_\beta)$, whose magnitude depends on the geometry adopted by the radical around the orbital containing the unpaired electron.[7]



Scheme 1. Complexation equilibrium between the α-carboxyalkyl radical **1** and β-CD.

Starting from this result, we looked for a more persistent radical in which a combination of polar and conformational effects might give rise to large differences in the resonance frequencies of the included and free species. A good choice was represented by benzyl *tert*-butylnitroxide (**BTBN1**, Scheme 2). This probe, in the presence of different molecular hosts, afforded inclusion complexes

characterized by a significant change both in the value of the nitrogen hyperfine splitting, *a*(N), (due to the different polarity experienced in the host cavities), and in the coupling to the benzyl protons, $a(2H_\beta)$, (due to conformational changes upon complexation). Because of the presence of β-hydrogens, the life time of **BTBN1** is significantly shorter if compared to sterically hindered nitroxides like TEMPO and thus the radical must be generated *in situ* by oxidation of the parent amine with organic oxidants. This in principal could constitute a severe limitation in using **BTBN1** as spin probe, but the presence of *tert*-butyl group gives to it a persistency long enough (in normal conditions from several minutes to hours) to permit the recording of several EPR spectra before changing the sample.



Scheme 2. BTBN family of spin probes described here.

Another favourable property showed by **BTBN1** in the presence of most of the host systems so far investigated, is represented by the strong alternating EPR linewidth dependence which is observed above 298 K. This effect is due to the switch of the radical between the free and complexed form which occurs with a rate comparable to that of the EPR timescale.[4] Analysis of the EPR spectra, therefore, makes possible to determine not only the molar ratio between the complexed and free radical, and thus of the equilibrium constant for the complexation process, but also to get information on the kinetic of the inclusion.

2.2. Cyclodextrins (CDs)

Cyclodextrin hosts were the first molecules investigated by our group with BTBN1.[8] With both unsubstituted and substituted β-CD and γ-CD the nitrogen *a*(N) and β-protons $a(2H_\beta)$ hyperfine splittings in free and included **BTBN1** differed significantly and determination of equilibrium constants for the complexation of the radical probe was quite straightforward. As mentioned in the introduction, EPR spectra of **BTBN1** show selective line broadening indicating that the lifetime of the radical in the CD cavity and in solution is comparable to the EPR timescale. Under these conditions, computer simulation of the spectra enabled us to determine the single rate constants for the association and dissociation (see Figure 1). While a large number of equilibrium constants for the formation of CD complexes were known from the literature, only few determinations of the *rates* of association and dissociation for CD complexation, mostly obtained by using complex stopped-flow or temperature-jump experiments, were available at that time.[9]

As a first example, the EPR method was employed for studying the effect of aliphatic alcohols on the kinetic of complexation by CDs.[10] In this work we also found that substitution of the *tert*-butyl hydrogens of **BTBN1** by deuterium atoms significantly improve the spectral resolution of EPR lines and, thus, the accuracy in measuring the kinetic rate constants.

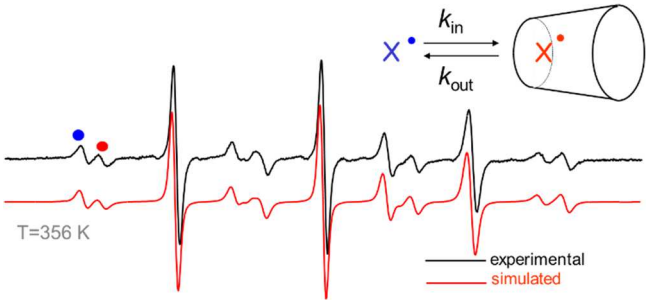


Figure 1. EPR spectrum of **BTBN1** recorded in water in the presence of β-CD 3.2 mM at 356K. The theoretical simulation (in red) was obtained by using $k_{in} = 2.3\times10^9$ M^{-1} s^{-1}, $k_{out} = 8.7\times10^6$ s^{-1}.

In a second example the combined use of **BTBN1** and EPR provided the partitioning rates of the organic probe in CD–sodium dodecyl sulfate (SDS) micelle systems (see Figure 2).[11] Because of the significant differences in the EPR parameters shown by **BTBN1** when it experiences water, CD cavity or micellar environments it was possible, for the first time, to simultaneously measure the concentration of the organic spin probe in these three different "pseudo-phases". The residence time of the probe in each environment determined by EPR was also used to predict the electrophoretic behaviour of the diamagnetic carbonyl analogue of BTBN, benzyl–*tert*–butyl ketone, in CD-micellar systems employed for separation of chiral organic solutes.[12]

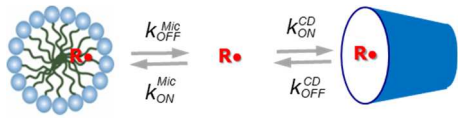


Figure 2. Kinetic scheme describing the exchange of the radical probe R● between the water phase and both the CD cavity and the micellar pseudo-phase. All the kinetic constants were determined by line width analysis of the corresponding EPR spectra.

We also investigated the kinetic of CD inclusion of BTBN derivatives (**BTBN7**-**BTBN8**) substituted at the aromatic ring. In this case we could distinguish different geometries

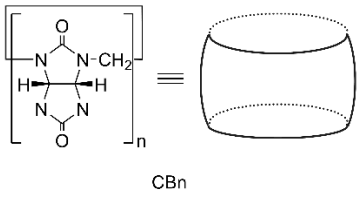
of inclusion complexes providing a data set for substituent effects on EPR parameters and association rate constants of complexation.[13] This type of analysis can provide useful information when the direction of incorporation of a guest into a host and the relative rates are important for the construction of rotaxane based molecular machines.[14]

The internal hydrophobic cavity of cycledxtrin is chiral and for this reason CD are largely employed as chiral selectors in separation techniques in water medium. By synthesizing enantiomeric pairs of chiral nitroxides deriving from BTBN of general structure $PhCH_2N(O\bullet)CH(R)R'$ (Scheme 2) it was also possible to investigate by EPR the relationship between the stereochemistry of eptakis-(2,6-O-dimethyl)-β-CD complexes and the thermodynamics of complexation.[15] This was done by correlating EPR data with ^1H-^1H NOE measurements carried out on the complexes containing the amine precursors of nitroxides. NOE data suggested that inclusion of the stereogenic center in the CD cavity occurs only when the R substituent linked to the chiral carbon holds an aromatic ring. Molecular dynamic calculations performed on these complexes, indicated that the observed chiral selectivity depends on the depth of penetration of the stereogenic center into the CD cavity which in turn is determined by the nature of the second substituent at the asymmetric carbon.

If a symmetric nitroxides like dibenzylnitroxide or di-*tert*-amylnitroxide, are employed as spin probes in the presence of the unsubstituted β-cyclodextrin, it is also possible to observe the formation of a supramolecular structure with a higher level of organization in which the radical guest is bound to two molecules of β-CD.[16] The life time of the nitroxide is sufficiently long to allow reversibly switching from 1:1 to 1:2 complexes many times by repeating acid–base treatments or by changing the temperature without occurrence of any side reaction. To the best of our knowledge, these 1:2 complexes represented the first examples of three-component, organic free radical supramolecular entities, which can be reversibly formed by changing pH or temperature.

2.3. Cucurbiturils (CBs)

Cucurbit[n]urils (CBn, n=5–8, 10) are a family of synthetic macrocycles that, similarly to CDs, are characterized by a hydrophobic cavity which is able to form a wide range of host–guest complexes with organic and inorganic compounds in water.[17]



Scheme 3. Structure of Cucurbit[n]uril (CBn).

The first EPR investigation exploring the binding properties of this relatively new class (at that time) of macrocyclic hosts was reported by our group in 2007 by using both **BTBN1** and TEMPO spin probes.[18] CB7 was selected because, the dimension of its cavity, being comparable to that of β-cyclodextrin, allows the formation of very strong inclusion complexes with many organic guests As already observed with CDs, the values of the nitrogen, a(N), and β-proton, $a(2H_\beta)$, splittings decrease significantly upon inclusion into the less polar environment of the CB7 host cavity, giving rise to the remarkable differences in the resonance frequencies of the included and free species. This favourable spectroscopic feature was employed to study the effect of alkali metal cations on the complexation of the probe. Addition of latter ones to the solution containing CB7 and **BTBN1** led to the appearance of signals of a new species identified as a radical hosted in the CB7 cavity in which one metal cation is in close contact with the nitroxidic oxygen and two neighbouring CB7 portal oxygen atoms (See Figure 3). The formation of the coordination complex results in a substantial increase in the electron spin density on the nitrogen in inverse order with respect to the size of the cation owing to the increased localisation of negative charge on the oxygen atom from bonding to the alkali cation. Analysis of the EPR linewidth made it possible to measure, for the first time, the kinetic rate constants for the metal exchange between bulk water and the coordination complex.

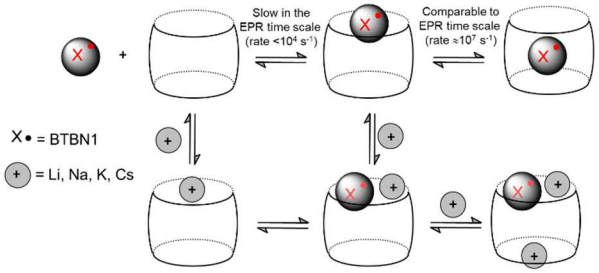


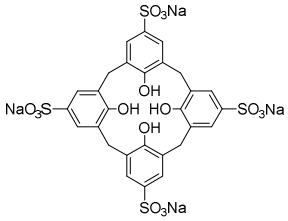
Figure 3. Equilibria involved in the complexation of BTBN1 by CB7 in the presence of alkali metal cations.

In the same work the behavior of "conventional" TEMPO nitroxide probe was also investigated. The complexed TEMPO radical shows significant smaller nitrogen hyperfine splitting than the corresponding free species. Although this effect was expected because of the less polar environment experienced by the nitroxyl group within the CB7 cavity, the spectral resolution of the signals due to the free and included radical resulted much higher than that generally observed in water with other macrocyclic host, such as CD. Analysis of EPR spectra provided the value of the binding constant at room temperature for TEMPO complexation as 25000 M^{-1}, that is about one order of magnitude larger than that measured for the same guest with β-cyclodextrin (2950 M^{-1}).[19] After this paper, both us and several other research groups reported EPR investigations on CB derivatives.[20] In

this context, it is worth recalling that the high stability of nitroxide-CB complexes was favourably exploited by the group of Paul Tordo in Marseille to increase the life-time of nitroxide radical under reductive conditions.[21] In particular, they found that the half-life of TEMPO in the presence of CB7 and 2mM ascorbate anion is increased of sixty times respect to that of the free nitroxide being about 254 min. at room temperature. This value is exceptionally high and CB should be considered a very interesting host system for *in vivo* applications that need long lifetimes of nitroxides.

2.4. Calixarenes

While a voluminous literature reporting the synthesis and characterization of calixarenes[22] soluble in organic solvents, relatively few examples of water soluble calixarenes were known in the 90s. They had been mainly employed to study the inclusion of charged organic species and very little was known on their inclusion properties towards neutral aromatic guest species.



Scheme 4. Structure of water soluble calixarene whose complexation properties were investigated by using BTBN1.

In 2000 we reported the use of **BTBN1** for studying the complexaion properties of a tetrahydroxycalix[4]arene tetrasulfonate (Scheme 4).[23] Differently to what observed with CDs and CB7 the inclusion produced marked differences in the $a(2H_\beta)$ hyperfine splitting constants while similar $a(N)$ values were found for the free and included species. This indicated that the nitroxide group in the radical is exposed to bulk water, and that inclusion induces a marked change in the geometry adopted by the included radical compared to the free probe. The EPR spectra also showed selective line broadening effects due to the exchange between free and included nitroxide which allowed us to measure for the first time the kinetic rate constants and the activation parameters for the inclusion of a neutral molecule in a calixarene host in water.[23]

3. Self-assembly

3.1. Nanoparticles

Well established long-standing collaboration with the group of Lucia Paquato at the University of Trieste allowed us to investigate, by means of functionalised BTBN, the properties of different type of water-soluble protected gold nanoparticles (AuNPs). In these systems, the organic monolayer of the nanoparticle, coupled with the radial nature of the gold core, creates ‘‘hydrophobic pockets’’ inside the monolayer where organic solutes can be partitioned very efficiently.[24] When a nitroxide probe is located in these low polar compartments the EPR nitrogen hyperfine splitting a(N) is significantly smaller than that measured in water and it is possible to distinguish a different signal for the radical located in the monolayer in equilibrium with the free nitroxide and thus to measure the affinity of the organic probe as a function of the monolayer composition or nanoparticle diameter. Probes used in these studies contain an aliphatic hydrocarbon or fluorinated chain at the *para* position of the aromatic ring while one of the methyl group is substituted with a hydroxymethyl group (**BTBN2-6**, Scheme 1).

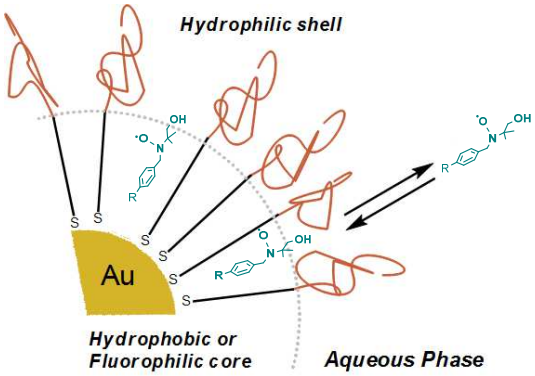


Figure 4. Schematic representation of the equilibrium involving the spin probe in the presence of water-soluble protected gold nanoparticles.

Similarly to those recorded with BTBN1, the EPR spectra of these probes were characterized by a strong alternating linewidth dependence on temperature, indicating that the lifetime of the nitroxide in the monolayer and in the bulk water is comparable to the EPR timescale. Thus, by analysis of the corresponding lineshape it was possible to obtain information on the kinetics of the exchange process.
NPs protected with monolayer of amphiphilic thiolates featuring hydrocarbon[25] or perfluorocarbon chains[26] (H- and F-chains) terminating with a short poly(oxoethylene) unit were investigated with this technique. Because of the different polar environment experienced, the nitrogen and hydrogen splittings were different when the radical probe was partitioned in the H- or F-chains. The favourable property of ‘‘distinguishing’’ the different nature of the monolayer by EPR was successfully exploited to study the topology of soluble gold NPs coated with a mixture of F- and H-amphiphilic thiolates in different ratios.[27] With these studies it was possible to demonstrate that in heteroligand monolayers phase separation can occur forming islands of homoligands triggered by the lipophobicity of perfluorocarbons.[28] These results were particularly useful

to confirm that the monolayer shows well defined regions due to the low mobility and to the specific orientation of the constituent thiols, contrary to micelles where it is impossible to know precisely how the monomers aggregate due to the disorder of the system.

3.2. Halogen Bonding

The halogen bond (XB) term is commonly utilized for defining non-covalent interaction comprising halogens as electron acceptors and electron donors. XB can be represented by the general scheme D-X–Y, in which X is the halogen (Lewis acid, XB donor), D is any electron donor (Lewis base, XB acceptor), and Y is carbon, nitrogen, halogen, etc.[29] We showed for the first time in solution that nitroxide functional group can work as a good electron donor, D, with several iodoperfluorocarbon halogen bond donors (X-Y) and that XB strength is comparable to hydrogen bonding[30] (in terms of equilibrium constants and other thermodynamic parameters).[31] The formation of an X–bonded TEMPO was manifested primarily as an increase in the nitrogen hyperfine coupling a(N), consistently with an increase in spin density at the nitrogen nucleus of the nitroxide. An even more convincing evidence for the formation of the halogen-bonded complex was provided by the marked broadening of the EPR lines observed when the nitroxide spectra were recorded in the presence of C_6F_5I or $C_8F_{17}I$ solvents (Fugure 5a).

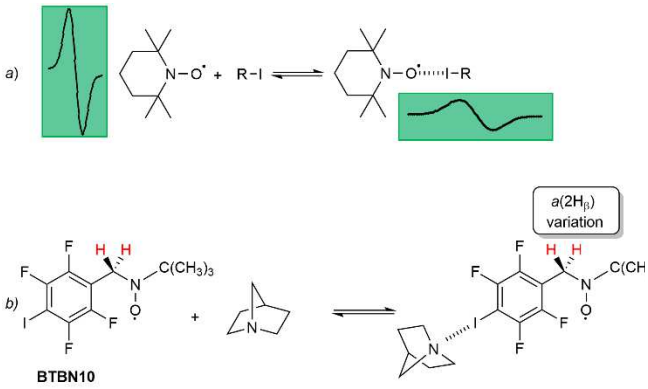


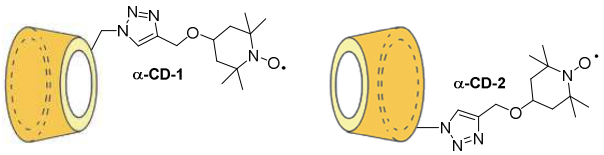
Figure 5. Schematic representation of XB-complex with TEMPO (a) and **BTBN10** (b). In the first case the XB complex formation was manifested by EPR line broadening, in the second case by variation of $a(2H_\beta)$ EPR couplings.

Similar results were successively observed by using an isoindoline nitroxide (TMIO) in the presence of pentafluoro-iodobenzene.[32] More recently XB interaction was detected by EPR by using a nitroxide radical belonging to BTBN family containing a iodoperfluoroaromatic recognition unit (**BTBN10**, scheme 2).[33] In this case, the formation of a XB complex was manifested by a significant change in the value of the benzylic hyperfine splitting upon complexation (see Figure 5b). This probe allowed also the measurement of the equilibrium constant for the formation of a XB complex with diamagnetic competitive electron donor D like chloride anions. The proposed procedure constituted the first direct methodology providing a reliable EPR measurement of the strength of XB in solution.[33]

4. Spin labelled host macrocycles

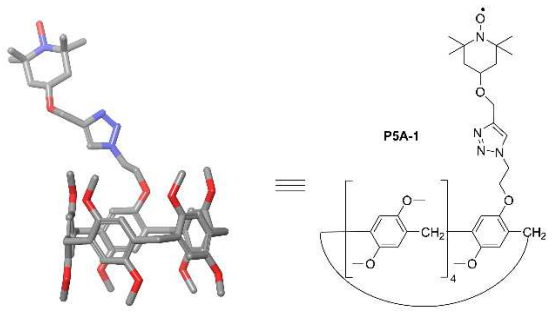
A large set of spin-labelled macrocyclic hosts have been synthesized and characterized in the last decades.[34] The main advantage of these spin probes consists in the possibility of using EPR spectroscopy for detecting binding event still in the presence of diamagnetic guests. Another stimulating opportunity is represented by the incorporation of a spin-labelled macrocycle in molecular interlocked molecules, MIMs, such as rotaxanes or catenanes, and the possibility of displacing the radical center between different recognition units by reversible external stimuli (see next paragraph).

Huisgen 1,3-dipolar cycloaddition reaction, also known as copper(I)-catalyzed click azide-alkyne reaction (CuAAC),[35] well tolerate aminoxyl functionalities and for this reason it has been successfully proposed by our group for the functionalization of different macrocycles. A first example comes from the TEMPO-monofunctionalization of α-CDs. The spin probe was introduced both at the C6 position of the primary rim (**α-CD-1**, Scheme 5) or at the larger secondary rim (C3 position, **α-CD-2**, Scheme 5) by CuI-catalyzed 1,3-dipolar cycloaddition of the monoazidodeoxy-α-CD with 4-propargyloxy-TEMPO.[36] These cyclodxtrins were successively employed for the preparation of a bis-labelled [2]rotaxane (see next paragraph).



Scheme 5. Spin-labelled cyclodextrins synthetized by our group.

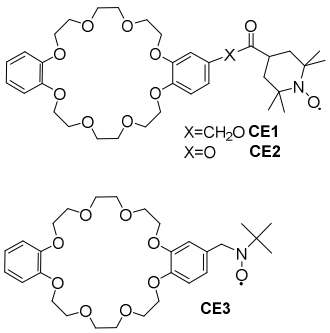
By employing the same “click” reaction it was also possible to introduce TEMPO spin label in a pillar[5]arenes (**P5A-1**, Scheme 6),[37] a member of a relatively new family of pillar-shaped hosts which have attracted considerable attention in host-guest chemistry due to their simple symmetrical structure and the electron-donating properties of the internal cavity that allows the formation of inclusion complexes in organic solvents with several electron-accepting molecules, such as viologen, pyridinium derivatives and imidazolium cations.[38]



Scheme 6. Spin-labelled pillar[5]arene **P5A-1**.

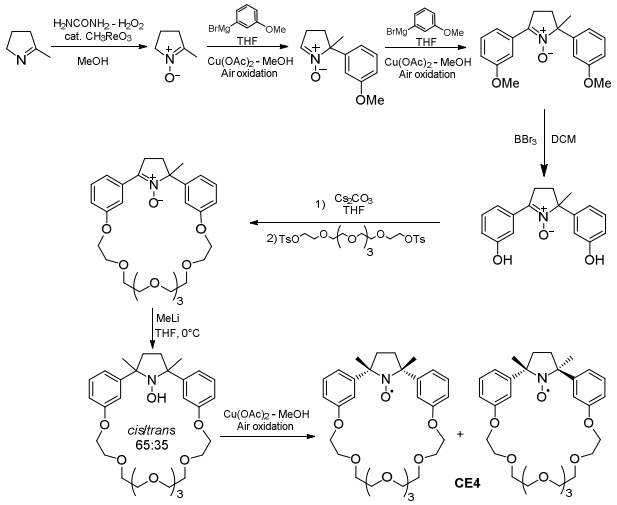
The host properties of **P5A-1** were tested in the presence of the cationic model guest 4-methyl-N-butylpiridinum. In $CHCl_3$ at 298 K we measured an association constant of 2572 M^{-1} which compared to the value of 2197 M^{-1} for the unfunctionalized pillar[5]arene, indicated that the paramagnetic arm does not hinder complexation processes. [37]

More recently, two paramagnetic crown ethers differing for the presence of a methylene unit connecting the aromating ring and the TEMPO spin label, were reported (**CE1**-**CE2**, Scheme 7).[39]-[40] They were prepared in good yield by reacting dibenzo[24]crown-8 ring alcohol with 4-carboxy-TEMPO derivatives in the presence of N,N'-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine as the condensing reagents. These spin labelled crown ethers revealed to be remarkably useful for detecting the movement in [2]rotaxanes containing the paramagnetic wheels and in elucidating their geometries (see next paragraph).



Scheme 7. Spin-labelled dibenzo[24]crown-8-ethers **CE1-3**.

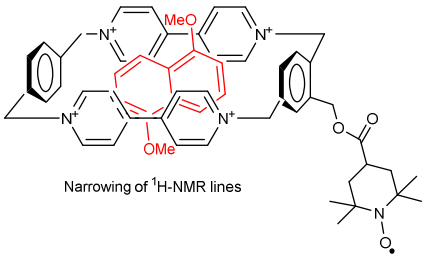
Unfortunately, they did not provide any EPR information on the complexation of metal cation by the ethereal oxygen. To this scope, we decided to employ the favourable features of BTBN family as EPR reporter for detecting cation complexation. The nitroxide **CE3** (Scheme 7), prepared by *in situ* oxidation of the corresponding amine with 3-chloroperbenzoic acid, was characterized by *a*(2H) values very responsive to binding events, this allowing us to distinguish between different cationic guests.[41] Amounts of cations in the order of mM concentrations could be easily detected by this method. The general good agreement with quantitative data determined with traditional techniques suggested that the proposed probe can be usefully employed to study complexation by crown ethers in supramolecular systems of higher complexity when traditional methods based on NMR or fluorescence cannot be applied.



Scheme 8. Reaction sequence for the preparation of the paramagnetic macrocycle **CE4**.

An alternative synthetic procedure for making a spin-labelled macrocycle consists in preparing a spin labelled fragment that is successively inserted by a cyclization reaction in the final ring structure. This approach was followed (by adapting the procedure reported by Keana *et al.*[42] for the preparation of a smaller ring) to obtain a new crown-ether wheel in which the nitroxidic was incorporated in the crown ether-like frame (**CE4**, Scheme 8).[43] The synthesis led to a mixture of *cis* and *trans* isomers in 6.5:3.5 molar ratio, as assessed by ^{1}H NMR on the corresponding hydroxylamine. The prevalence of *cis* isomer was attributed to the attack of MeLi to the nitrone intermediate occurring more easily from the opposite side of the bulky phenyl group. Also this spin labelled crown ethers was successfully incorporated in a bi-stable paramagnetic [2]rotaxane (see next paragraph).

Finally, a spin-labelled, *p*-electron-deficient, tetracationic cyclophane ring, cyclobis-(paraquat-*p*-phenylene) ($CBPQT^{4+}$), one of the most versatile hosts involved in the synthesis of molecular devices,[44] was prepared by our group (Scheme 9).[45]. Complexation of electron rich guest molecules like dimethoxynaphthalene was demonstrated by measuring line narrowing of nuclear magnetic resonances of $CBPQT^{4+}$ due to the displacement of the nitroxide probe from the host cavity after the binding event.



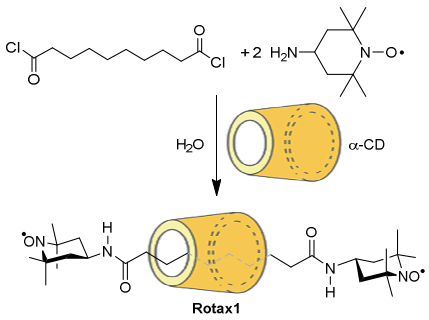
Scheme 9. Schematic representation of the complex between DMN and spin-labelled ($CBPQT^{4+}$).

5. Spin labelled rotaxanes

5.1. CD- and CB-based rotaxanes

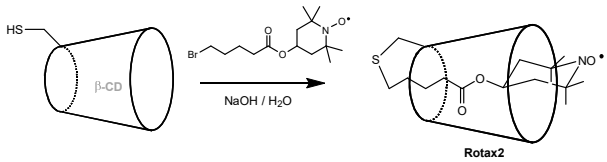
Most of the spin labelled host macrocycles described above were successfully employed as component of paramagnetic mechanically interlocking architectures (MIMs)[46] like rotaxanes and catenanes. These fascinating structures represent the typical prototype of molecular machines in which it is possible to have quasi-mechanical movements in response to specific stimuli. While a rotaxane consists of a linear molecule which is threaded through a ring with the ends of the thread, or axle, capped in such a manner that the ring cannot slip off, in a catenane two or more macrocycles are mechanically interlocked similarly to links in a chain. In both cases the single components are held together as a consequence of their topology and cannot be separated without breaking covalent bonds.[46]

While the earliest example of nitroxide spin-labelled [2]catenane was reported in 2003,[47] we described the first example of a spin-labelled [2]rotaxane containing α-CD as the wheel (**Rotax1**, Scheme 10) in 2006.[48] In this example, TEMPO nitroxide radicals behave as stoppers of an alkyl chain axle that was mechanical trapped inside the cavity of a α-CD. EPR spectrum of **Rotax1** showed complete suppression of through-space spin exchange between TEMPO end units which is instead clearly visible in the flexible free bis-axle. This observation provided a strong evidence for the formation of the MIM structure.



Scheme 10. Synthesis and structure of **Rotax1**.

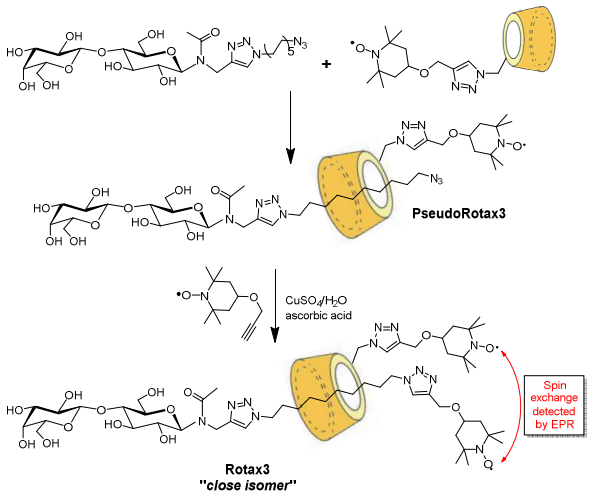
When a 6-mercapto-β-cyclodextrin was reacted in alkaline water with a sterically hindered nitroxide containing an alkylbromide functionality, it was possible to mechanically trap inside the cavity of CD, by a covalent link, the radical center, affording a rare example of a self-included [1]rotaxane (**Rotax2**, Scheme 11).[49]



Scheme 11. Synthesis and structure of the [1]rotaxane **Rotax2**.

Evidences for the formation of a true [1]rotaxane were obtained both by measuring NOE interactions between the CD internal H3 and H5 protons and the piperidine ring in the reduced N-hydroxyl form and by comparing the EPR spectrum of **Rotax2** with that of the analogous spin-labelled β–CD having the radical arm exposed to bulk water.

Some years later, we described a novel α-CD-based [2]rotaxane in which the axle and the wheel were both spin-labelled. (**Rotax3**, see Scheme 12).[50] By following threading-stoppering approach, the bis-labelled rotaxane was prepared by reacting the corresponding pseudorotaxane, consisting of a lactose-blocked C-10 half thread carrying an azido group, with an alkyne-TEMPO stopper unit. Although two possible structures can be predicted on the basis of the orientation of the CD along the thread, rotaxanation provided only one main isomer (the so-called "*close isomer*", see Scheme 12) whose geometry was determined both by the EPR measure of through-space spin exchange between the two mechanical assembled nitroxide units and by molecular dynamic calculations.



Scheme 12. Synthesis and structure of the bis-labelled rotaxane **Rotax3**.

We also synthetize and characterize an example of a bis-labelled cucurbit[6]uril-based [3]rotaxane (**Rotax4**, Figure 6).[51] In the present case PELDOR experiments were employed for the first time to measure the end-to-end distance between the two TEMPO groups in a MIM.[52] PELDOR[53] is a pulsed-EPR methodology that is now frequently used to determine distance distributions between native or synthetically introduced spin centres within a macromolecular structure. With such data, conformational flexibility of macromolecules can be depicted, structural models can be validated or geometry of macromolecular complexes can be determined.[53] In our case, a very narrow distribution of the end–end distances between the two TEMPO groups was obtained by the analysis of PELDOR data. This led to the conclusion that rotaxanation by two CB6 macrocycles has a dramatic effect on the conformation flexibility of the diradical.

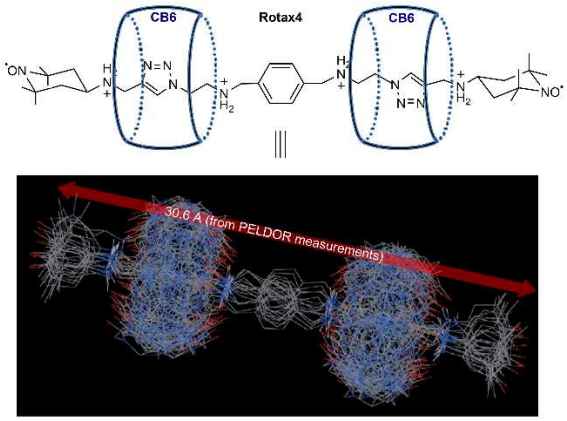
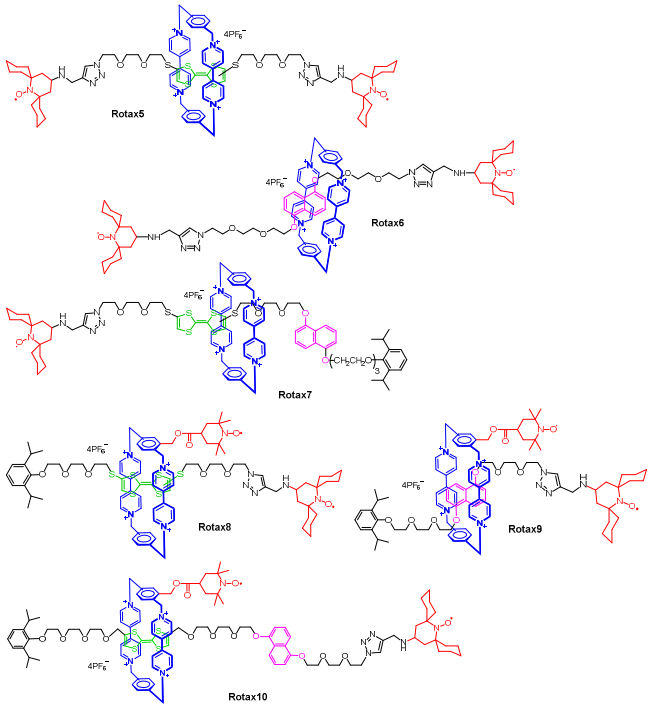


Figure 6. Structures of the [3]rotaxane **Rotax4** and molecular dynamics simulations in water (15 structures, corresponding to 30000 ps of simulation).

5.2. CBPQT- based rotaxanes

One of the most investigated programmable molecular switch is certainly represented by the "Stoddart–Heath type" bistable [2]rotaxane.[54] This consists of an aromatic shuttle, the cyclobis-(paraquat-*p*-phenylene) ($CBPQT^{4+}$) ring, and a molecular axle containing the two molecular stations tetrathiafulvalene (TTF) and 1,5-dioxynaphthalene (DNP). This rotaxane shows a reversible on/off switching which arises from moving the wheel between TTF and DNP by one-electron oxidation and back-reduction of the TTF moiety.[54]



Scheme 13. Spin-labelled CBPQT-based [2]rotaxanes synthetized by our group.

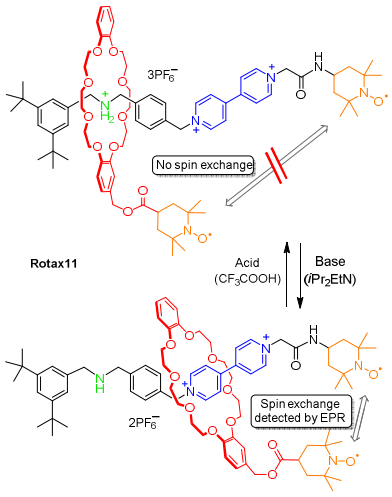
The series of spin-labelled [2]rotaxanes based on $CBPQT^{4+}$ wheel synthetized by our group[55] are reported in Scheme 13 (**Rotax5**-**Rotax10**). In all cases a more hindered nitroxide containing spirocyclohexyl substituents at 2 and 6 positions of the piperidine-N-oxyl ring was used as a terminal radical in place of classical TEMPO unit because this is not large enough to prevent unthreading of the wheel in CBPQT-based rotaxanes.

By EPR it was shown that through-space spin–spin interactions are the result of combined effects due to the movement of the wheel and the flexibility of the thread chain that in turn can be controlled by rotaxanation.[55] The possibility of reversibly moving rotaxane components provides an effective new strategy for the development of a novel generation of polyradicals where the magnetic interactions can be turned on/off by application of an appropriate stimulus.

5.3. Crown ether-based rotaxanes

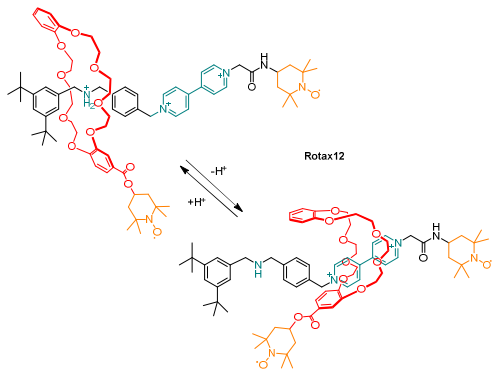
A collaborative project with the group of Alberto Credi inspired the incorporation of spin-labelled crown ether macrocycles **CE1**-**CE2** described in the previous paragraph in [2]rotaxanes possessing in the axle a dialkylammonium and a 4,4'-bipyridinium (BPY^{2+}) recognition sites and TEMPO radical as one of the terminal stopper unit (**Rotax11-12,** Schemes 14-15).[39]-[40] The proposed mechanical switch is based on the well-known process of movement of the crown ether after a change in the pH of the

solution.[56] Deprotonation of the dialkylammonium group results in a quantitative displacement of the ring to the BPY^{2+} recognition site, a process that can be reversed by acid treatment.



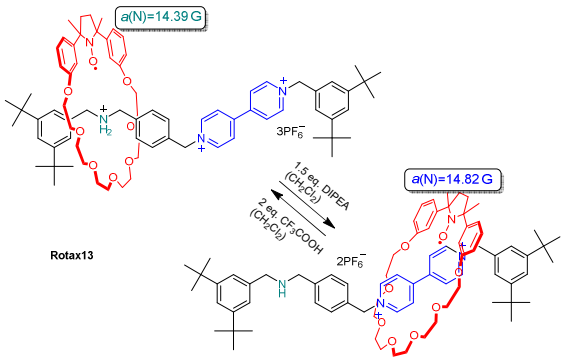
Scheme 14. Switching process in **Rotax11** detected by measuring spin exchange between the two spin labels.

With **Rotax11** the displacement of spin-labelled macrocycle **CE1** was followed by measuring through-space spin exchange between TEMPO radicals connected to the ring and dumbbell components (see Scheme 14). Base/acid treatments allowed to reversibly convert the system between noncoupled and coupled spin states.[39] This process occurred several times without an appreciable loss of the EPR signal, indicating this molecular machine is capable of switching on/off magnetic interactions by chemically driven reversible mechanical effects.



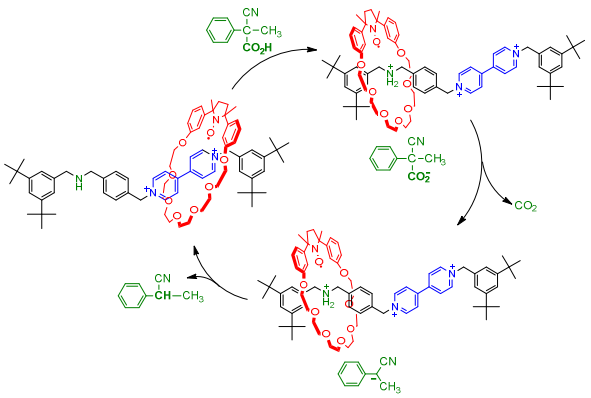
Scheme 15. Macrocycle conformational changes in the switching process of **Rotax12** assumed by measuring spin label distance distribution by PELDOR.

With **Rotax12** instead the reduced flexibility of the spin label allowed to monitor the shuttling process by performing PELDOR experiments supported by molecular dynamics (MD) calculations.[40] While the distance distribution between the nitroxide labels is not significantly affected by ring shuttling a significant geometrical change of the crown ether was observed when passing form the ammonium site to the bipyridinium site (see Scheme 15).Spin-labelled crown ether **CE4** was also successfully mechanical interconnected with the axle containing dialkylammonium and 4,4`-bipyridinium units by following the traditional threading-stoppering approach (**Rotax13**, see Scheme 16).[43] But unlike previous examples, the shuttling process of the paramagnetic ring between the two recognition sites was monitored by just measuring the nitrogen hyperfine coupling *a*(N). This was possible because the nitroxide group of the macrocycle strongly interacts with the bipyridinium unit of the thread by a charge-dipolar interaction. This leads to an increase in the spin density of the nitrogen atom and thus of the corresponding EPR nitrogen splitting.



Scheme 16. Switching process in **Rotax13** detected by variation of *a*(N).

Very recently Di Stefano group described an example of molecular machine based on MIM which is able to exploit acid/base stimulus to pass from an initial state to another and return to the initial one without necessity of a counterstimulus as instead occurs in all examples see before where subsequent additions of base and acid is required to perform the whole cycle of motions from the initial state to the final state and back again.[57] The reported example was based on a of Sauvage-type catenane, which can move back and forth when one eq. of 2-phenyl-2-cyanopropanoic acid is added. By treating **Rotax13** with one eq. of the same organic acid (see Scheme 17), we were able to fuel the back and forth motions of the paramagnetic crown ether in an autonomous cycle and to monitor this movement by EPR spectroscopy.[58]



Scheme 17. Switching motions of **Rotax13** triggered by 2-phenyl-2-cyanopropanoic acid.

6. Conclusion

We contributed to develop EPR based methodologies for detecting and identifying noncovalent assemblies in solution and for clarifying their structure and properties. Although paramagnetic probes must be added to the samples, the considerable amount of information that can be extracted by EPR on noncovalent systems justify the great deal of work, sometime necessary, to synthetize spin-labelled molecular structures.

The development of mechanically interlocked paramagnetic molecules in which the nitroxide moiety could behave as a recognition site represents a stimulating research topic that surely need further investigations and so are the development of advanced spin traps assembled with macrocyclic compounds and the use of host–guest chemistry to tune free radical properties.

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[1] M. Gomberg, *J. Am. Chem. Soc.* **1900**, *22*, 757-771.
[2] *Encyclopedia of Radicals in Chemistry, Biology and Materials* (Eds.: C. Chatgilialoglu, A. Studer), Wiley, Cheichester, **2012**.
[3] *Nitroxide Spin Labels: Reactions in Biology and Chemistry* (Eds.: N. Kocherginsky, H. M. Swartz), CRC Press, Boca Raton, **1995**.
[4] *Electron Paramagnetic Resonance: a practitioner's toolkit*, (Eds.: M. Brustolon, E. Giamello), John Wiley & Sons Inc., Hoboken, **2009**.
[5] a) E. G. Bagryanskaya, S. R. A. Marque, *Electron Paramag. Reson.,* **2017**, *25,* 180-235; b) E. Mezzina, R. Manoni, F. Romano, M. Lucarini, *Asian J. Org. Chem.*, **2015**, *4*, 296-310; c) M. Lucarini in *Encyclopedia of Radicals in Chemistry, Biology and Materials*, *Vol. 1* (Eds.: C. Chatgilialoglu, A. Studer, Wiley, Chichester, **2012**, p. 229-248; d) D. Bardelang, M. Hardy, O. Ouari, P. Tordo in *Encyclopedia of Radicals in Chemistry, Biology and Materials*, *Vol. 4 (*Eds.: C. Chatgilialoglu, A.Studer, Wiley, Chichester, **2012**, p. 1965-2016; e) M. Lucarini, E. Mezzina, *Electron Paramagn. Reson.*, **2010**, ***22***, 41-70; f) M. Lucarini, L. Pasquato, *Nanoscale*, **2010**, *2*, 668-676; g) P. Franchi, M. Lucarini, G. F. Pedulli, *Curr. Org. Chem.*, **2004**, *8*, 1831-1849.
[6] M. Lucarini, B. P. Roberts, *Chem. Commun.*, **1996**, 1577-1578.
[7] C. Heller, H. M. McConnell, *J. Chem. Phys.*, **1960**, *32*, 1535-1539.
[8] M. Lucarini, B. Luppi, G. F. Pedulli, B. P. Roberts, *Chem. Eur. J.*, **1999**, *5*, 2048-2054.
[9] As an example see: N. Yoshida, A. Seiyama, M. Fujimoto, *J. Phys. Chem.* **1990**, *94*, 4246-4253.
[10] P. Franchi, G. F. Pedulli, M. Lucarini, *J. Phys. Chem. A.*, **2008**, *112*, 8706-8714.
[11] E. Mileo, P. Franchi, R. Gotti, C. Bendazzoli, E. Mezzina, M. Lucarini, *Chem. Comm.*, **2008**, 1311-1313.
[12] C. Bendazzoli, E. Mileo, M. Lucarini, S. Olmo, V. Cavrini, R. Gotti, *Microchim. Acta*, **2010**, *171*, 23-31.
[13] P. Franchi, C. Casati, E. Mezzina, M. Lucarini, *Org. Biomol. Chem.*, **2011**, *9*, 6396-6401.
[14] As an example see: T.Oshikiri,Y. Takashima, H.Yamaguchi, A.Harada, *J. Am. Chem. Soc.*, **2005**, *127*, 12186-12187.
[15] P. Franchi, M. Lucarini, E. Mezzina, G. F. Pedulli, *J. Am. Chem. Soc.*, **2004**, *126*, 4343-4354.
[16] P. Franchi, M. Lucarini, G. F. Pedulli, *Angew. Chem. Int. Ed.*, **2003**, *42*, 1842-1845.
[17] *Cucurbiturils. Chemistry, Supramolecular Chemistry and Applications* (Eds: K. Kim, J. Murray, N. Selvapalam, Y. Ho Ko, I. Hwang), World Scientific Publishing Europe Ltd, London, **2018**
[18] E. Mezzina, F. Cruciani, G. F. Pedulli, M. Lucarini, *Chem. Eur. J.*, **2007**, 13, 7223-7233.
[19] M. Okazaki, K. Kuwata, *J. Chem. Phys.* **1984**, *88*, 3163-3165.
[20] a) E. Mileo, E. Mezzina, F. Grepioni, G. F. Pedulli, M. Lucarini, *Chem. Eur. J.*, **2009**, *15*, 7859-7862; b) I. Kirilyuk, D. Polovyanenko, S. Semenov, I. Grigor'ev, O. Gerasko, V. Fedin, E. G. Bagryanskaya, *J. Phys. Chem. B*, **2010**, *114*, 1719-1728; c) N. Jayaraj, M. Porel, M. F. Ottaviani, M. V. S. N. Maddipatla, A. Modelli, J. P. Da Silva, B. R. Bhogala, B. Captain, S. Jockusch, N. J. Turro, V. Ramamurthy, *Langmuir*, **2009**, *25*, 13820-13832.
[21] D. Bardelang, K. Banaszak, H.Karoui, A. Rockenbauer, M. Waite, K. Udachin, J. A. Ripmeester, C. I. Ratcliffe, O. Ouari, P. Tordo, *J. Am. Chem. Soc.*, **2009**, *131*, 5402-5404.
[22] *Calixarenes and Beyond* (Eds.: P. Neri, J. L. Sessler, M.-X. Wang), Springer International Publishing, Switzerland, **2016**.
[23] P. Franchi, M. Lucarini, G. F. Pedulli, D. Sciotto, *Angew. Chem. Int. Ed.* **2000**, *39*, 263-266.
[24] A. G. Kanaras, F. S. Kamounah, K. Schaumburg, C. J. Kiely, M. Brust, *Chem. Commun.* **2002**, 2294-2295.
[25] a) M. Lucarini, P. Franchi, G. F. Pedulli, P. Pengo, P. Scrimin, L. Pasquato, *J. Am. Chem. Soc.*, **2004**, *126*, 9326-9329; b) M. Lucarini, P. Franchi, G. F. Pedulli, C. Gentilini, S. Polizzi, P.

Pengo, P. Scrimin, L. Pasquato, *J. Am. Chem. Soc.*, **2005**, *127*, 16384-16385.
[26] C. Gentilini, F. Evangelista, P. Rudolf, P. Franchi, M. Lucarini, L. Pasquato, *J. Am. Chem. Soc.*, **2008**, *130*, 15678-15682.
[27] C. Gentilini, P. Franchi, E. Mileo, S. Polizzi, M. Lucarini, L. Pasquato, *Angew. Chem., Int. Ed.* **2009**, 48, 3060-3064.
[28] a) P. Posocco, C. Gentilini, S. Bidoggia, A. Pace, P. Franchi, M. Lucarini, M. Fermeglia, S. Pricl, L. Pasquato, *ACS Nano*, **2012**, *6*, 7243-7253; b) M. Boccalon, P. Franchi, M. Lucarini, J. J. Delgado, F. Sousa, F. Stellacci, I. Zucca, A. Scotti, R. Spreafico, P. Pengo, L. Pasquato, *Chem. Commun.*, **2013**, *49*, 8794-8796; c) M. Boccalon, S. Bidoggia, F. Romano, L. Gualandi, P. Franchi, M. Lucarini, P. Pengo, L. Pasquato, J. *Mater. Chem. B*, **2015**, 3, 432-439.
[29] For recent reviews on XB, see: a) G. Cavallo, P. Metrangolo, R. Milani, T. Pilati, A. Priimagi, G. Resnati, G. Terraneo, *Chem. Rev.* **2016**, *116*, 2478-601; b) H. Wang, W. Wang, W. J. Jin, *Chem. Rev.* **2016**, *116*, 5072-5104.
[30] P. Franchi, M. Lucarini, P. Pedrielli, G. F. Pedulli, *ChemPhysChem*, **2002**, *3*, 789-793.
[31] V. Mugnaini, C. Punta, R. Liantonio, P. Metrangolo, F. Recupero, G. Resnati, G. F. Pedulli, M. Lucarini, *Tetrahedron Lett.*, **2006**, *47*, 3265-3269.
[32] G. R. Hanson, P. Jensen, J. McMurtrie, L. Rintoul, A. S. Micallef, *Chem. Eur. J.*, **2009**, *15*, 4156-4164
[33] L. Gualandi, E. Mezzina, P. Franchi, M. Lucarini, *Chem. Eur. J.*, **2016**, *22*, 16017-16021.
[34] As an example with CD see: a) R. M. Paton, E. T. Kaiser, *J. Am. Chem. Soc.*, **1970**, *92*, 4723-4725; b) G. Ionita, V. Chechik, *Org. Biomol. Chem.,* **2005**, *3*, 3096-3098; c) R. K. Strizhakov, E. V. Tretyakov, A. S. Medvedeva, V. V. Novokshonov, V. G. Vasiliev, V. I. Ovcharenko, O. A. Krumkacheva, M. V. Fedin, E. G. Bagryanskaya, *Appl. Magn. Reson.,* **2014**, *45*, 1087-1098; d) D. Bardelang, A. Rockenbauer, L. Jicsinszky, J.-P. Finet, H. Karoui, S. Lambert, S. R. A. Marque, P. Tordo, *J. Org. Chem.*, **2006**, *71*, 7657-7667; e) Y. Han, B. Tuccio, R. Lauricella, F. A. Villamena, *J. Org. Chem.*, **2008**, *73*, 7108-7117; f) M. Hardy, D. Bardelang, H. Karoui, A. Rockenbauer, J.-P. Finet, L. Jicsinszky, R. Rosas, O. Ouari, P. Tordo, *Chem. Eur. J.*, **2009**, *15*, 11114-11118; g) F. Poulhès, E. Rizzato, P. Bernasconi, R. Rosas, S. Viel, L. Jicsinszky, A. Rockenbauer, D. Bardelang, D. Siri, A. Gaudel-Siri, H. Karoui, M. Hardy, O. Ouari, *Org. Biomol. Chem.*, **2017**, *15*, 6358-6366; h) F. Caglieris, L. Melone, F. Canepa, G. Lamura, F. Castiglione, M. Ferro, L. Malpezzi, A. Mele, C. Punta, P. Franchi, M. Lucarini, B. Rossi, F. Trotta, *RSC Adv.*, **2015**, *5*, 76133-76140.
[35] V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2002**, *41*, 2596-2599.
[36] C. Casati, P. Franchi, R. Pievo, E. Mezzina, M. Lucarini, *J. Am. Chem. Soc.* **2012**, *134*, 19108-19117.
[37] R. Manoni, P. Neviani, P. Franchi, E. Mezzina, M. Lucarini, *Eur. J. Org. Chem.*, **2014**, 147-151.
[38] *Pillarenes* (Ed. T. Ogoshi), Royal Society of Chemistry, Cambridge, **2016**.
[39] V. Bleve, C. Schäfer, P. Franchi, S. Silvi, E. Mezzina, A. Credi, M. Lucarini, *ChemistryOpen,* **2015,** *4*, 18-21.
[40] P. Franchi, V. Bleve, E. Mezzina, C. Schäfer, G. Ragazzon, M. Albertini, D. Carbonera, A. Credi, M. Di Valentin, M. Lucarini, *Chem. - Eur. J.*, **2016**, *22*, 8745-8750.
[41] L. Gualandi, E. Mezzina, P. Franchi, M. Lucarini, *Chem. Eur. J.*, **2016**, *22*, 16017-16021.
[42] J. F. W. Keana, J. Cuomo, L. Lex, S. E. Seyedrezai, *J. Org. Chem.*, **1983**, *48*, 2647-2654.
[43] V. Bleve, P. Franchi, E. Konstanteli, L. Gualandi, S. M. Goldup, E. Mezzina, M. Lucarini, *Chem. Eur. J.*, **2018**, *24*, 1198-1203.
[44] V. Balzani, A. Credi, F. M. Raymo, J. F. Stoddart, *Angew. Chem., Int. Ed.*, **2000**, *39*, 3348-3391.
[45] A. Margotti, C. Casati, M. Lucarini, E. Mezzina, *Tetrahedron Lett.*, **2008**, *49*, 4784-4787.
[46] C. J. Bruns, J. F. Stoddart, *The Nature of the Mechanical Bond: From Molecules to Machines*, John Wiley & Sons, Inc., Hoboken, New Jersey, **2017**.
[47] G. Jeschke, A. Godt, *ChemPhysChem,* **2003**, *4*, 1328-1334.
[48] E. Mezzina, M. Fanì, F. Ferroni, P. Franchi, M. Menna, M. Lucarini, *J. Org. Chem.*, **2006**, *71*, 3773-3777.
[49] P. Franchi, M. Fanì, E. Mezzina, M. Lucarini, *Org. Lett.,* **2008**, *10*, 1901-1904.
[50] C. Casati, P. Franchi, R. Pievo, E. Mezzina, M. Lucarini, *J. Am. Chem. Soc.,* **2012**, *134*, 19108-19117.
[51] E. Mileo, C. Casati, P. Franchi, E. Mezzina, M. Lucarini, *Org. Biomol. Chem.*, **2011**, *9*, 2920-2924.
[52] R. Pievo, C. Casati, P. Franchi, E. Mezzina, M. Bennati, M. Lucarini, *ChemPhysChem*, **2012**, *13*, 2659-2661.
[53] G. Jeschke, *Annu. Rev. Phys. Chem.* **2012**, *63*, 419-446.
[54] R. A. Bissell, E. Cordova, A. E. Kaifer, J. F. Stoddart, *Nature*, **1994**, *369*, 133-137.
[55] a) R. Manoni, F. Romano, C. Casati, P. Franchi, E. Mezzina, M. Lucarini, *Org. Chem. Front.*, **2014**, *1*, 477-483; b) F. Romano, R. Manoni, P. Franchi, E. Mezzina, M. Lucarini, *Chem. - Eur. J.*, **2015**, *21*, 2775-2779; c) V. Bleve, P. Franchi, L. Gualandi, F. Romano, E. Mezzina, M. Lucarini, *Org. Chem. Front.*, **2018**, *5*, 1579-1585.
[56] P. R. Ashton, R. Ballardini, V. Balzani, I. Baxter, A. Credi, M. C. T. Fyfe, M. T. Gandolfi, M. Gumez-Lupez, M. V. Mart.nez-D.az, A. Piersanti, N. Spencer, J. F. Stoddart, M. Venturi, A. J. P. White, D. J. Williams, *J. Am. Chem. Soc.* **1998**, *120*, 11932-11942.
[57] J. A. Berrocal, C. Biagini, L. Mandolini, S. Di Stefano, *Angew. Chem., Int. Ed.* **2016**, *55*, 6997-7001.
[58] P. Franchi, C. Poderi, E. Mezzina, C. Biagini, S. Di Stefano, M. Lucarini, *J. Org. Chem.* **2019**, *84*, 9364-9368.