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Complex formation of chlorhexidine gluconate with hydroxypropyl-β-cyclodextrin (HPβCD) by proton nuclear magnetic resonance spectroscopy (^{1}H NMR)

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ABSTRACT

The complex formation of chlorhexidine digluconate (CHX-G_2) with hydroxypropyl-β-cyclodextrin (HPβCD) was studied using NMR spectroscopy. The results revealed that this surfactant agent shows an monomer/aggregate equilibrium, which is dependent on the concentration of this drug. This equilibrium can be modified by the presence of HPβCD, which reduces the aggregation of the CHX-G_2 molecules. An inclusion process of the CHX-G_2 aromatic residue within the cyclodextrin cavity was confirmed by 2D ROESY spectroscopy. ^{1}H NMR titration studies of CHX-G_2 with HPβCD in D_2O confirmed the formation of higher order complexes between CHX-G_2 and HPβCD. Moreover, the addition of HPβCD into CHX-G_2 solutions forms insoluble aggregates. Such insoluble aggregates may result in the stacking of CHX-G_2 molecules on the surface of the CHX-G_2:HPβCD complexes.

1. Introduction

Chlorhexidine (CHX, Fig. 1), 1,1′-hexamethylene-bis-5-(4-chlorophenyl) biguanidide, is a wide spectrum antimicrobial agent that is effective against both Gram-positive and Gram-negative bacteria, yeast, and virus.[1,2]

The presence of two symmetrically positioned basic chlorophenyl guanide groups attached to a lipophilic hexamethylene chain aid in rapid absorption through the outer bacterial cell wall, causing irreversible bacterial membrane injury, cytoplasmic leakage, and enzyme inhibition.[3] It is a common ingredient in various formulations ranging from skin disinfectants in healthcare products to antiplaque agents in dentistry.[4–6]

Because the free base of CHX is essentially insoluble and only exists at very low hydrogen ion concentrations (pH >12), CHX is used as the salt form, commercially available as CHX diacetate (CHX-Ac_2), dihydrochloride (CHX-Cl_2), or digluconate (CHX-G_2). Saturated solutions of CHX diacetate (CHX-Ac_2) and dihydrochloride (CHX-Cl_2) have concentrations of CHX of 2% and 0.2% (w/v), respectively.[7] The digluconate salt is available at a much higher concentration of 20% (w/v) and is used clinically as a diluted solution (2%).[8]

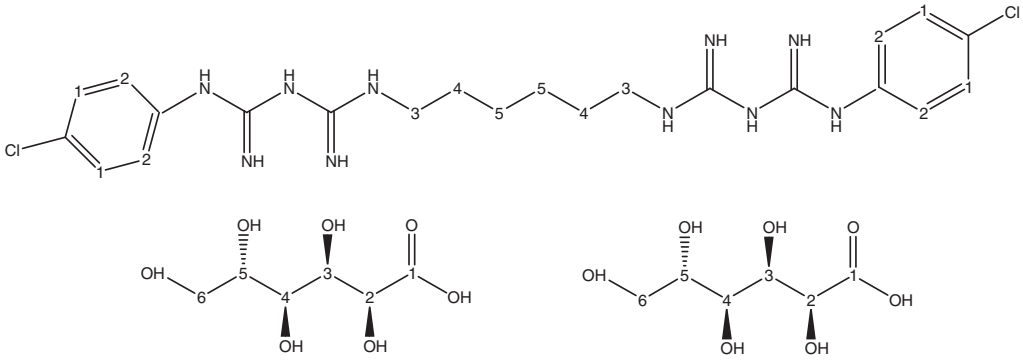

Figure 1. Structure of the chlorhexidine digluconate.

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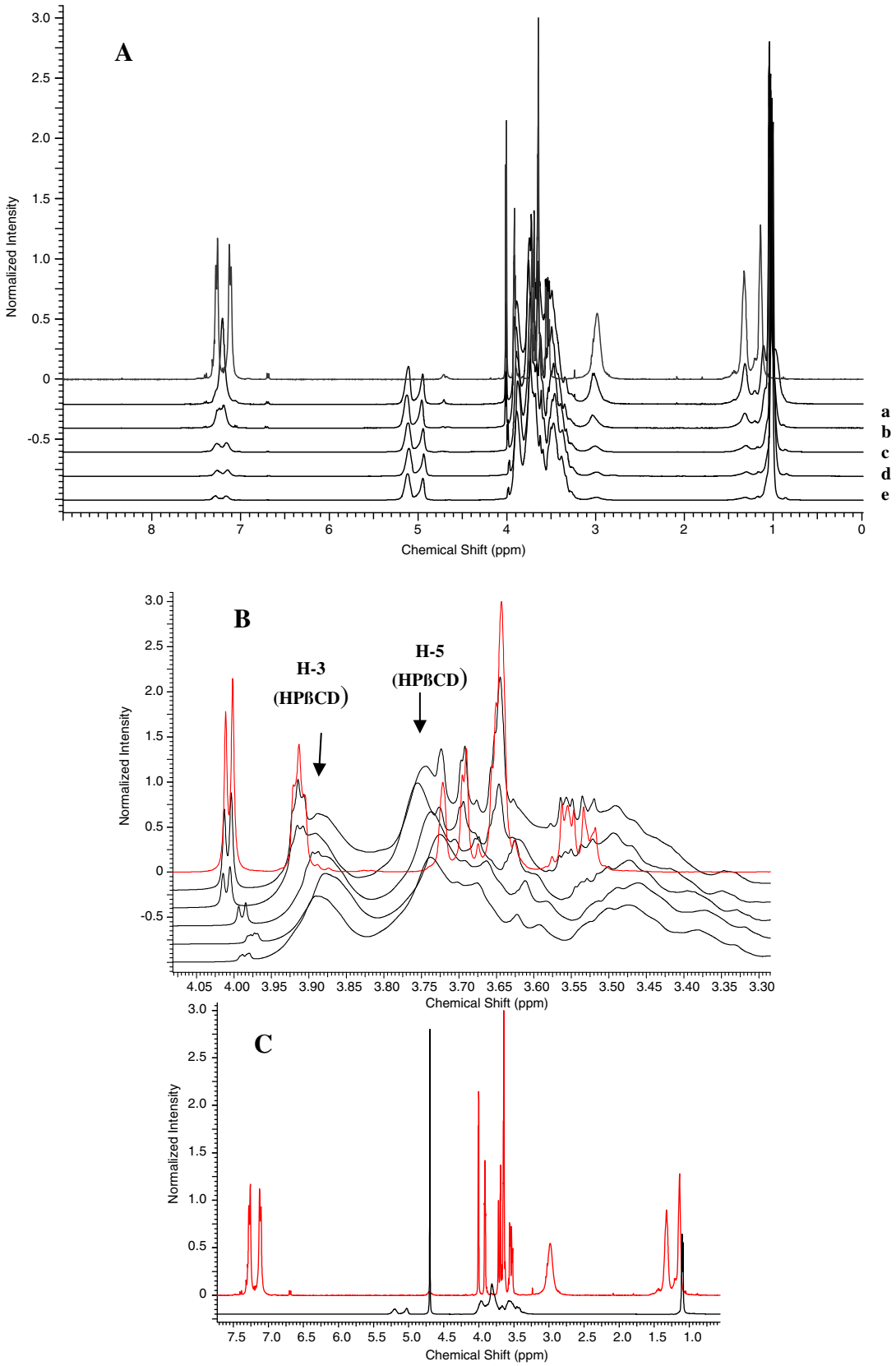

Figure 2. (A) ^{1}H NMR spectra in D_2O at 25 °C for free CHX-G_2 (upper line) and different molar ratios of CHX-G_2 and HPβCD (a) 1:1, (b) 1:2, (c) 1:3, (d) 1:6 and (e) 1:9. (B) Expansion of ^{1}H NMR spectra (4.1–3.3 ppm) in D_2O at 25 °C for free CHX-G_2 (red line) and different molar ratios of CHX-G_2 and HPβCD. (C) ^{1}H NMR spectra in D_2O at 25 °C for free CHX-G_2 (red line) and HPβCD.

It has been reported that CHX forms aggregates in solution similar to the self-association of planar dyes.[9] It was proposed that the aggregation of CHX may involve the association of the phenyl group of one CHX with the hexamethylene chain of a second

CHX and thereby forms a staggered chain,[10] being the concentration for the onset of association at 7 and 11 mM for the diacetate and digluconate solutions, respectively.[9]

Cyclodextrins (CDs) are toroidally shaped polysaccharides with a highly hydrophobic central cavity that allows them to form inclusion complexes with many organic substrates.[11] CDs are able to form host–guest complexes with most surfactants with high binding constants by including surfactants into CD cavities.[12–16] Because the outer surfaces of these cavities are hydrophilic, the resultant surfactant/CD complexes disfavor forming aggregates and are quite dissolvable in water. That is to say, CDs can remove surfactant molecules from aggregates. For single surfactant systems, the removal of surfactant molecules will break or 'weaken' aggregates.[17,18]

Of particular interest is the effect of CDs on the self-organization process of surfactants in which the presence of these macrocycles introduces a new equilibrium which competes with the aggregation.

In this paper, we report on the complex formation of CHX-G_2 with hydroxypropyl-β-cyclodextrin (HPβCD) using NMR techniques including ^{1}H NMR, diffusion, ^{1}H–^{13}C heteronuclear HSQC-DEPT and nuclear Overhauser enhancement (NOE) analysis.

Table 1
^{1}H NMR chemical shifts of clhorhexidine digluconate (CHX-G_2) in the presence or absence of HPβCD

Hydrogen	δ_{CHX}	$\Delta\delta_{CHX:HP\beta CD}$[a]				
		1:1	1:2	1:3	1:6	1:9
1	7.28	0.08	0.05	0.02	0.03	0.02
2	7.13	−0.09	−0.07	−0.04	−0.03	−0.04
3	2.99	0.01	−0.01	0.02	0.04	0.03
4	1.34	0.01	0.01	0.02	0.03	0.01
5	1.15	0.03	−0.06		−0.02	−0.03

[a] $\Delta\delta = \delta_{CHX\text{-}G2} - \delta_{CHX\text{-}G2:HP\beta CD}$.

2. Experimental

2.1. Materials

Chlorhexidine digluconate (20%, w/v) was obtained from Parafarm® (Córdoba, Argentina). HP-β-CD (MW = 1325–1400; degree of molar substitution, 7.0) was a gift from Ferromet S.A. (agent of Roquette in Argentina). For NMR experiments Chlorhexidine digluconate was initially frozen and then dried over night under high vacuum. It was then reconstituted with D_2O to yield a series of concentrations.

2.2. Nuclear magnetic resonance spectroscopy

NMR experiments were recorded on Bruker Avance II 400 spectrometer, operating at 400.16 MHz and equipped with a Broad Band Inverse probe (BBI) and a Variable Temperature Unit (VTU). In all experiments the probe temperature was maintained at 25 °C and standard 5 mm NMR tubes were used. ^{1}H NMR experiments were obtained by the use of the sequence commercially available on Bruker spectrometer. ^{1}H NMR spectra were achieved using the WATERGATE technique for suppression of the residual water signal.

The HSQC-DEPT spectra were recorded with 2D H-1/X correlation via double inept transfer, using sensitivity improvement phase sensitive using Echo/Antiecho-TPPI gradient selection, with decoupling during acquisition using trim pulses in inept transfer with multiplicity editing during selection step using shaped pulses for all 180 degree pulses on F_2—channel with gradients in back-inept. Size of FID TD(F_2) = 2048 TD(F_1) = 256, p_1 = 7.90 useg.

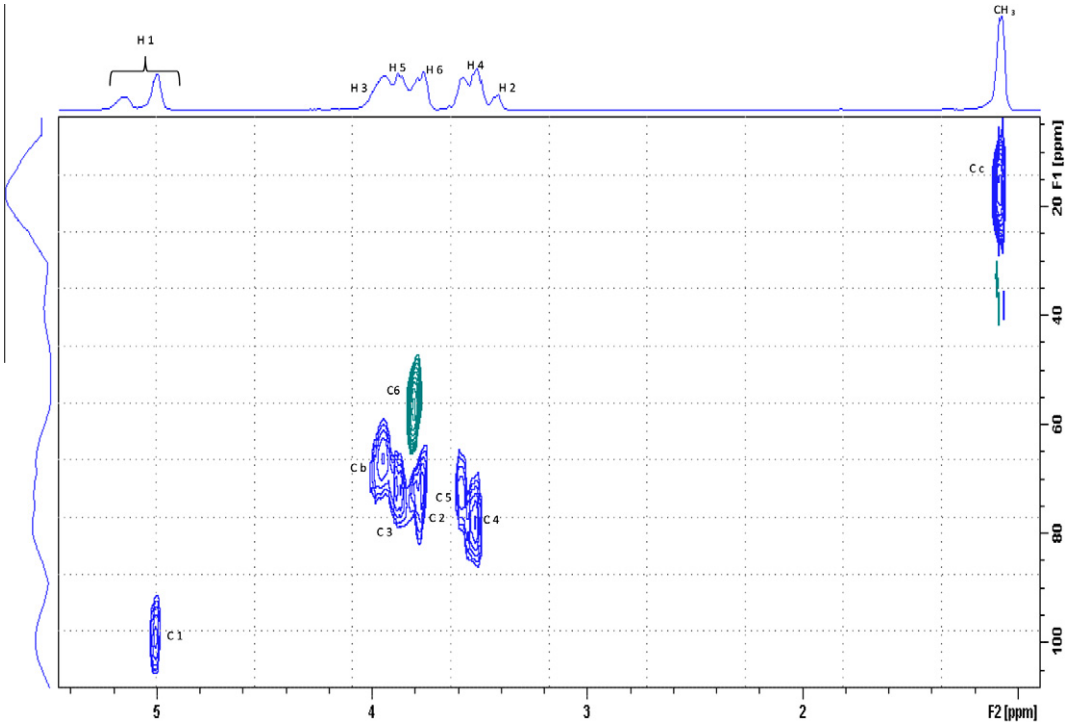

Figure 3. HSQC-DEPT 2D NMR spectrum of HPβCD.

The two-dimensional Rotating frame Overhauser experiments (2D ROESY) spectra (mixing time 250 ms) were acquired using standard experiments from the spectrometer library. Diffusion-ordered spectroscopy (DOSY) experiments were performed using the simulated echo with a bipolar gradient pulse pair sequence, modified with an introduction of a presaturation pulse for solvent signal suppression (STEBPGP1S).

^{1}H NMR titrations were made in D_2O with a constant CHX-G_2 concentration (6 mM) and by progressively increasing the amount of HP-β-CD (0–96 mM) or using a set of solutions containing the same concentration of HPβCD (12 mM) and different concentrations of CHX-G_2 (19.5–250 mM).

The stoichiometry of the complex was obtained using the continuous variation method of Job.[19] ^{1}H NMR spectra were obtained for a series of CHX-G_2:HPβCD mixtures in which the total initial concentration of the two species was kept constant (6 mM) but the mol fraction of each component was varied from 0 to 1. Using this method, the value for $\Delta\delta$ reaches a maximum at the stoichiometric point.

3. Results and discussion

Inclusion of a guest molecule into the hydrophobic cavity of a cyclodextrin molecule generally modifies the environment of the protons of the guest moiety which is included and of the host cavity. Therefore, ^{1}H NMR can provide direct information on the complex structure from the comparison of the proton chemical shifts observed for the guest/host mixture with those found for the individual species.

Because of the symmetric nature of the molecule of CHX (Fig. 1), there are only five unique peaks in the reported proton spectrum of

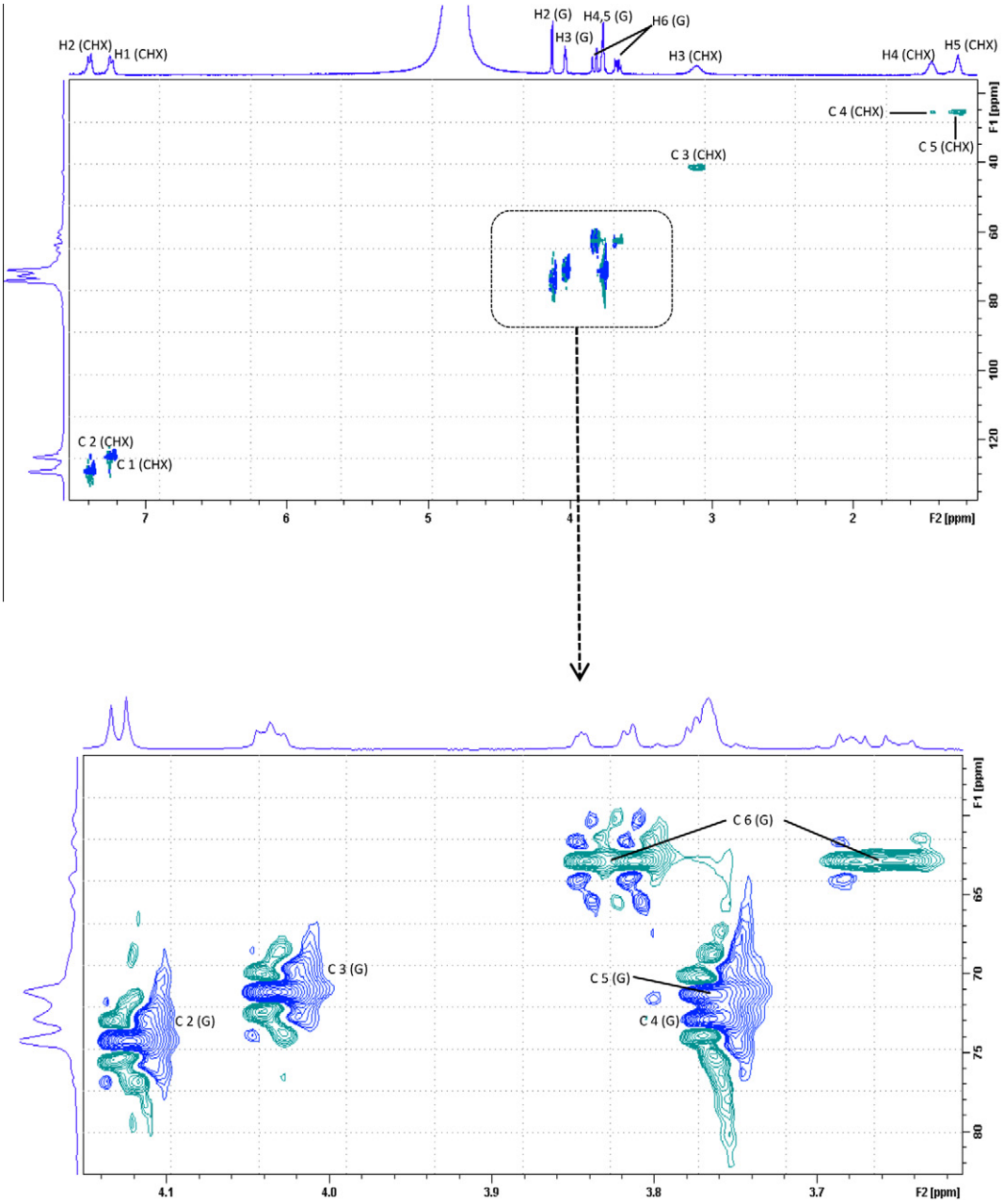

Figure 4. HSQC-DEPT 2D NMR spectrum of CHX-G_2.

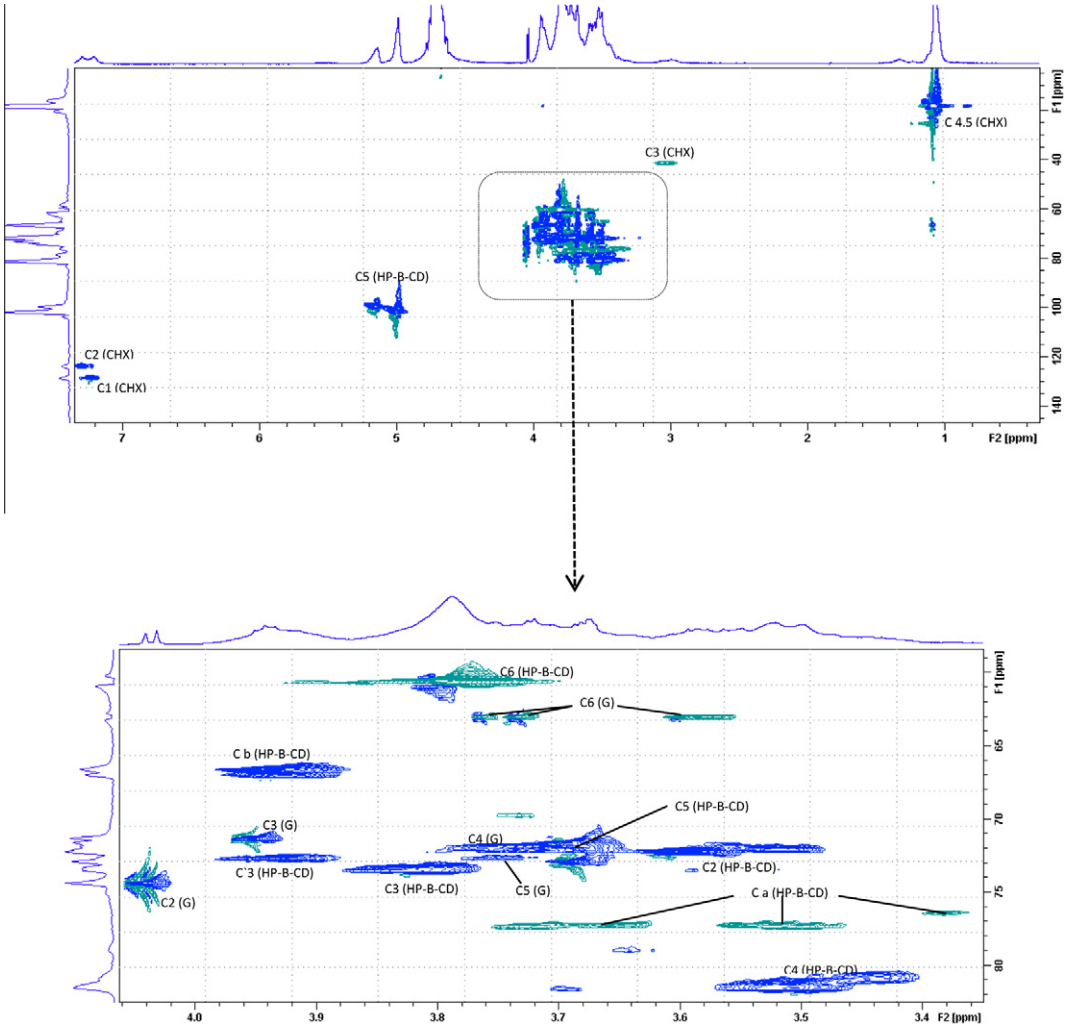

Figure 5. HSQC-DEPT 2D NMR spectrum of CHX-G_2:HPβCD system.

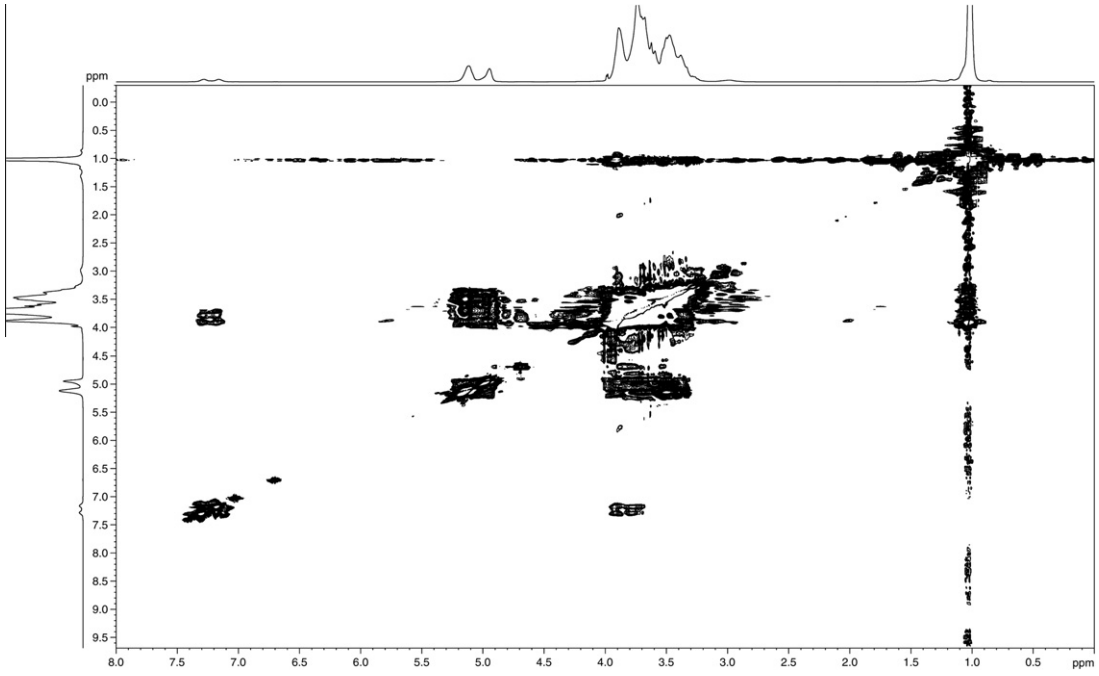

Figure 6. 2D ROESY spectrum of CHX-G_2:HPβCD system (400 MHz, in D_2O).

CHX. Two peaks near 7 ppm arise from the four protons on the *para*-chlorophenyl group and the three remaining peaks at about 2.8, 1.3, and 1.1 ppm from the 12 protons on the symmetric hexamethylene chain in the center of the molecule. The protons associated with the biguanidine group exchange with the D_2O and are not observed.[20]

^1H NMR experiments were performed to evaluate the changes in the chemical shifts ($\Delta\delta$) among pure CHX-G_2 with its inclusion complexes with HPβCD. Figure 2 shows the spectral changes of the CHX-G_2 protons with the addition of HPβCD.

By comparing the ^1H NMR spectra of the inclusion complexes, at molar ration 1:1, 1:2, 1:3, 1:6, and 1:9 of CHX-G_2:HPβCD, to the pure CHX-G_2 spectrum (Fig. 2), modifications in the chemical shifts of the all CHX-G_2 protons can be clearly seen. (Table 1).

It is interesting to note significant changes of the chemical shifts of the *para*-chlorophenyl group signals, the decrease in their resolution, and coalescence phenomena. These protons together with the signals of protons of the hexamethylene chain in the position-5 were more affected.

An unambiguous resonance assignment of the CHX-G_2, HPβCD, and CHX-G_2:HPβCD complex resonance protons was required to ascertain which one of the protons of CHX-G_2 and HPβCD are involved in the complexation. Thus, several ^1H–^{13}C HSQC-DEPT experiments were carried out in order for us to be able to fully assign the spectra. Owing to the observation of superimposed signals

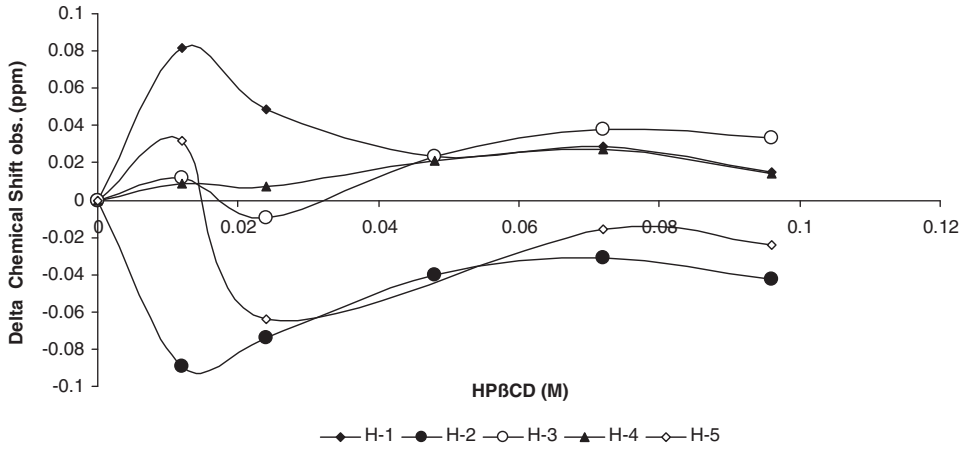

Figure 7. Change in the proton chemical shift values of CHX-G_2, in D_2O at 25 °C, as a function of the HPβCD concentration.

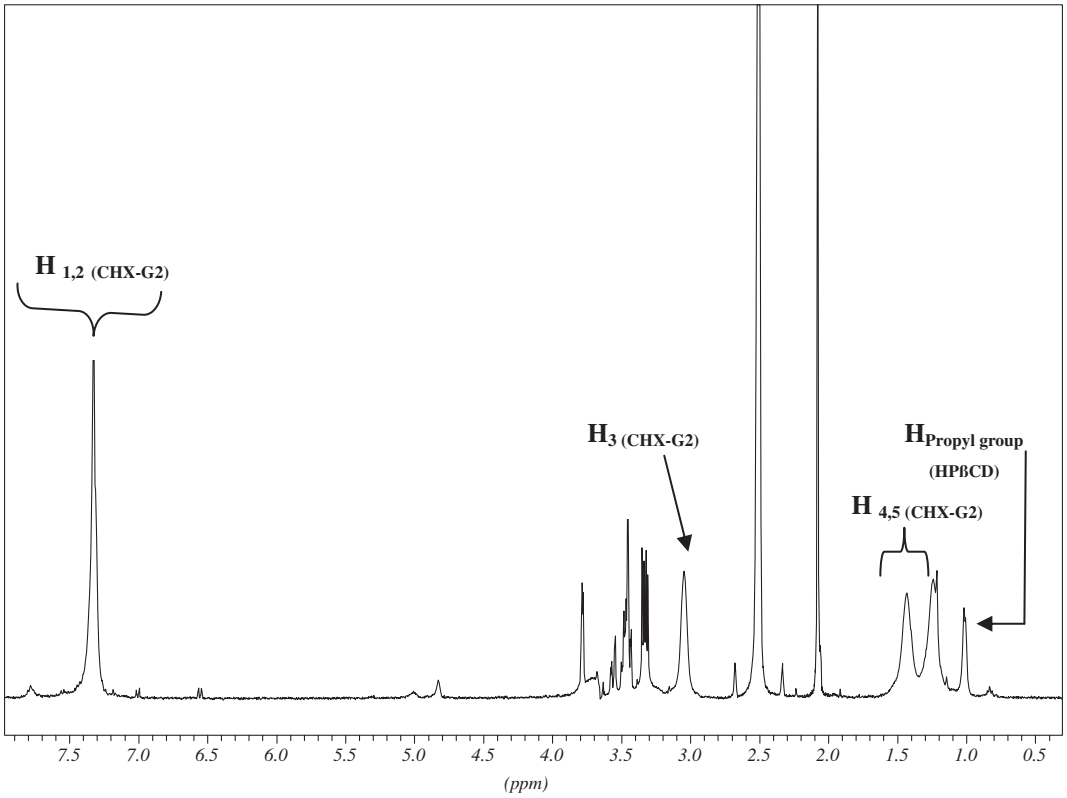

Figure 8. ^1H NMR spectra in DMSO-d_6 at 25 °C of the precipitate, obtained by filtration, formed in the mixture between CHX-G_2 and HPβCD.

in the HPβCD ^{1}H spectrum, a ^{1}H–^{13}C HSQC-DEPT measurement was obtained, Figure 3, in order to provide a tentative assignment. As commercial HPβCD contains a mixture of hydroxypropyl β-CD derivatives with different substitution degrees, a special characteristic of this spectrum is the presence of signals spread over a range of δ values. ^{1}H–^{13}C HSQC-DEPT played an instrumental role in this, because it has pulse sequences to place the CH_2 coupling systems into opposite phase from that of CH and CH_3 coupling systems. This differentiates all the protons attached to C-6 from those attached to C-5. In Figure 3, all correlation peaks in green are related to the H-6 resonances that correlate with C-6 resonances. This spectrum is in good agreement to that reported previously by Schönbeck et al.[21] The CHX resonance proton signals were shown to be well resolved from those of the HPβCD resonance protons signals. However, all the resonances of the gluconate protons of CHX-G_2 appeared superimposed with some protons signals of the HPβCD (Fig. 2). Therefore, we performed the ^{1}H–^{13}C HSQC-DEPT spectra of CHX-G_2 (Fig. 4) and CHX-G_2:HPβCD system (Fig. 5), in the same conditions as those used for the ROESY spectrum.

An accepted criterion for the detection by ^{1}H NMR of the formation of inclusion complexes in cyclodextrins is the NOE between the protons of the guest and the host internal cyclodextrin protons H-3 and H-5. The 2D ROESY experiments allowed observation of the spatial proximity at the 5 Å maximal limit. The formation of inclusion complexes between CHX-G_2 and HPβCD was confirmed by ROESY experiments, which show NOEs between H-1 and H-2 protons of the *para*-chlorophenyl group of CHX-G_2 with the internal cyclodextrin protons H-3 and H-5 situated inside the cavity (Fig. 6). Resonance signals of protons H-3 and H-5 of HPβCD are located around 3.93 and 3.78 ppm, respectively (Fig. 2C). Fig. 2B shows the chemical shift variations of H-3 and H-5 protons of HPβCD (Fig. 2C) with [host]:[guest] mole ratio variations. These data demonstrate the penetration of CHX-G_2 into the HPβCD cavity.

In order to gain better insight into the nature of the inclusion complexes between HPβCD and CHX-G_2 we performed ^{1}H NMR titration experiments. A 6 mM solution of CHX-G_2 was titrated with increasing amounts of HPβCD, and the chemical shifts of CHX-G_2 protons were monitored by ^{1}H NMR (Fig. 7).

Titration curves obtained were unusual, which are consistent with perturbation of CHX-G_2 protons by contribution of more than two species present at equilibrium. From a theoretical point of view, if we consider that CHX-G_2 only exists in the unimeric form in water (i.e., the work concentration is lower than the critical aggregate concentration), the HPβCD can form various inclusion complexes with CHX-G_2 with one or two hydrophobic binding sites, in this case, multiple equilibria may exist for the formation of inclusion complexes between HPβCD and CHX. Taking into account that the CHX-G_2 concentration in the HPβCD solutions used was less than its cac (critical aggregation concentration) and therefore, the self-association of CHX is negligible, we can suggest the formation of complexes of higher stoichiometries between HPβCD and CHX-G_2. Denadai et al.[22] reported the formation of a highly ordered supramolecular complex between CHX and βCD, which a 1:4 CHX:βCD stoichiometry.

Increasing difficulty was encountered during collection of the data points due to the appearance of precipitate in the NMR tubes. Taking into account that both CHX-G_2 and HPβCD are independently soluble in the molality ranges explored, the solid phase observed could not otherwise be composed of a complex precipitate. An explanation of this phenomenon may be the existence of attractive interaction between CHX-G_2:HPβCD complexes and free CHX-G_2 molecules leading to the formation of mixed CHX-G_2 and cyclodextrin complexes aggregates, the water solubility of which may be much lower than those of the separate species. According to this hypothesis, the crystal formed should be composed of a mixture of CHX-G_2:HPβCD complexes with free CHX-G_2 molecules. The presence of these components in the isolated precipitate was confirmed by ^{1}H NMR observation upon dissolution of the isolated precipitate by centrifugation in DMSO-d_6 (Fig. 8).

Job's diagrams based on the induced chemical shifts of CHX-G_2 in ^{1}H NMR were plotted (Fig. 9). The obtained results were contradictory. The plots constructed for protons H-1, H-2 and H-3 of CHX-G_2 on the ^{1}H resonance signals were ambiguous because the maxima observed in the Job plots were in range of 0.3–0.7 and thus are difficult to interpret. The plot having a maximum at 0.5 suggested the existence of a 1:1 complex, whereas the other maximums at 0.33 and 0.67 guest/(host + guest) ratio correspond to 1:2 and 2:1 host–guest stoichiometries, respectively, and suggest that there is not only one uniform complex present in the aqueous solution. A possible rationale for this unexpected behavior could be the presence of multiple equilibria in solution. We suggest the coexistence in solution of 1:1, 1:2 and 2:1 CHX-G_2:HPβCD complexes.

CHX is a symmetric polydiguanide molecule. It is amphiphilic in nature, due to the hydrophilic aromatic rings and urea chain, and the hydrophobic chain in the center of the molecule. Free energy in aqueous media may be minimized when the hydrophobic portion is away from water, while the chlorophenol rings are in the vicinity of water. Therefore, its most stable arrangement should

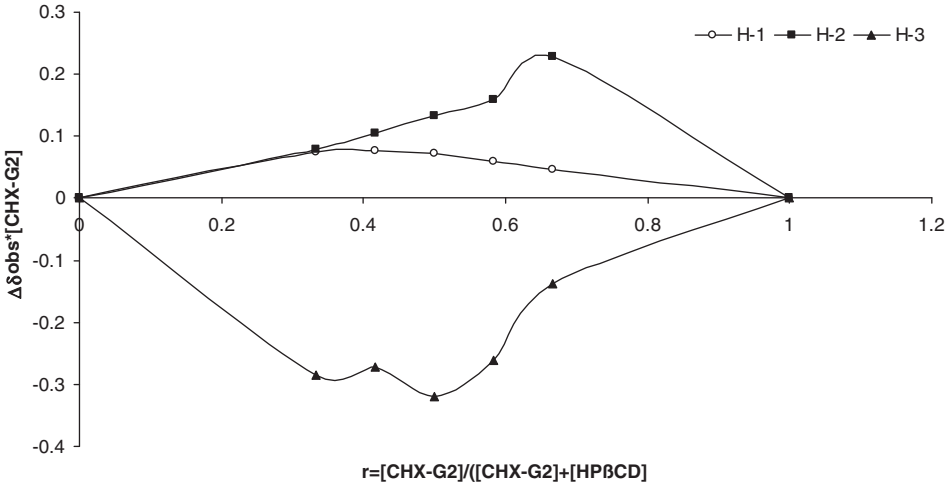

Figure 9. Continuous variation plots (Job's plot) for the CHX-G_2:HPβCD system.

the ends of the same CHX find each other through trans-gauche isomerism of the hexamethylene linker, this energy might be sufficient to hold the CHX in a bent configuration. The 2D ROESY spectrum of CHX-G_2 in D_2O (Fig. 10) supports in favor of this configuration due to correlations between the protons of the hexamethylene chain in the position-3 and positions-4 and -5, respectively were observed.

On the other hand, CHX is known to self-associate in aqueous solution with the formation of dimers or higher aggregates. Zeng et al.[10] suggested that the aggregation of CHX may involve association of the phenyl group of one CHX with the hexamethylene chain of a second CHX and thereby form of a staggered chain. However, 2D ROESY NMR spectra (Fig. 10) obtained from samples of different CHX-G_2 concentrations did not shown intermolecular cross-peak between protons of the phenyl group of one CHX with the protons of the hexamethylene chain of a second CHX. On the contrary, some cross-peaks between the protons of position-3 and position-4 and -5 of the hexamethylene group of CHX were observed. However, it is interesting to note that the intensities of these cross-peaks are decreased as a function of the CHX-G_2

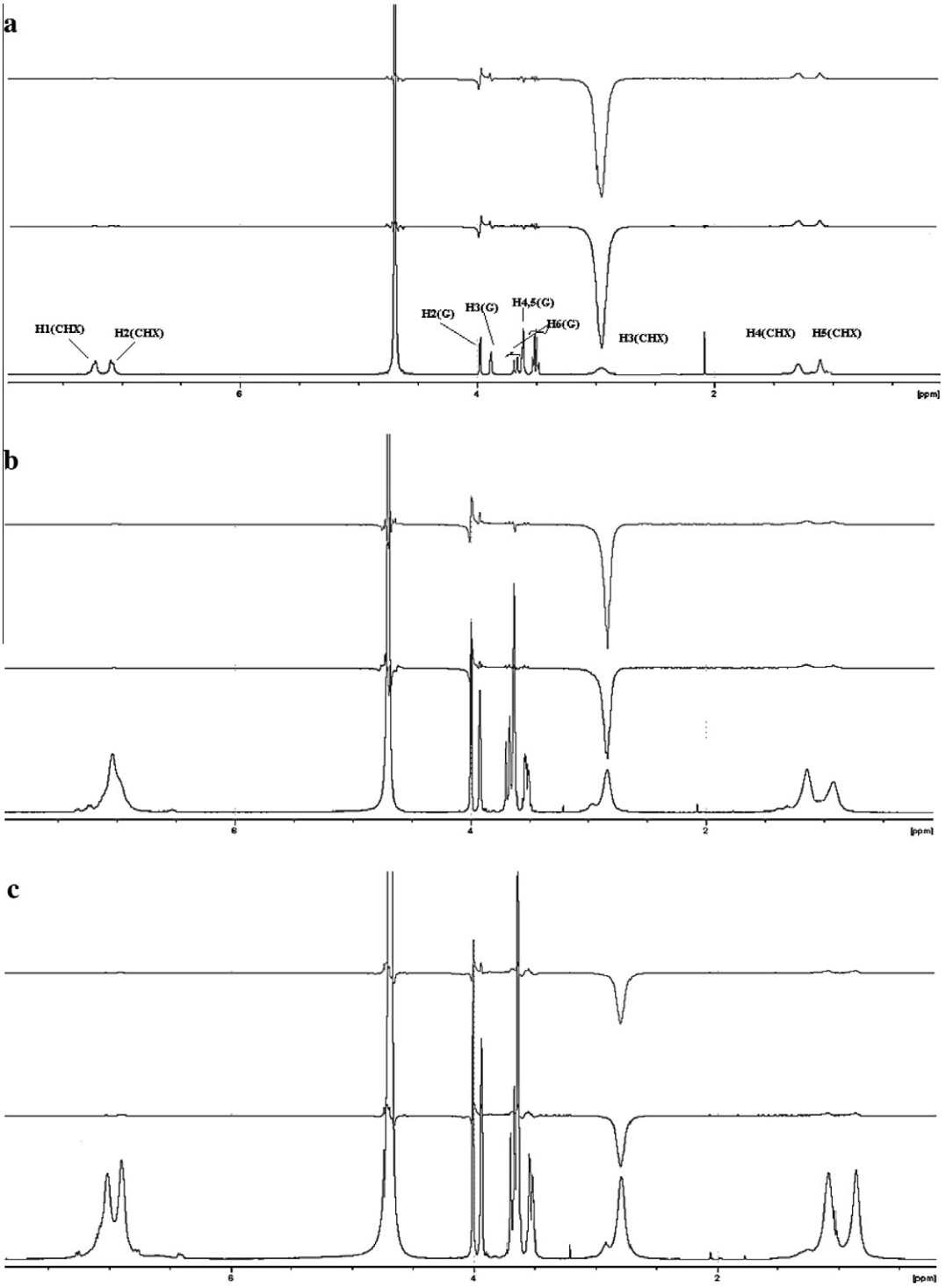

Figure 10. 2D ROESY spectrum of CHX-G_2 in D_2O at 25 °C at (a) 19.5 mM, (b) 82.5 mM and (c) 200 mM.

concentration. This may be due to the changes on the conformation of these molecules at different concentrations as a consequence of molecular aggregation leading to the formation of differently packed supramolecular assemblies. In such assemblies, the number of molecules, the orientations in the aggregate and their mutual interactions and 'tightness' of association should vary as a function of concentration which in turn should manifest in the altered chemical shifts.

To get deeper insight into the effect of HPβCD on the self-aggregation process of CHX-G_2, DOSY experiments were carried out. The variation of molecular size is a crucial parameter in the evaluation of aggregate or complex formation. On the basis

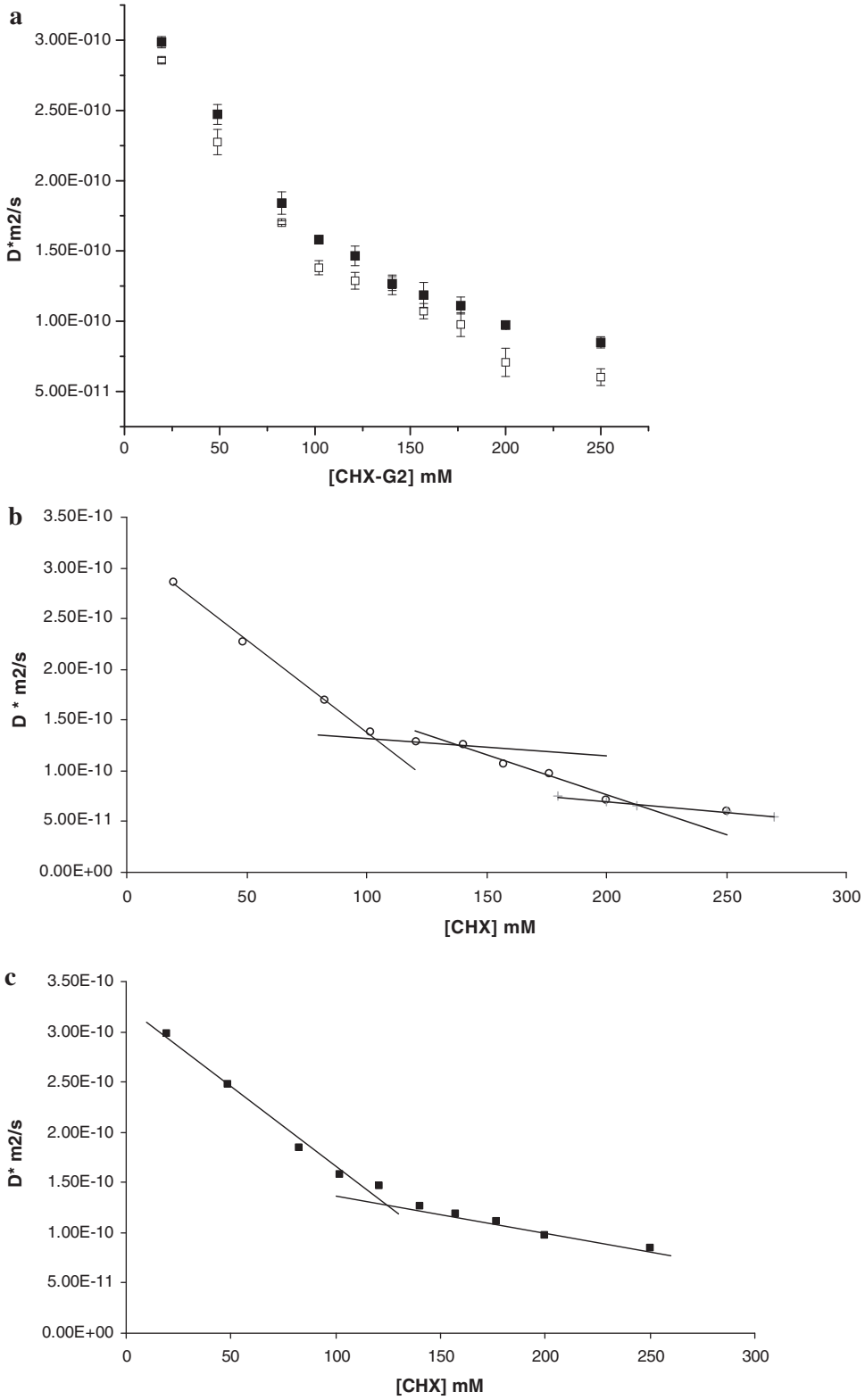

Figure 11. Self-diffusion coefficients from DOSY experiments for (□) CHX-G_2 and (■) CHX-G_2:HPβCD system plotted against the CHX-G_2 concentration.

of the Stokes–Einstein equation, the connection between molecular size and diffusion coefficient can be used to analyze the structural properties of the formed aggregates or complexes. The decreased diffusion coefficient of CHX-G_2 means increased molecular size and decreased molecular mobility (aggregates and/or supramolecular inclusions). The measured values of the diffusion coefficients of these systems were plotted versus the CHX-G_2 concentration (Fig. 11).

The inspection of Figure 11 reveals that, in the absence of HPβCD, CHX-G_2 diffuses more slowly with the concentration as a result of the self-aggregation. Its diffusion profile displays three singular inflection points, corresponding to the concentrations of ~100, ~150 and ~210 mM, respectively. We may identify these concentrations as the first, second, and third critical aggregation concentrations (cac). These critical aggregation concentrations were graphically determined from the CHX-G_2 diffusion curve (Fig. 11b) by intersection of the two straight lines above and below each break point. The cac's for CHX-G_2 were 111, 140, and 213 mM. These data suggest that the aggregation process of CHX-G_2 is a stepwise process involving different species as a function of the CHX-G_2 concentration. On the other hand, as can be seen in Figure 11a, the diffusion coefficients of CHX-G_2 slightly increase upon addition of HPβCD. Figure 11c displays only a single point of inflection in the diffusion profile of CHX-G_2 in the presence of HPBCD. The observed break in the curve may be ascribed to the cac, which was determined to be 125 mM. This curve shows a slight, continuous decrease, proving gradual aggregation. This in turn indicates that complexation with HPβCD breaks up the aggregates of the drug. These results imply that, the formation of the aggregates, in the presence of HPβCD, is shifted toward a higher drug concentration with respect to the values obtained with the drug in the absence of HPβCD. This may be explained by the capacity of HPβCD to modify the aggregation equilibrium existing in different molecules, producing individual molecules of the solute.

4. Conclusions

There is particular interest in the interaction of surfactants with cyclodextrins, both because of the potential for modulating surfactant properties, and because of the possible importance of mixed surfactant/cyclodextrin systems in commercial formulations.

CHX-G_2 is a dicationic surfactant. Its salts are well known for their antibacterial activity and have been used in aqueous based oral compositions to counter dental plaque formation and caries caused by bacteria in the oral cavity. However, CHX and its derivatives have the disadvantage of staining the teeth following repeated use and have furthermore a bitter taste. As strategy to reduce these drawbacks may be the use of cyclodextrins to obtain controlled or sustained release systems of CHX and its derivatives.

In this study, different NMR experiments have been conducted to study the effects of the complexation with HPβCD on the aggregation process of CHX-G_2.

CHX-G_2 undergoes self-association in aqueous solution, most likely forming aggregates that increased in size with increasing concentration. In the concentration range investigated here, CHX-G_2 shows a stepwise association process. Through DOSY analysis it was found three cac's CHX-G_2 values (111, 140 and 213 mM). On the contrary, an exponential decay of the diffusion coefficient values for CHX-G_2 in the presence of HPβCD was observed. Analysis of the diffusion data reveals that an increase in the HPβCD concentration makes this supramolecular system diffuse more quickly than the pure CHX-G_2 molecule. Only one cac value for CHX-G_2 was estimated from the observation of the CHX diffusion profile in the presence of HPβCD (125 mM). Addition of HPβCD increases the first cac's CHX-G_2 value as a consequence of the disruption of the aggregates, modifying the self-aggregation exhibited by this drug. This increase is ascribed to the reduction of free CHX-G_2 concentration, caused by the complexation of CHX-G_2 by HPβCD.

The supramolecular interaction between CHX-G_2 and HPβCD involves the inclusion of the phenoxy groups of CHX-G_2 inside the host central void, as has been evidenced by 2D ROESY observations. ^{1}H NMR titration studies of CHX-G_2 with HPβCD in D_2O and the continuous variation method (Job's plot) confirmed the formation of higher order complexes between CHX-G_2 and HPβCD. Also, the addition of HPβCD into CHX-G_2 solutions forms insoluble aggregates. Such insoluble aggregates may result in the stacking of CHX-G_2 on the surface of the CHX-G_2:HPβCD complexes.

Acknowledgements

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