ELSEVIER

Carbohydrate Research 310 (1998) 261–267

CARBOHYDRATE RESEARCH

Non-covalent associations of cyclomaltooligosaccharides (cyclodextrins) with *trans*-β-carotene in water: evidence for the formation of large aggregates by light scattering and NMR spectroscopy

Andrea Mele [a], Raniero Mendichi [b], Antonio Selva [a,*]

[a] *CNR-Centro di Studio sulle Sostanze Organiche Naturali[1], Dipartimento di Chimica del Politecnico di Milano, Via Mancinelli, 7, I-20131 Milano, Italy*

[b] *CNR-Istituto di Chimica delle Macromolecole, Milano, Italy*

Received 6 May 1998; accepted 13 July 1998

Abstract

Light scattering and NMR experiments provide evidence for the formation of large aggregates, like micelles, from β-carotene complexes with β- and γ-cyclodextrin in water. High-resolution NMR spectra of the system γ-cyclodextrin/β-carotene in D_2O point out guest-induced chemical shift variation of the sugar protons, thus suggesting host–guest interaction in solution.

Keywords: Cyclodextrins; β-Carotene; Noncovalent associations; Micelles; Light Scattering; NMR spectroscopy

1. Introduction

Cyclomaltooligosaccharides (cyclodextrins, CDs), the natural macrocyclic oligosaccharides, present a torus-shaped structure with a rigid hydrophobic cavity into which lipophilic guest molecules of appropriate size can be hosted. The resulting non-covalent inclusion or host–guest (h–g) complexes are of current scientific and technological interest for their peculiar physical, chemical, and biological properties. Such noncovalent associations can actually improve the guest stability, bioavailability and water solubility; they can also regulate the release of the volatile guest molecules [1,2]. Hence CD h–g complexes and in particular those of βCD, which is available as a high-purity and low-cost commercial product recently approved [3] as food additive by the Food and Drug Administration (FDA) in the United States, can fulfill most relevant requirements for applications to the pharmaceutical and food industry [1,2].

In connection with our research programme on h–g complexes [4] and related multicomponent

* Corresponding author. Fax: +39 2 23993080; e-mail: selva@dept.chem.polimi.it

[1] Associated to the Istituto Nazionale di Coordinamento 'Chimica dei Sistemi Biologici'.

0008-6215/98/$—see front matter
PII S0008-6215(98)00193-1

systems [5] of CDs with drugs or molecules of interest to food chemistry, we are presently investigating [6,7] the noncovalent associations of CDs with *trans*-β-carotene (βCAR), a natural vitamin A precursor, which is currently used [8,9] as a yellow coloring agent for foods and as an ultraviolet screen. Actually, water-soluble inclusion complexes of such an absolutely hydrophobic molecule, which could also protect βCAR from the well-known easy inactivation by light and oxygen, should likely find extensive application [8,9] to the industrial preparations of pharmaceuticals, cosmetics, foods, and beverages.

As preliminarily reported [6,7] we prepared orange-pink water solutions from mixtures of βCAR and βCD or γCD in various molar ratios. A most recent high-resolution mass spectrometry study [6] on the white or very pale solid samples obtained by freeze-drying the colored water solutions supported the presence of complexed βCAR, based upon its lower volatility with respect to the free molecule. An earlier study [7] of these βCAR complexes with CDs was performed by electrospray-ionization mass spectrometry. The gaseous 1:1 βCAR/CDs intact charged species, associated to more or less solvent molecules, were detected from 1:1 (v/v) water/methanol + 1% acetic acid solutions of the solid samples, and the best results were obtained operating in the negative-ion mode [7]. These results could have suggested the presence in water of 1:1 βCAR/CDs h–g complexes. However, ^{1}H NMR spectra of the freeze-dried sample mentioned above, dissolved in D_2O, indicated [6] a strong tendency of the system to form larger complex/aggregates, possibly micelles, which could hamper the structural characterization of the βCAR/CDs complexes.

In the present paper we report and discuss light scattering (LS) and most recent NMR results, which provide a significant contribution to the illustration of such non-covalent associations in water.

2. Experimental

Preparation of the samples.—The samples of βCAR/CD complexes were prepared by the kneading procedure previously described [6]. A typical detailed preparation protocol is reported in the case of γCD: 22 mg (4.1×10^{-2} mmol) of commercial βCAR (Fluka) were mixed in a mortar with 213 mg (0.164 mmol, 4 equiv) of commercial γCD (Aldrich). The solid mixture (9.4% w/w of βCAR) was kneaded until a homogeneous dust was obtained. Kneading was continued after adding 2 mL of distilled water, thus obtaining a dark red slurry. The mixture was allowed to stand overnight under a nitrogen atmosphere, and then it was resuspended in 200 mL of warm (40 °C) distilled water. The orange suspension was stirred for 20 min at 40 °C and subsequently twice filtered under vacuum with standard filter paper, eventually affording an orange opalescent solution. The aqueous phase was purged with nitrogen in order to remove the dissolved oxygen. The solid association γCD/βCAR was isolated by freeze-drying. An aliquot of the solution, stored at 4 °C, did not show any sediment or precipitate after several days.

βCAR quantitation in organic extracts of γCD/βCAR water solution.—γCD/βCAR water solution (100 mL) was extracted with *n*-hexane (3×30 mL) and with *n*-pentane (2×30 mL). The organic layer was dried with Na_2SO_4, the organic solvent was removed under vacuum, and the residue was dissolved in 50 mL of spectroscopy grade CH_2Cl_2. Quantitation of β-carotene was achieved by VIS spectroscopy, monitoring the absorbance at 460 nm. According to this protocol, the solvent extraction removed 0.2% w/w of β-carotene.

Light scattering.—The LS measurements were performed with a multiangle light scattering (MALS) photometer Dawn DSP-F from Wyatt (S. Barbara, CA) in H_2O solvent at room temperature operating in the static off-line mode. A flow cell (F2) was preferred in order to reduce the scattering volume. The MALS photometer used a vertically polarized laser (He–Ne) of 632.8 nm of wavelength. The MALS measured simultaneously, by means of an array of photodiodes, the intensity of the scattered light at fifteen fixed angular locations ranging in H_2O solvent from 8.9 to 171.1°. In the data analysis we have used 13 angles with θ that ranged between 21.2 and 158.8°. The photodiodes number 4, $\theta = 8.9°$, and number 18, $\theta = 171.1°$, have been discharged because too noisy.

The calibration constant to transform the detector voltages in Rayleigh factor [$R(\theta)$] was calculated using toluene as a standard [$R(\theta) = 1.406\cdot10^{-5}$ cm^{-1}], where θ denotes the angle between the detector and the primary incident light. The angular normalization of the 15 photodiodes was carried out by means of a concentrated

solution of bovine serum albumin (BSA) globular protein (Sigma, A7888), assumed as isotropic scatterer. Details of the MALS hardware and software have been described in detail elsewhere [10]. Besides the methodology to get reliable results from a MALS photometer have been described previously [11].

Each sample solution was prepared by mixing a weighed amount of the complex with the solvent. One half of each solution was filtered through 0.2-μm cellulose acetate filters, and the residue solution was filtered through 0.45-μm filters.

NMR spectroscopy.—The high-resolution NMR spectra of the complex/aggregate of βCD and γCD with βCAR were recorded with a Bruker ARX 400 spectrometer in D_2O at a nominal temperature of 315 K. Standard water presaturation techniques were applied to suppress the signal of residual HOD. The chemical shifts were referenced to external DSS.

3. Results and discussion

Mixing βCAR with CDs we achieved intensely pink-orange colored, opalescent water solutions, which appeared to be rather stable, particularly that prepared from βCAR and γCD (see Experimental).

At first an attempt to determine the amount of βCAR, removable by extraction with apolar organic solvents from water solution of γCD/βCAR, was carried out using VIS spectroscopy for quantitation as described in the Experimental section. According to this procedure, the solvent extraction did remove just 0.2% w/w of β-carotene and, most interestingly, the aqueous phase still showed an intense pink-orange colouration, suggesting that a significant amount of βCAR had not been extracted. MALDI-TOF mass spectrometry analysis of the aqueous phase after solvent extraction revealed a strong signal at m/z 536, corresponding to the molecular ion of βCAR, thus confirming that the presence of cyclodextrin caused the retention of carotene in water, even in the presence of absolutely apolar solvents as *n*-hexane and *n*-pentane. Unfortunately, a quantitative analysis of carotene in water cannot be accomplished by VIS spectroscopy, as no data of absorbance and molar extinction can be obtained in water, because of the complete insolubility of βCAR in that solvent.

Light scattering.—Static LS is a convenient method for the study of large particles in solution as macromolecules, aggregates or micelles. In a LS experiment following the treatment of Zimm [12] the reciprocal of the reduced excess of the Rayleigh factor, $R(\theta)$, may be expressed by the equation:

$$\frac{K\cdot c}{R(\theta)} = \frac{1}{M_W\cdot P(\theta)} + 2A_2\cdot c$$

where $K = \left(2\pi^2 n_0^2 (dn/dc)^2\right)/\left(\lambda_0^4 Na\right)$ denotes the optical constant, n_0 the refractive index of the solvent, dn/dc the refractive index increment of the solute with respect to the solvent, λ_0 the wavelength of the light in the vacuum, Na the Avogadro's number, c the concentration of the sample (g/mL), M_w the weight-average molar mass, A_2 the second virial coefficient and $P(\theta)$ the intramolecular scattering function or "form factor". Debye showed [13] that the form factor $P(\theta)$ may be approximated by:

$$P(\theta) = 1 - \frac{1}{3}\cdot\mu^2\cdot\langle s^2\rangle + \ldots$$

where, $\mu = (4\pi/\lambda)\cdot\sin(\theta/2)$ is a function of the angle (θ) and of the wavelength (λ) of the light in the medium and $< s^2 >$ denotes the mean-squares radius of the molecules [10–13]. As a result from a single MALS experiment could be obtained three important molecular data: M_w, A_2 and the z-average-root-mean squares radius $< s^2 >_z^{\frac{1}{2}}$.

In our specific case there are two particular problems: First, there is a limited solubility of the samples, and more important, part of the sample remains on the filter. As a consequence it is difficult to know the exact concentration of the solutions. Second, connected to the first, we do not know the refractive index increment of the complex, dn/dc. Fortunately, in order to show the existence of complexes (large molecules), it is sufficient to measure the dimension of the molecules in solution.

It was demonstrated [10] that in the limit of very low concentration and scattering angle the dimension $< s^2 >^{\frac{1}{2}}$ of the molecules can be obtained independent of dn/dc, M_w and c. All that is required is to collect data at sufficiently small scattering angle and very low concentration. This fact is important because it allows us to overcome the problems described before.

First results are of the solutions filtered with 0.2-μm filters. The intensities of the scattering of

these solutions do not differ significantly from the scattering of the reference solution of cyclodextrin alone. Samples are soluble but obstruct completely the 0.2-μm filters.

The results are different with 0.45-μm filters. Fig. 1 shows a comparison between the scattering of three samples filtered with 0.45-μm filters. First sample (●) is the reference, pure βCD. Second sample (■) is a complex between γCD and βCAR in the molar ratio 4:1. Finally the third sample (◆) is a complex between βCD and βCAR in the molar ratio 4:1. The nominal concentration of the dilute solutions was very low: approximately 9.0×10^{-5} g/mL for the complex and 9.0×10^{-4} g/mL for βCD.

These light-scattering results are very interesting. First, the dilute βCD solution, as well as the γCD solution, do not show the presence of large aggregates. In fact, the signal is low, consistent with the relatively low molar mass of the βCD sample, and there is no meaningful angular variation of the scattering. Second, the dilute solutions of the complexes show high signal and large angular variation of the scattering that mean high molar mass and large dimensions of the molecules. Finally, at constant nominal concentration of the solutions the signal of the βCD complex, with respect to the γCD complex, is much higher. Hence, at least from a qualitative point of view, we could affirm that in the aqueous dilute solutions of the complexes there are aggregates of very large dimensions.

In regard to the dimensions of these aggregates, we can also estimate some quantitative values. Fig. 2 shows the angular variation of the scattering, in the form of $P(\theta)^{-1}$ against $\sin^2(\theta/2)$ plot, for the two complexes βCD/βCAR and γCD/βCAR. From the initial slope of the $P(\theta)^{-1}$ versus $\sin^2(\theta/2)$ plot, where $P(\theta)^{-1}= R(\theta=0°)/R(\theta)$, with a linear fitting we obtain an estimate of the z-average of the root-mean squares radius, $< s^2 >_z^{\frac{1}{2}}$, value of the aggregates. Details of the data analysis algorithms and of the estimation of the uncertainties can be found in the reference [10].

Table 1 reports data of the dimensions of the two complexes between βCAR and γCD or βCD. The $< s^2 >_z^{\frac{1}{2}}$ value of the two complexes, filtered through 0.45 μm filter, was 110.2 ± 1.4 nm for the γCD complex and 123.4 ± 1.8 nm for the βCD complex. Hence, the dimension of the βCD complex is approximately 10% larger than that of the γCD complex.

Eventually, as a control experiment, a sample was prepared following the protocol procedure described above (see Experimental) mixing βCAR with soluble starch (ISO Merck, mol. weight 6500–8000) in the place of cyclodextrin: the LS measurements performed on the resulting, very slightly colored water solution, did not provide any evidence of formation of large-dimension aggregates.

NMR spectroscopy.—The NMR spectra of both the samples of complex/aggregate of βCD and

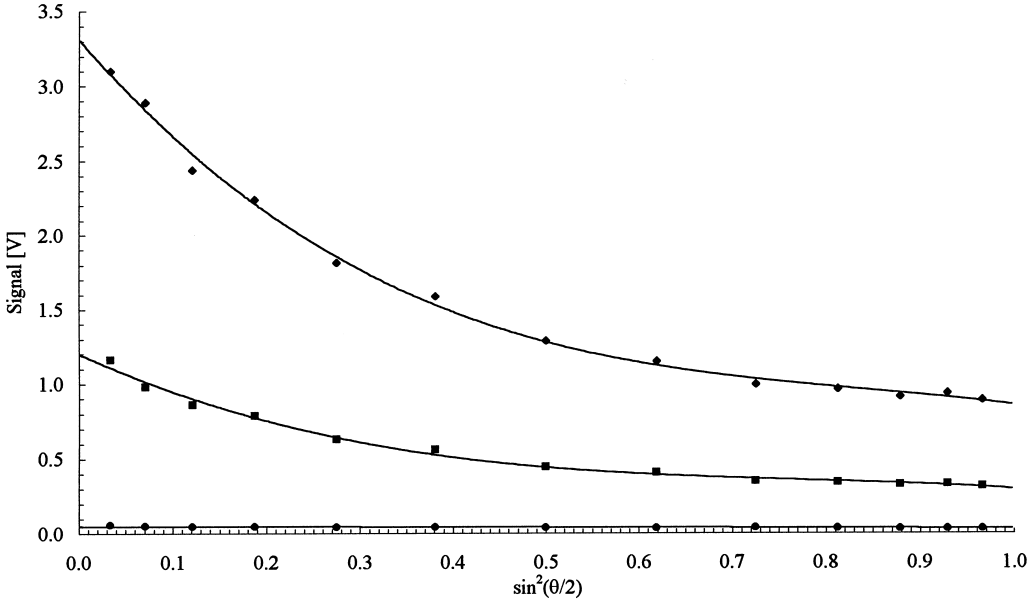

Fig. 1. Comparison of the scattering intensities of three samples: (●) β-cyclodextrin; (■) complex γ-cyclodextrin/β-carotene; (◆) complex β-cyclodextrin/β-carotene.

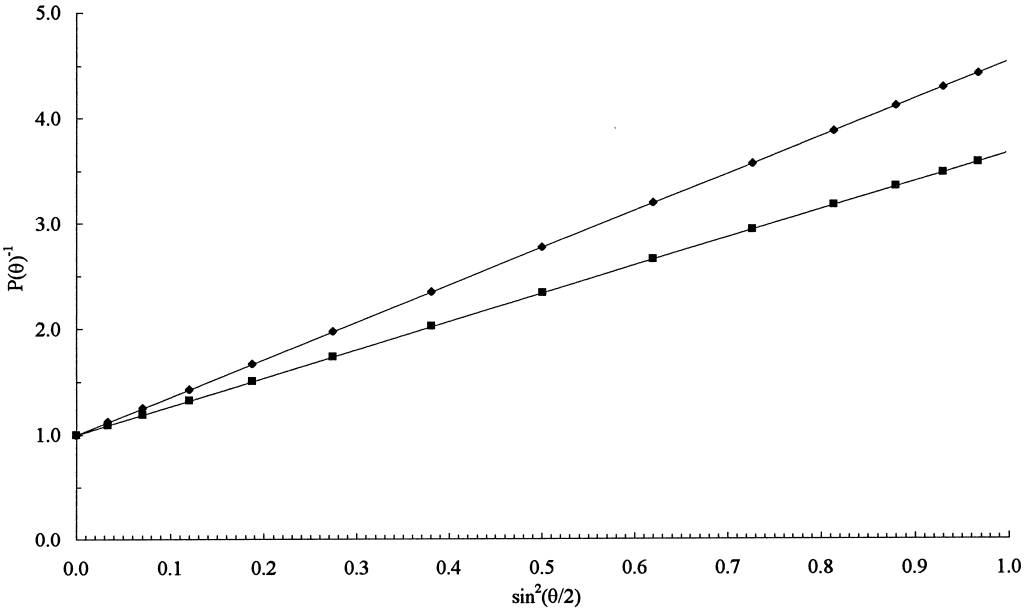

Fig. 2. $P(\theta)^{-1}$ against $\sin^2(\theta/2)$ plot of two complexes: (■) γ-cyclodextrin/β-carotene; (◆) β-cyclodextrin/β-carotene.

γCD with βCAR dissolved in D_2O showed only the resonances of the glucose units giving rise to sharp, high-resolution multiplets, whilst no peaks assignable to βCAR could be detected, neither in the low-field nor in the high-field region of the spectrum, confirming what was previously reported in the case of the βCD/βCAR system only [6]. These data can be interpreted taking into account the findings obtained by the static LS experiments described above.

The observed NMR signals are only due to the free β and γCD molecules, which are in fast exchange, on the NMR timescale, with the micelle-bound cyclodextrin molecules. Interestingly, although both βCD and γCD exhibit the same dynamic behaviour in the presence of βCAR, chemical shifts of the glucose protons of native βCD do not show any appreciable variation in the presence of βCAR, whilst significant chemical shift variations of the glucose signals of γCD are observed in the γCD/βCAR system compared to native γCD. The values of the chemical shifts of the assigned protons of native γCD and in the presence of βCAR are reported in Table 2.

Table 1
Root-mean squares radius, $< s^2 >^{\frac{1}{2}}$, of the two complexes, filtered 0.45 μm

Complex	$< s^2 >^{\frac{1}{2}}$ nm
γ-cyclodextrin/β-carotene	110.2 ± 1.4
β-cyclodextrin/β-carotene	123.4 ± 1.8

The analysis of the chemical shift variations induced by the complexation is one of the classical tools for the investigation of cyclodextrin–guest inclusion complexes [14]. The formation of an inclusion complex is known to shield the protons of CDs that are inside the hydrophobic cavity (H-3 and H-5), and deshield the protons of the guest molecule directly interacting with the cavity [15]. Chemical shift variations of the outer protons of cyclodextrin, such as H-1, H-2 and H-4, have been ascribed to h–g association at the exterior of the cavity and deformation of the macrocyclic ring of cyclodextrins [16,17]. The observed chemical shift variations of all the γCD protons in the γCD/βCAR system indicate that the interaction between γCD and βCAR is not specific, as it could be in a genuine 1:1 inclusion complex. On the micelle surface γCD molecules are likely to undergo distortion of the equilibrium conformation of their macrocyclic ring by interaction with βCAR and thus experience chemical shift variation with respect to uncomplexed γCD. The γCD molecules at the surface of the micelle are, in turn, in fast exchange with those in the bulk solvent, giving rise to the observed chemical shift variation reported in Table 2. The different results obtained with βCD are probably due to the higher conformational rigidity of the seven-membered ring structure of βCD with respect to that of γCD [2].

Table 2
Chemical shift (δ) of native and complexed γ-cyclodextrin protons. Values in ppm from external DSS (D_2O solution, 315.0 ± 0.1 K)

	H-1	H-2	H-3	H-4	H-5-H-6[b]
Free γ-CD	5.101	3.649	3.919	3.574	3.886–3.809
γ-CD/β-CAR	5.057	3.638	3.933	3.567	3.850–3.791
Difference[a]	0.044	0.011	−0.014	0.007	0.017[c]

[a] Difference = δ(γCD free)-δ (γCD with βCAR).
[b] Considered as a multiplet.
[c] The difference is calculated on the average point of each multiplet.

4. Conclusions

The present LS results on βCAR complexes with β and γCD in water are in agreement with the representation we proposed previously [6] only for βCD/βCAR. Also, we show in the present paper that NMR spectra of γCD/βCAR association in D_2O solution point out chemical shift variation of the γCD protons upon complexation, indicating that h–g interaction is taking place in solution. The pattern of chemical shift variation is, however, different from that expected in the case of classical inclusion complexes of defined stoichiometry (e.g., a 1:1 complex), indicating that the formation of a true inclusion compound between γCD and βCAR could be only one of the possible mechanisms of interaction.

Actually, the possible CDs/βCAR h–g associations can be reasonably expected to have a neat amphiphilic character due to the hydrophilic hosts and the hydrophobic guest. Just as it was already predicted [6], the present LS results confirm that in water the hydrophobic moiety (βCAR) of such inclusion complexes should tend to self-associate forming larger supramolecular aggregates, like micelles, with the hydrophilic CDs molecules arranged outside and in fast exchange, on NMR timescale, with the free CD molecules in solution.

Besides, the present LS data show that pure βCD and γCD do not form large self-aggregates in water, at least in our experimental conditions. For instance, this observation is in agreement with recent NMR studies showing that pure βCD is a monomer in water solution [18], ruling out previous contrary conclusions from LS experiments [19].

Acknowledgements

The authors thank Mr. Alberto Giacometti Schieroni (CNR) for valuable technical assistance.

References

[1] J. Szejtli, *Cyclodextrin Technology*, Kluwer Academic Publishers, Dordrecht, 1988.
[2] H. Fromming and J. Szejtli, *Cyclodextrins in Pharmacy*, Kluwer Academic Publishers, Dordrecht, 1994.
[3] FDA, *Federal Register*, Vol. 61, No. 164, Sept. 20, 1996.
[4] A. Selva, E. Redenti, M. Zanol, P. Ventura, and B. Casetta, *Org. Mass Spectrom.*, 28 (1993) 983–986; A. Selva, E. Redenti, M. Zanol, P. Ventura, and B. Casetta, *Eur. Mass Spectrom.*, 1 (1995) 105–106; A. Selva, E. Redenti, M. Zanol, P. Ventura, and B. Casetta, *Eur. Mass Spectrom.*, 1 (1995) 330; A. Mele and A. Selva, *J. Mass Spectrom.*, 30 (1995) 645–647; T.F. Favino, G. Fronza, C. Fuganti, D. Fuganti, P. Grasselli, and A. Mele, *J. Org. Chem.*, 61 (1996) 8975–8979; G. Fronza, A. Mele, E. Redenti and P. Ventura, *J. Org. Chem.*, 61 (1996) 909–914.
[5] A. Selva, E. Redenti, M. Pasini, P. Ventura, and B. Casetta, *J. Mass Spectrom.*, 30 (1995) 219–220; A. Selva, E. Redenti, P. Ventura, M. Zanol, and B. Casetta, *J. Mass Spectrom.*, 31 (1996) 1364–1370; A. Mele, W. Panzeri, and A. Selva. *J. Mass Spectrom.*, 32 (1997) 807–812.
[6] A. Mele and A. Selva, *11th Int. Symp. Carotenoids*, Leiden, The Netherlands, 18–23 August 1996, Abstr. Posters, p. 90; A. Mele and A. Selva, *Eur. Mass Spectrom.*, 3 (1997) 161–163.
[7] A. Selva, A. Mele, and G. Vago, *Eur. Mass Spectrom.*, 1 (1995) 215–216.
[8] A. Poznankaja, E. Malakhova, T. Korsova, and V. Gunar, *7th Int. Cyclodextrins Symp.*, Tokyo, April 25-28 1994, book of abstracts, p 31.
[9] T. Murao, T. Maruyama, Y. Takahashi, Y. Komatsu, and Y. Yamamoto. (1994), *Jpn. Kokai Tokkyo Koho JP* 06 25,156; C. A., 121 (1994) 7844d.
[10] P.J. Wyatt, *Anal. Chim. Acta*, 272 (1993) 1–40.
[11] R. Mendichi and A. Giacometti Schieroni, *Proc. 9th ISPAC*, Oxford UK, B22, 1996.
[12] B.H. Zimm, *J. Chem. Phys.*, 16 (1948) 1099–1116.
[13] P. Debye, *J. Phys. Colloid Chem.*, 51 (1947) 18–31.

[14] P.V. Demarco and A.L. Thakkar, *J. Chem. Soc., Chem. Commun.*, (1970) 2–4.
[15] Y. Inoue, *Ann. Rep. NMR Spectrosc.*, 27 (1993) 59–101.
[16] S. Li and W.C. Purdy, *Anal. Chem.*, 64 (1992) 1405–1412.
[17] A. Botsi, K. Yannakopoulou, B. Perly, and E. Hadjoudis, *J. Org. Chem.*, 60 (1995) 4017–4023.
[18] N. Azaroual-Bellanger and B. Perly, *J. Magn. Reson.*, 32 (1994) 8–11.
[19] A.W. Coleman, I. Nicolis, N. Keller, and J.P. Dalbiez, *J. Inclus. Phenom.*, 13 (1992) 139–143.