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Dedicated to Professor Mihaela Hillebrand
on the occasion of her 85th anniversary

EFFECT OF ALKYL CHAIN LENGTH ON THE INCLUSION OF CARBOXYLATED NITROXIDE RADICALS IN β- AND γ-CYCLODEXTRINS: AN EPR STUDY**

Muhammed ALKALI,[a] Alexandru Gabriel BUCUR,[a] Adrian FIFERE,[b] Ioana MOLEAVIN TURIN,[b] Florenta Daniella SAVONEA,[a] Victorita TECUCEANU [c] and Gabriela IONITA[a]*

[a] "Ilie Murgulescu" Institute of Physical Chemistry, 202 Splaiul Independenței, Bucharest, 060021, Roumania
[b] "Petru Poni" Institute of Macromolecular Chemistry, Alea Grigore Ghica Voda 41-A, 700487 Iasi, Roumania
[c] "Costin D. Nenițescu" Institute of Organic and Supramolecular Chemistry of the Roumanian Academy, 202B Splaiul Independentei, 060023 Bucharest, Roumania

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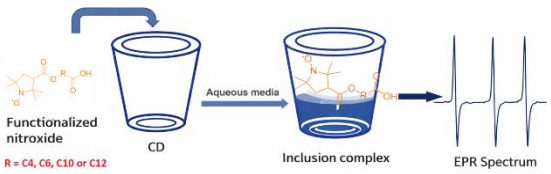
A series of novel nitroxide radicals of 3-carboxy PROXYL derivatives bearing alkyl chains of varying length (**C-4, C-6, C-10**, and **C-12**) were synthesised and investigated for their host–guest interactions with β- and γ-cyclodextrins using X-band EPR spectroscopy. The results from EPR spectra show a systematic decrease in the nitrogen hyperfine coupling constant (a_N) upon complexation with cyclodextrin in water, indicating transfer of the nitroxide moiety from a polar aqueous environment into the less polar cyclodextrin cavity. Generally, this effect was consistently more pronounced for γ-cyclodextrin, thereby indicating the stronger shielding and deeper inclusion associated with it due to its larger cavity size. The rotational correlation times (τ) for the spin probes increased by approximately 1 order of magnitude upon complexation compared to the free radicals. This confirmed the substantial motional restriction of the radicals in the inclusion complex. Although the increase of the alkyl chain length enhances both the rigidity and hydrophobic interactions, the cyclodextrin cavity size appears to play a dominant role in controlling the inclusion depth and dynamic confinement.



INTRODUCTION

Nitroxide compounds are stable free radicals that generated lots of interest in supramolecular chemistry due to their paramagnetic properties, which allow them to be useful molecular probes for characterising complex formation accompanied by changes in local environments through electron paramagnetic resonance (EPR) spectroscopy. Their signals are sensitive to changes in motion, polarity, and molecular interactions.[1] Cyclodextrins (CDs) are cyclic oligosaccharides with ring-shaped molecules with a hydrophobic inner cavity and a hydrophilic outer surface that allow them to form host-guest inclusion complexes. Among them, β-cyclodextrin has a medium-sized cavity consisting of 7 glucose units, while γ-cyclodextrin is larger, having 8 glucose units. These cavities provide a

* Corresponding author: gabi2ionita@yahoo.com
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hydrophobic microenvironment ideal for encapsulating non-polar guests, while the hydrophilic exterior enhances water solubility. The cavity size itself determines selectivity; for instance, β-CD favours aromatic or medium-sized alkyl guests, while γ-CD accommodates larger molecules.[2] When a nitroxide enters a cyclodextrin cavity, its EPR spectrum changes, usually by showing line broadening and shifts in hyperfine splitting. These effects indicate how the radical is included within the host and, as such, is exploited to characterize host-guest interactions.[3–6]

Nitroxides functionalized with alkyl chains bearing carboxylic moieties can introduce amphiphilicity, where the lipophilic tail promotes inclusion into the CD cavity, and the polar head enables additional electrostatic or hydrogen-bonding interactions. It is expected that varying alkyl chain length will modulate the depth and stability of inclusion, thereby influencing binding constants. This phenomenon was seen in a previous work referenced in,[6] by Tan *et al.* where they studied biradicals having 1, 2 or 4 C-C bond linkers and association constants were highest for the spin probe with 4 C-C linkers. This was also demonstrated in an analogous study referenced in[7] where it was established that there existed the competition between the alkyl chains and nitroxide functionalities for the hydrophobic cyclodextrin cavities and this led to the formation of different types of inclusion complexes. The ability of cyclodextrin to form inclusion complexes based on different structural frameworks of guest molecules makes it promising for drug delivery, for enhancing solubility and bioavailability of lipophilic therapeutics, and in spin-labelling for biomolecular studies.[8–10]

The current work examines how derivatives of 3-carboxy-PROXYL with varying carbon chain lengths shown in Fig. 1 namely 3-[(3-carboxy propan-2-yl)oxycarbonyl]-2,2,5,5-tetramethylpyrrolidin-1-oxyl; 2-[(2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-carbonyl) oxy]hexanoic acid; 10-[(2,2,5,5-tetramethyl pyrrolidin-1-oxyl-3-carbonyl)oxy]decanoic acid and 12-[(2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-carbonyl)oxy] dodecanoic acid for C4, C6, C10 & C12 respectively interact with β- and γ-CDs in water using EPR measurements taken at different cyclodextrin concentrations. We studied how systematic variation in the alkyl chain length of carboxylic acid-terminated nitroxides affects host-guest interactions with β- and γ-CDs, thereby providing insights into how supramolecular assemblies can be affected by these factors.

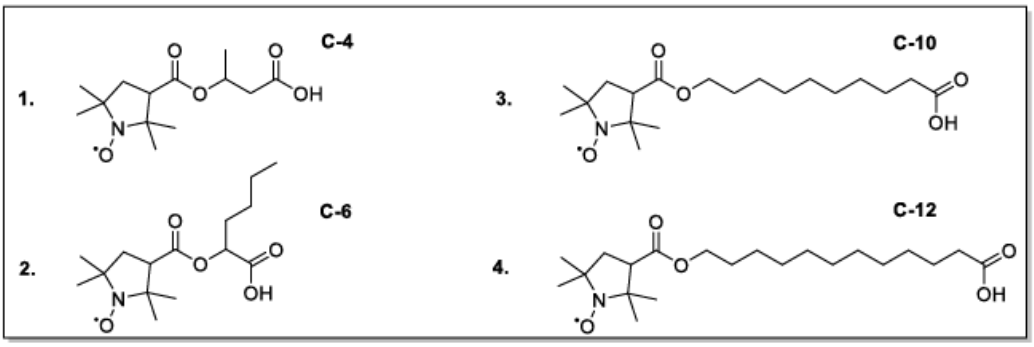


Fig. 1 – The structure of the new spin probes bearing alkyl chain.

EXPERIMENTAL

Materials and methods

The cyclodextrins as well as the starting materials for the synthesis of the compounds; 3-carboxy PROXYL, 3-hydroxybutanoic acid, 2-hydroxyhexanoic acid, 10-hydroxydecanoic acid and 12-hydroxydodecanoic acid, were purchased from Sigma-Aldrich and used as delivered.

The cyclodextrin solutions were made in water in varying molar concentrations (5×10^{-4}, 10^{-3}, 3×10^{-3}, 7×10^{-3} and 10^{-2}), whereas for the radicals, 10^{-2} M stock solutions in ethanol were prepared from which appropriate amount was taken and mixed thoroughly with cyclodextrin solution in water after evaporating the ethanol solvent, ensuring a uniform ~10^{-4} M concentration of the radical within each cyclodextrin solution.

The EPR measurements were recorded at 20 ºC using a JEOL FA100 X-band EPR spectrometer, equipped with a cylindrical type resonator TE011. The frequency modulation was 100 kHz, microwave power 1 mW, modulation amplitude of 1 G, sweep time 240 s, and the sweep width of 100 G. Simulation of the experimental spectra were

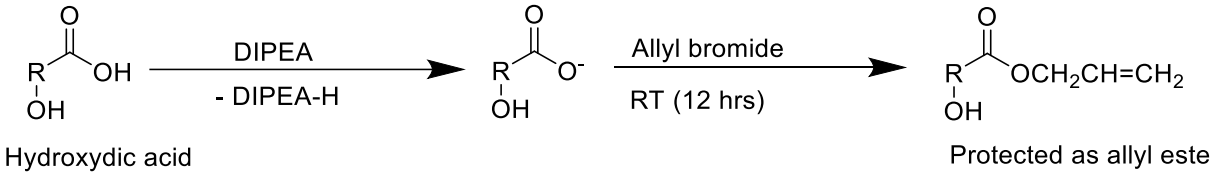
done with EasySpin[11] software using garlic core function for isotropic and fast-motion continuous wave EPR spectra.

Synthesis

The nitroxide compounds (1–4) listed in Fig. 1 were prepared via Steglich esterification, involving firstly, protection of the acid group from the radical as an allyl ester, followed by esterification before the final deprotection step. This was done by slightly modifying the methodologies employed elsewhere[12] through the following:

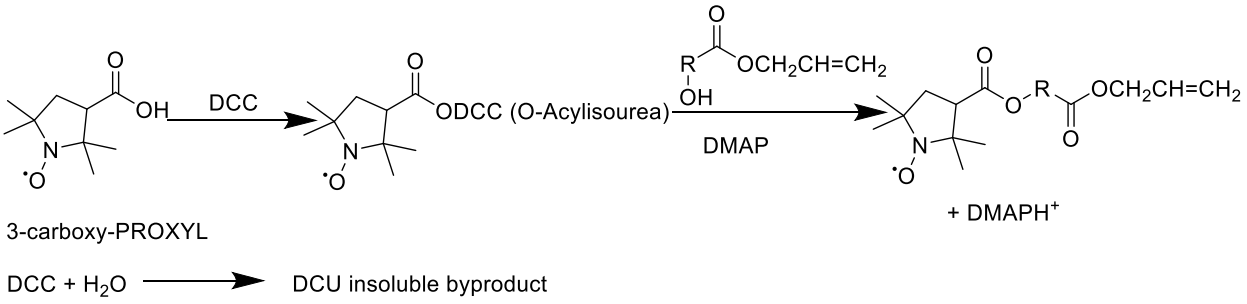
a) Allyl protection

To a stirred solution of the hydroxydic acid (1 eq.) in DMF was added DIPEA (2 eq.), then allyl bromide (2 eq.). The reaction was left at room temperature for 12 hours. After this time, the reaction was quenched by the addition of water and the product was extracted 3 times with diethyl ether. The combined organic extracts were washed with brine, dried with $MgSO_4$, filtered, and concentrated to give the allyl ester.



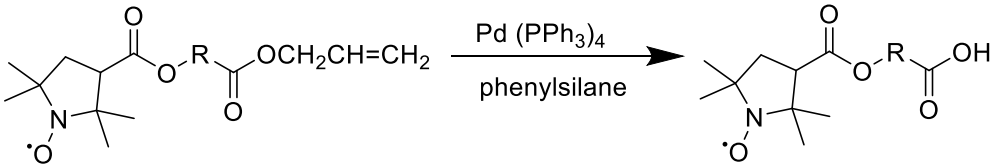
b) Steglich esterification.

To a stirred solution of 3-carboxy-PROXYL (1 eq.) in DCM, was added the alcohol, i.e. protected allyl ester (2 eq.), DMAP (0.5 eq.) and finally DCC (1.5 eq.). The reaction was left at room temperature overnight. After this time, the reaction was filtered and diluted with DCM. The combined organic phase was washed with HCl 1M, saturated $NaHCO_3$ and brine, then dried with Na_2SO_4, filtered, and concentrated to give the ester after purification on silica gel (Toluene/AcOEt).



c) Allyl deprotection

To a stirred solution of allyl esters (1 eq.) in DCM was added Pd $(PPh_3)_4$ (0.01 eq.), then phenyl silane (2 eq.). The reaction was stirred for 1h at room temperature, and after this time, the solvent was evaporated, and the crude was dissolved in DCM. The solution was filtered and purified on silica gel to give the free carboxylic acid.



The final nitroxide type compounds were obtained in good yields, and the radicals were confirmed by EPR and structurally characterised by ESI-MS. The figures of the mass spectra for the compounds are provided in the ESI (Figs. S1A-F).

C-4 ($C_{13}H_{22}NO_5^*$) Yellow solid (Yield: 67%). MS(m/z) $[M]^+ + Na = 295.1$ (Calculated: 295.14).
C-6 ($C_{15}H_{26}NO_5^*$) Yellow solid (Yield: 65%). MS(m/z) $[M]^+ + Na = 323.2$ (Calculated: 323.17).

C-10 ($C_{19}H_{34}NO_5^*$) Yellow solid (Yield: 70%). MS(m/z) $[M]^+ + Na = 379.2$ (Calculated: 379.23).
C-12 ($C_{21}H_{38}NO_5^*$) Yellow solid (Yield: 75%). MS(m/z) $[M]^+ + Na = 407.3$ (Calculated: 407.26).

RESULTS AND DISCUSSION

The results obtained for the EPR analysis of the radicals in water (Table 1) show that as the alkyl chain length increases, *i.e.* from **C-4** to **C-12**, the hyperfine coupling constant (a_N) decreases slightly, which is an indication that the nitroxide group experiences a slightly less polar environment due to an increase in the non-polar carbon chain length. In other words, the longer alkyl chains tend to increase the hydrophobic character of the molecule, which partially shields the nitroxide from the surrounding water molecules. At the same time, the rotational correlation time (τ) increases steadily, meaning the radicals display a reduced mobility in solution. This happens because longer chains increase molecular size and hydrodynamic volume, and they can also promote hydrophobic interactions. Overall, by increasing the chain length, the radical becomes more hydrophobic, slightly less exposed to water, and significantly less mobile.

Table 1

EPR parameters of the new spin probes in water.

Radical	C-4	C-6	C-10	C-12
a_N (G)	16.14	16.13	16.08	16.07
τ (s)	2.1×10^{-10}	2.7×10^{-10}	3.8×10^{-10}	4.5×10^{-10}

When the radicals were complexed with CDs, the analysis of the EPR spectra (Fig. 2) of all the spin probes indicates that the a_N decreases relative to the free radicals in water, and this decrease is found to be more pronounced for γ-CD than for β-CD, which indicates a deeper inclusion of the radical into the γ-CD cavity. EPR spectra were recorded at constant radical concentration while varying cyclodextrin concentration. The complete concentration-dependent analysis is provided in the ESI (Table S1).

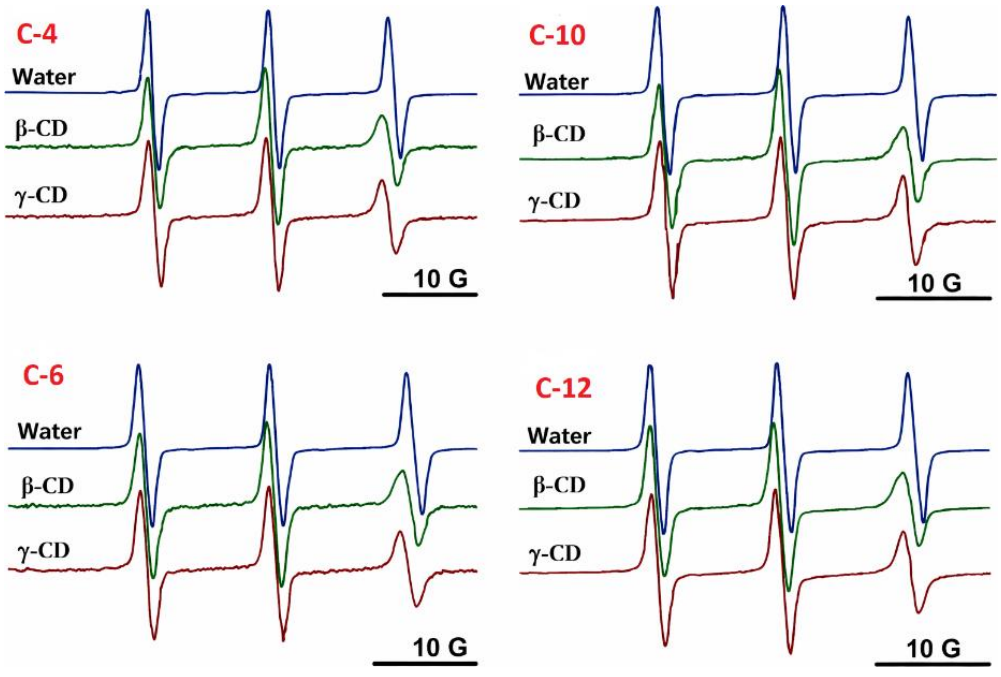


Fig. 2 – EPR spectra of the spin probes in water and cyclodextrin solutions (10^{-2}M).

Hyperfine coupling and host complexation

All the four nitroxide probes exhibited a clear decrease in their nitrogen hyperfine coupling constant (a_N) values upon addition of cyclodextrin, which is an indication of inclusion into a more hydrophobic microenvironment. In water, the a_N values are highest (~16.14–16.07 G), while in β-cyclodextrin (β-CD), they fall to ~15.65–15.62 G, and in γ-cyclodextrin (γ-CD), they become even

lower to ~15.41–15.37 G (ESI Table S2). This monotonic decrease (water > β-CD > γ-CD) is consistent with classical polarity effects, where it is understood that inclusion into the cyclodextrin cavity tends to shield the nitroxide moiety from aqueous solvent, thereby changing the charge distribution on the N-O bond and thus lowering the a_N. Similar trends have been reported in the literature: for example, Ionita *et al*. have shown in their study that inclusion into CD produces distinct changes in the EPR hyperfine splitting, which they attributed to the alteration of electrostatic (polar) interactions with water.[13]

From the simulations of the EPR spectra, the relative proportions of the complexed species were extracted as a function of cyclodextrin concentration. These data were then used to quantify the binding affinities between the investigated radicals and cyclodextrins. Accordingly, the association constant values (Table 2) were determined using Eq. (1).

$$K = \frac{[complexed\ species]}{[free\ species]\times[free\ cyclodextrin]} \qquad (1)$$

Table 2

Association constants values K (M^{-1})

Radical	β-CD	γ-CD
C4	241 ±5	302 ±6
C6	246 ±5	307 ±3
C10	253 ±4	316 ±2
C12	258 ±2	323 ±2

Effect of alkyl chain length

The carbon chain length incorporated into the radicals subtly influences these inclusion effects. Even in water, a_N decreases slightly with longer chains (16.14 → 16.07 G, from **C-4** to **C-12**), which may arise from partial hydrophobic shielding or mild self-association of the longer-tailed probe. Upon complexation, a similar trend is observed: for β-CD, the a_N drop from water is ~0.49 G for **C-4** and ~0.45 G for **C-12**, and for γ-CD, the drop is ~0.73 G (**C-4**) versus ~0.70 G (**C-12**). In other words, the a_N values generally became slightly smaller with an increase in the carbon chain length (see Fig. S2 in ESI for a graphical illustration). Prior EPR studies have indicated the existence of competition between alkyl chains and nitroxide functionalities for cyclodextrin binding. For instance, Tan *et al*. studied certain biradicals with β-CD and γ-CD derivatives and showed that the linker chain length between the radicals influences the binding constants.[6]

An overall slight increase in the association constants is observed with increasing alkyl-chain length (Table 2). This trend suggests a progressive enhancement of host–guest inclusion, likely governed by changes in the solvation properties of the radicals. Specifically, the increase in carbon-chain length is accompanied by greater hydrophobicity, which may facilitate the transfer of the radical species from the polar aqueous medium into the comparatively less polar cavity of cyclodextrin, thereby promoting stabilization of the resulting inclusion complex.[7]

β-CD *vs* γ-CD effects

Structurally, β-CD (7 glucoses) has an internal diameter of roughly 0.78 nm, whereas γ-CD (8 glucoses) is about 0.95 nm. This difference allows γ-CD to be able to accommodate bulkier guests. Indeed, it is well-established that β-CD preferentially binds relatively small or medium-sized hydrophobic guests, while γ-CD can include larger or more extended molecules.[2,3,14] For this new spin probes, the results obtained show that γ-CD's larger cavity likely allows deeper or more complete inclusion of the nitroxide moiety (and possibly part of the chain), leading to a more hydrophobic microenvironment proved by a consistently decrease of a_N compared to β-CD. Quantitatively, the γ-CD complexes show a_N values of ~0.23–0.25 G lower than those of the corresponding β-CD complexes (see Fig. S3 in ESI for a graphical illustration of this trend). This indicates the stronger polarity damping by γ-CD. The consistency of this difference across all chain lengths implies that for γ-CD, the nitroxide experiences a comparably nonpolar environment in each case, whereas in β-CD, the inclusion may be somewhat shallower or more solvent-exposed. In other words, γ-CD, with its larger cavity, likely immerse the nitroxide probe more fully in, compared to that of β-CD, which may have only partially included the nitroxide.[15,16] Study of the association constants further collaborates that complexation with γ-cyclodextrin is favored for all radicals.

Rotational correlation time (τ)

To probe rotational dynamics, simulations were used to determine the rotational correlation time (τ) of the spin probes, which increases by approximately one order of magnitude upon complexation compared to the free radicals,

indicating that cyclodextrin binding restricts radical mobility. Slightly larger τ values (Table 3) are observed for γ-CD complexes than for β-CD complexes, which is attributable to the larger size of γ-CD relative to β-CD. We found that τ increases significantly upon complexation with β- or γ-CD, especially for the longer alkyl-chain nitroxides, indicating a slower tumbling of the spin label within the host. In other words, binding to the cyclodextrin cavity constrains the radical's motion (as a larger τ indicates more restricted reorientation). This trend is consistent with previous reports: for instance, in the study where TEMPO and TEMPONE had their τ values increased by 5 and 3-fold, respectively, upon complexation with β-CD.[17]

Table 3

Correlational rotational time τ values (10^{-10} s)

Radical	Water (free)	β-CD (complexed)	γ-CD (complexed)
C4	2.1	18.7	31.2
C6	2.7	19.2	31.7
C10	3.8	19.8	32.5
C12	4.5	20.1	33.2

CONCLUSION

This study has successfully demonstrated how the host–guest complexation of 3-carboxy PROXYL derivatives with β- and γ-cyclodextrins is strongly influenced by both alkyl chain length and type of cyclodextrin (cavity size), as revealed by EPR spectroscopy. The systematic decrease in the a_N values upon complexation confirms the transfer of the nitroxide moiety into a less polar environment of the cyclodextrin cavity, while the increase of rotational correlation times (τ) further indicates that spin probes have a restricted motion within the complex. In general, γ-CD consistently induced greater shielding and stronger motional confinement than β- CD, due to its larger cavity and deeper inclusion capability. Also, we established that by increasing the alkyl chain length, the hydrophobic interactions and intrinsic rigidity are enhanced, even though the host cavity ultimately dominates the dynamic behaviour of the bound radical.

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