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Microcalorimetric investigation on the formation of supramolecular nanoassemblies of associative polymers loaded with gadolinium chelate derivatives

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ABSTRACT

In this study, isothermal titration microcalorimetry (ITC) and molecular modeling were used to investigate the mechanism of formation of supramolecular nanoassemblies prepared by mixing aqueous solutions of two associative polymers (i.e. polymerised β-CD (pβ-CD) and dextran grafted with lauryl side chains (MD)). Their capacity to entrap a contrast agent for magnetic resonance imaging (a gadolinium (Gd^{3+}) derivative) has been determined by the same methods. ITC experiments have been employed to evaluate the stoichiometry of interaction (N), association constants (K) and thermodynamic parameter variation associated with complexation between hosts and guests involved in this system. The inclusion compounds studied were: as hosts, β-CD and pβ-CD, and as guests, MD, adamantyl amine, and a Gd^{3+} complex functionalized with adamantane. It has been demonstrated that pβ-CD cavities tend to interact more favourably with MD ($K = 25{,}000\ M^{-1}$) than with adamantly amine ($K = 3650\ M^{-1}$) and Gd^{3+} complex ($K = 1460\ M^{-1}$), forming 1:1 complexes, as also confirmed by molecular modeling. Noteworthy, the Gd^{3+} derivatives, although incorporated in the supramolecular nanoassemblies (by inclusion into the β-CD cavities of pβ-CD), did not destabilize the pβ-CD–MD inclusion complexes, probably because the interaction between pβ-CD and MD was stronger. Finally, the analysis of thermodynamic parameters revealed that the interaction between MD and pβ-CD was entropy driven ($|\Delta H| < |T\Delta S|$) while the interactions of adamantly amine and Gd^{3+} complex with β-CD and pβ-CD were enthalpy driven and dominated by van der Walls forces ($|\Delta H| > |T\Delta S|$).

1. Introduction

Magnetic resonance imaging (MRI) is a powerful, non-invasive clinical imaging modality with high spatial resolution, which has become widely used in the diagnosis of human diseases around the world. The contrast of an MR image is the result of a complex interplay between instrument parameters and intrinsic differences in the relaxation rates of tissue water protons. Currently, 40–50% of MRI exams include the use of a contrast agent (CA), which can dramatically improve the contrast by locally modifying the proton relaxation times (Bellin, 2006). The magnitude of this effect on the longitudinal relaxation time T_1 (or transverse relaxation time T_2) is measured as relaxivity r_1 (or r_2 respectively) normalized to 1 mM concentration at a given magnetic field strength and is used to evaluate the efficacy of the contrast agent. Among the CAs arsenal, Gd^{3+} chelates have become commonplace in medical diagnostics, due to the unique magnetic properties of the Gd^{3+} ion which has seven unpaired electrons (Caravan et al., 1999). The Gd^{3+} ion disturbs the relaxation of nearby water protons, causing decreases of both T_1 and T_2 relaxation times, the effects on T_1 relaxation times being more pronounced in the range of the concentrations used in clinical practice (Mathur-de Vre and Lemort, 1995). Shortening of T_1 relaxation time in tissues, as observed after administration of the standard 0.1 mmol/kg dose Gd^{3+}, produces an increase of signal intensity (positive enhancement).

Gadolinium chelates are the most widely used extracellular, non-specific contrast agents. Currently, seven Gd^{3+} chelates are approved for clinical use in the international market: Magnevist® (gadopentetate dimeglumine; Schering AG), Dotarem® (gadoterate meglumine; Guerbet), Omniscan® (gadodiamide; Nycomed), ProHance® (gadoteridol; Bracco SpA), Gadovist® (gadobutrol; Schering AG), MultiHance® (gadobenate dimeglumine; Bracco SpA) and OptiMARK® (gadoversetamide; Mallinkrodt) (Bellin, 2006). Among all these compounds, only MultiHance® is not only an extracellular contrast agent, but also a liver specific product.

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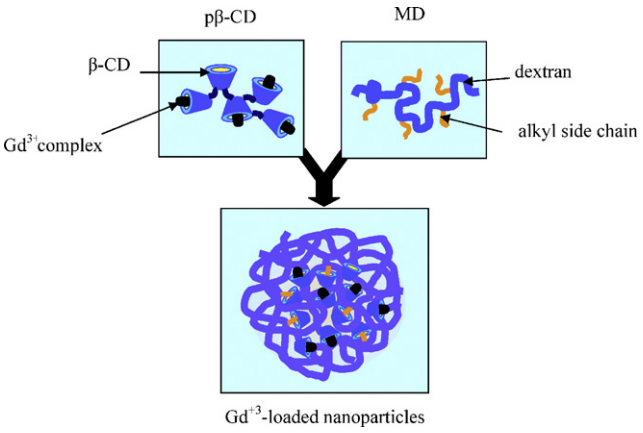

Fig. 1. Schematic representation of the formation of the supramolecular nanoassemblies loaded with Gd^{3+} complex by mixing the two associative polymers MD and pβ-CD.

Indeed, most of the Gd^{3+}-based extracellular agents are non-specific. This means that in order to achieve enough contrast in an MR image, concentrations of CA higher than 50 μM have to be reached in a localized area (Sosnovik and Weissleder, 2007; Zhang et al., 2005). Thus, a very active research area is focussed on the tissue targeting of CAs (Sosnovik and Weissleder, 2007; Jasanoff, 2005), i.e. conceiving systems able to delineate lesions by the specific design of molecules reporter of a given pathology. As the concentration of the targets may be very low (typically 10^{-9} to 10^{-13} mol g^{-1} of tissue), it is compulsory to reach high concentrations of Gd^{3+} chelates with high relaxivity at the site of interest (Caravan, 2006). This goal may be pursued by (i) using polymers containing covalently bound CA units (Aime et al., 2001a; Langereis et al., 2007), (ii) exploiting self-assembly or non-covalent interactions between a suitably functionalized chelate and a macromolecular substrate (André et al., 1999; Aime et al., 2001b) and (iii) using nanocarriers such as liposomes (Erdogan et al., 2006) and nanoparticles (Zhu et al., 2006). Nanocarriers appear therefore as promising candidates for molecular imaging as their size and surface properties can be adapted for a given application, i.e. targeting of a specific tissue.

We recently developed a new nanoparticulate paramagnetic contrast agent with high Gd^{3+} payload and high relaxivity (Battistini et al., 2008). These supramolecular assemblies with a mean diameter of about 200 nm resulted from the association of two water soluble polymers: (i) dextran grafted with alkyl side chains (MD) and (ii) polymer of beta cyclodextrin (pβ-CD). The cohesion of these stable structures is based upon a "lock and key" mechanism; inclusion complexes are formed between the hydrophobic alkyl chains (lauryl) on MD and the molecular cavities contained in the pβ-CD (Gref et al., 2006). Numerous empty cyclodextrin units remained accessible for the inclusion of functionalized Gd^{3+} chelates. The loading of the Gd^{3+} derivatives into the nanoassemblies was performed by simply mixing pβ-CD aqueous solutions containing the Gd^{3+} derivatives with MD solutions (Fig. 1). In these conditions, a payload of 1.8×10^5 units of Gd^{3+} per nanoparticle and a relaxivity r_1 of 48.4 mM^{-1} s^{-1} at 20 MHz and 37 °C were obtained. These results were particularly promising, placing these nanoassemblies as good candidates for MRI, but raising also some issues that needed to be addressed.

Indeed, the mechanism of formation of these new Gd^{3+} loaded nanoassemblies has not been investigated yet and it is not clearly understood why the entrapment of Gd^{3+} derivatives in the supramolecular nanoassemblies, by inclusion into the β-CD cavities of pβ-CD, did not destabilize the system by competing with the alkyl side chains of MD. The key points are the thermodynamic parameters, the stoichiometry of the interactions in the system, and the constants of association of the inclusion complexes. In the present study, isothermal titration microcalorimetry (ITC) was used as a powerful tool enabling to assess all these parameters. The inclusion compounds studied were: as hosts, β-CD and pβ-CD, and as guests, MD, adamantly amine and the Gd^{3+} complex functionalized with adamantane (Fig. 2).

2. Materials and methods

2.1. Materials

β-CD (Cavamax® W7 Pharma) was purchased from Wacker Fine Chemicals, Burghausen, Germany. Pyrene, lauroyl chloride, pyridine, and 4-(dimethylamino)pyridine (DMAP), 1-adamantyl amine (97% purity) were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France) and were used as received without further purification. Lithium chloride (Acros Organics, Belgium) and dextran (average molecular weight 40,000 g/mol, Amersham, Sweden) were dried overnight under vacuum at 80 °C. Anhydrous grade *N*,*N*-dimethylformamide was from Aldrich Chemicals. The other solvents were of analytical grade.

Functionalized gadolinium complex: Gd^{3+} complex of the acid 1,4,7,10-tetraazacyclododecane-1-[(*N*-adamantyl)acetamide]-4,7,10-triacetics, was synthesized as described previously (Battistini et al., 2008). Briefly, the Gd^{3+} complex was obtained in a four-step process from triethyl ester of 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3AET) and 1-adamantyl amine which was first acylated with bromo acetylbromide in CH_2Cl_2 and a suspension of sodium carbonate. The bromo acetamide derivative was then used to alkylate the macrocycle DO3AET. Saponification of the tetra-alkylated cycle (10-[2-(1-adamantylamino)-2-oxoethyl]-DO3AET) by a strong anion exchange resin avoided the formation of salts during this step. The Gd^{3+} complex was formed under pH-controlled conditions with a stoichiometric amount of $GdCl_3$ at room temperature. Finally, special attention was paid to the removal of salts through gel filtration chromatography. The overall yield of synthesis of the Gd^{3+} complex was around 15%.

The pβ-CD polymer was prepared as previously described, by polycondensation of β-CD with epichlorohydrin under strong alkaline conditions (Renard et al., 1997; Daoud-Mahammed et al., 2009). The β-CD content in the polymer, as determined by ^{1}H NMR spectroscopy, was 70% (w/w). The molecular weight of pβ-CD was around 1.5×10^6 g/mol, as determined by gel permeation chromatography.

To synthesize MD, 4 g of dextran (40,000 g/mol) were solubilized in 100 mL of dimethylformamide containing 1 g of lithium chloride. Then, 0.175 mL lauryl chloride and 0.031 mL of pyridine were added to the dextran solution. The reaction was carried out at 80 °C for 3 h. The obtained MD was isolated by precipitation in isopropyl alcohol. It was further solubilized in deionised water, purified by dialysis for 48 h and finally freeze-dried. The substitution yield of MD was determined according to the ^{1}H NMR spectra in DMSO-d_6. It was 2.7% of glucose units.

2.2. Methods

2.2.1. Critical association concentration (CAC) of MD using pyrene as a fluorescent probe

Samples for spectroscopic analysis were prepared as follows: a pyrene-saturated solution in MilliQ® water was prepared by stirring overnight a suspension of pyrene in water, followed by filtration to remove excess of undissolved pyrene microcrystals. MD stock solution (15 g/L) was prepared in pyrene-saturated water. It was left to equilibrate under agitation over 24 h protected from

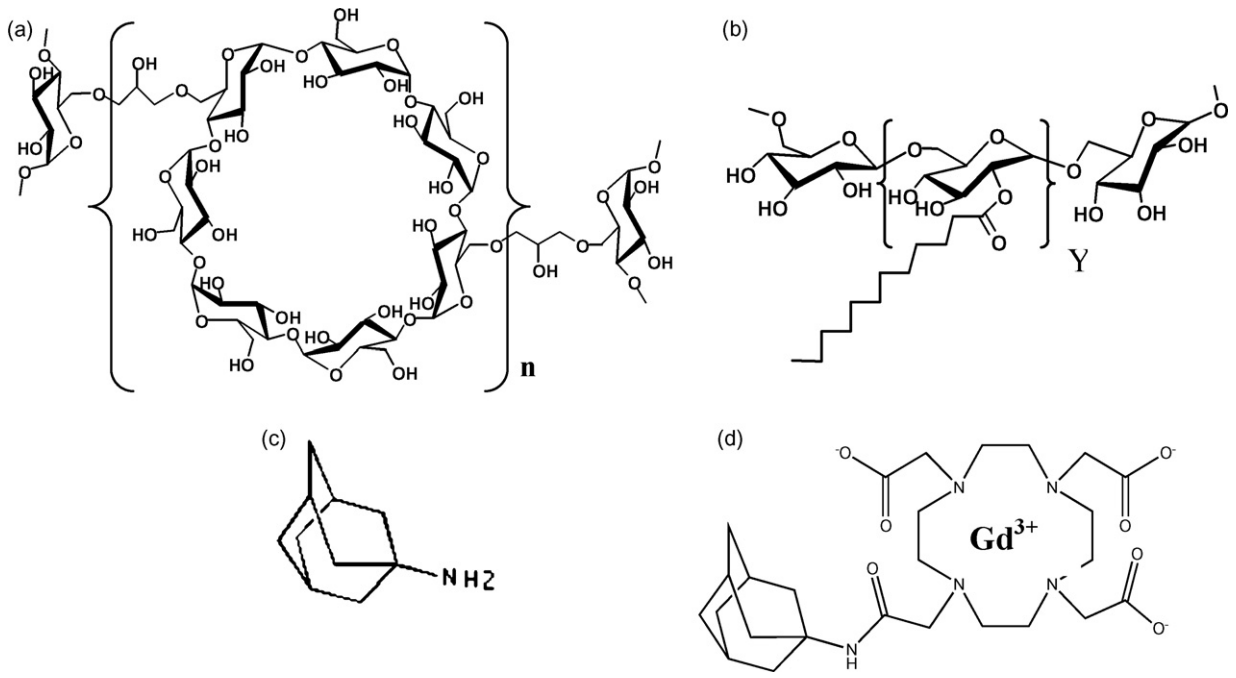

Fig. 2. Chemical structures of (a) the repetitive unit of pβ-CD, (b) the hydrophobized unit of MD, (c) adamantyl amine and (d) Gd^{3+} complex.

light. Subsequently, the stock solution was diluted with pyrene-saturated water to obtain solutions of varying concentrations (1.5×10^{-3}–7.5 g/L), which were further equilibrated under agitation for 24 h. An estimation of the CAC value was obtained by monitoring the changes in the ratio of the pyrene excitation spectra intensities (Francis et al., 2003) at $\lambda = 333$ nm (I_{333}) for pyrene in water and $\lambda = 336$ nm (I_{336}) for pyrene in the hydrophobic medium within the micelle core. Excitation spectra were monitored at emission wavelength $\lambda_{em} = 390$ nm. In this work, we defined the CAC values as the intercept of the tangent of the curve at the inflection point and of the tangent of the curve at high polymer concentration. Fluorescence spectra were measured at 23 °C with a SPEX Fluorolog FL1T11 fluorimeter controlled by computer (Spex Industries, Edison, USA).

2.2.2. Preparation of solutions for ITC experiments

The amount of water in each product (β-CD, pβ-CD and MD) used for the ITC experiments was accurately determined by weighting the samples before and after drying under vacuum at 105 °C during 24 h.

- β-CD and pβ-CD solution were prepared by dissolving the corresponding weight of β-CD or pβ-CD powder into MilliQ® water.
- MD solutions were prepared by dissolving the corresponding weight of MD powder into MilliQ® water. To allow complete polymer solubilization, solutions were magnetically stirred overnight.

2.2.3. Isothermal titration microcalorimetry studies

ITC (MicroCal Inc., USA) has been used for determining from a single titration curve the association constant and the enthalpy of the interaction between the guest (alkyl chains of MD, Gd^{3+} complex, or adamantyl amine) and the hosts (β-CD or pβ-CD solutions). The ITC instrument was periodically calibrated either electrically using an internal electric heater, or chemically by measuring the dilution enthalpy of methanol in water. This standard reaction was in excellent agreement (1–2%) with MicroCal constructor data.

In a typical experiment, aliquots of 10 μL of titrant (β-CD or pβ-CD solutions at a concentration of β-CD cavities = 10 mM), placed in the stirring syringe were delivered over 25 s into guest solutions: MD (0.33 mM in alkyl chains), Gd^{3+} complex (0.8 mM), or 1-adamantyl amine (0.8 mM), placed in the measurement cell at 298.15 K. The corresponding heat flow was recorded as a function of time. Intervals between injections were 600 s to allow complete equilibration and agitation speed was 394 rpm. A background titration, consisting in injecting the same cyclodextrin solution in solely MilliQ® water placed in the sample cell, was subtracted from each experimental titration to account for dilution effects.

Data consisting series of heat flows were collected automatically and when appropriate, the interaction process between the two species has been analysed by the mean of either a one-site or two-site binding model proposed in the Windows-based Origin 7 software package supplied by MicroCal. Based on the concentrations of the titrant and the sample, the software used a nonlinear least-squares algorithm (minimization of Chi^2) to fit the series of heat flows (enthalpograms) to an equilibrium binding equation, providing best fit values of the stoichiometry (N), the association constant (K) and the change in enthalpy (ΔH) (Bouchemal, 2008; Segura-Sanchez et al., 2009; Daoud-Mahammed et al., 2009).

2.3. Molecular modeling

Molecular modeling was used for a better understanding of the interaction of adamantyl amine and Gd^{3+} complex with β-CD. The β-CD structure was taken from Martin Chaplin web page from London South Bank University (Chaplin, 2009). The structures of different molecules have been drawn and presented to the β-CD molecule. Further, the dreiding force field was minimized with the software DS ViewerPro 6.0 (Accelrys Software Inc.) leading to the more likely supramolecular assembly. Bump monitorization, minimization of the dreiding force field (Mayo et al., 1990) and molecular rendering (solvent accessible surface) were also achieved with DS Viewer Pro 6.0 in the case of these optimized structures.

3. Results and discussion

It has been shown that in aqueous media MD and pβ-CD associate whatever their concentrations (Gref et al., 2006; Wintgens

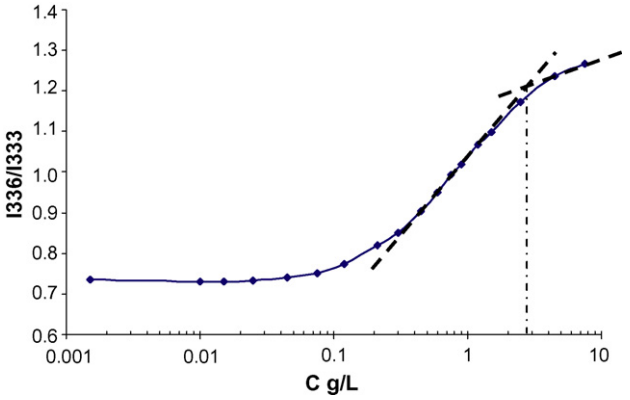

Fig. 3. Experimental determination of the CAC of MD (substitution yield 2.7%). Changes in the I_{336}/I_{333} ratio of pyrene fluorescence intensity as a function of MD concentration. The CAC of MD is 2.5 g/L equal to 0.4 mM of alkyl chain.

et al., 2008). However, stable monodisperse nanoassemblies could only be formed for a MD substitution yield higher than 4% (Gref et al., 2006). In these conditions, MD was used at concentrations higher than the CAC (Gref et al., 2006). In the present study, the CAC of the amphiphilic MD was estimated by fluorescence spectroscopy using pyrene, a hydrophobic fluorescence probe that preferentially partitions into the hydrophobic core of the micelle. The excitation spectrum undergoes a small shift to longer wavelengths as the probe passes from a hydrophilic to a hydrophobic environment (Zhao et al., 1990). This shift was quantified in terms of the ratio, I_{336}/I_{333}, of the fluorescence intensities at 336 nm and 333 nm. The intensity ratios of pyrene fluorescence were plotted against the logarithm of MD concentration (Fig. 3). The CAC was found equal to 2.5 g/L of MD, corresponding to 0.4 mM of alkyl chains. This is in agreement with previously published data of MD (2.2 g/L for MD with substitution yield of 2.9%) obtained by fluorescence spectroscopy using the ratio of first and third emission bands, I_1/I_3, of pyrene (Wintgens et al., 2008).

This means that at concentrations higher than the CAC (0.4 mM), MD, in the form of micelles, should demicellize to associate with pβ-CD. During ITC experiments, both the heats of demicellization and the heats of association CD-alkyl chains are measured. Thus, to determine the association constant between MD and pβ-CD by ITC, we used a concentration of 0.33 mM of alkyl chains grafted on MD, which was lower than the CAC (0.4 mM). In a preliminary study, we established that among a series of MD with substitution yields varying from 2.7% up to 7%, only MD with a substitution yield of 2.7% could be used, because it had the highest CAC, enabling to carry on ITC experiments with a good enough reliability (results not shown). One of the advantages of ITC is the direct estimation of binding enthalpy (ΔH), which in conjunction with the estimated association constant, allows the calculation of the free energy (ΔG) and entropy of binding (ΔS). Examination of these thermodynamic parameters led to a straightforward explanation of the dependence of association constant on the structural features of the alkyl chains or adamantly moieties.

A typical ITC data corresponding to the binding interaction of MD (degree of substitution of 2.7%) with pβ-CD at a concentration of 0.32 mM and at a temperature of 25 °C is presented in Fig. 4. The constant of association determined by ITC between MD and pβ-CD was found very high, 25,000 M^{-1}. Noteworthy, previously published data have indicated that inclusion of MD (substitution yield 4.2%) with single β-CD monomers let to association constants in the lower range of 1950 M^{-1}, as measured by ITC (Wintgens et al., 2008), i.e. about 13 times lower than the association constant that was found here with MD and pβ-CD. Thus, our data account for the high affinity of the alkyl chains for the CD cavities, when used as pβ-CD polymers. The higher association constant of MD-pβ-CD as compared with that of MD-β-CD could be explained by the proximity between C12 and CDs in the pβ-CD polymer; as soon as one inclusion complex is formed, the alkyl chains on MD become in proximity with other available CD cages in pβ-CD and the probabilities to form new complexes increase. Therefore, the association of the two polymers, MD and pβ-CD, can be seen as a "zip mechanism" and accounts for the previously reported excellent affinity between these two water soluble polymers (Gref et al., 2006).

The 1:1 complexation interaction of alkyl chains of MD with a cyclodextrin host (CD) may be written as follows:

$$\text{MD-alkyl}\cdot g\text{H}_2\text{O}+\text{CD}\cdot h\text{H}_2\text{O}\leftrightarrow\text{MD-alkyl}\cdot\text{CD}\cdot(g+h-i)\text{H}_2\text{O}+i\text{H}_2\text{O} \quad (1)$$

where g represents the number of water molecules interacting with the free guest, h the number of tightly bound hydration water molecules inside the free cyclodextrin cavity, and i the net displace-

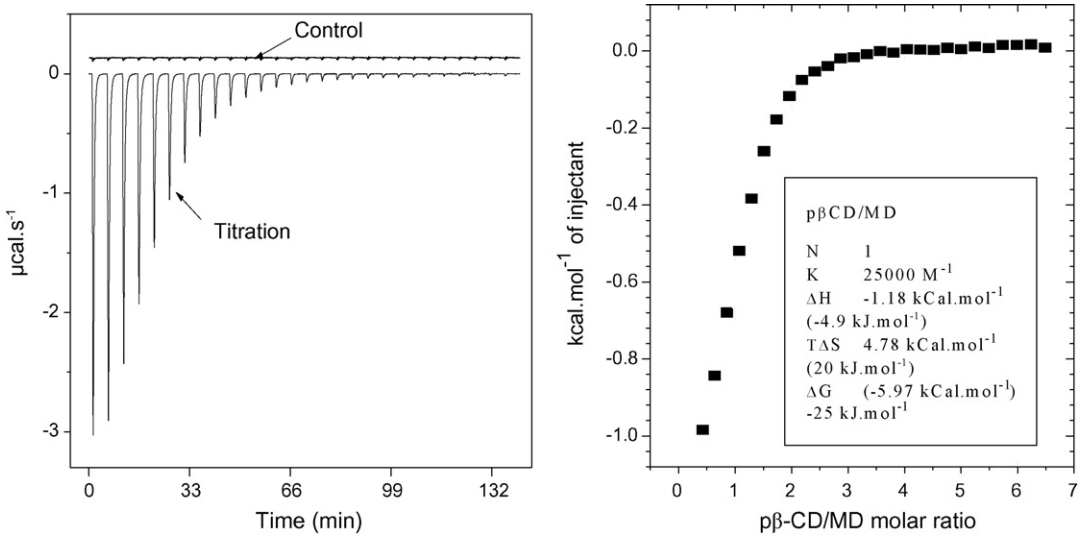

Fig. 4. Typical ITC data corresponding to the binding interaction of MD with a degree of substitution of 2.7% (0.32 mM) with pβ-CD at 25 °C (298.15 K). The left panel shows exothermic heat flows which are released upon successive injection of 10 μL aliquots of pβ-CD into MD. The right panel shows integrated heat data, giving a differential binding curve which was fit to a standard single-site binding model yielding the following parameters $N = 1$, $K = 25{,}000\ M^{-1}$ and $\Delta H = -4.96\ kJ\ mol^{-1}$. Control consisted in successive injections of the pβ-CD solution in solely MilliQ® water. Heat flows accounting for dilution effects were further subtracted from each experimental heat flows.

Table 1
Association constants (K) and thermodynamic parameters corresponding to inclusion complex formation between adamantyl amine (0.8 mM) and Gd^{3+} complex (0.8 mM) with β-CD (10 mM) and pβ-CD (10 mM).

Guest	Host	N	K_a (M^{-1})	ΔH (kJ mol^{-1})	$T\Delta S$[a] (kJ mol^{-1})	ΔG[a] (kJ mol^{-1})
Adamantyl amine	β-CD	1	17300 ± 200	−22.8 ± 3.6	1.4	−24.2
	pβ-CD	1	3650 ± 100	−15.6 ± 2.4	4.7	−20.3
Gd^{3+} complex	β-CD	1	11000 ± 150	−22.5 ± 3.5	0.6	−23.1
	pβ-CD	1.6	1460 ± 120	−9.5 ± 2.0	8.5	−18.0

[a] $\Delta G = -RT\ln K = \Delta H - T\Delta S$.

ment of water upon complexation (Rekharsky and Inoue, 1998). The association constant for a 1:1 complexation of the conjugated cyclodextrin with the guest molecule is expressed by Eq. (2):

$$K = \frac{[\text{MD-alkyl} \cdot \text{CD}]}{[\text{MD-alkyl}][\text{CD}]} \quad (2)$$

where [CD], [MD-alkyl] and [MD-alkyl·CD] are the concentrations of the cyclodextrin, modified dextran expressed in alkyl chains, and the inclusion complex respectively.

The ΔG and ΔS changes were obtained from the following equation:

$$\Delta G = -RT\ln K = \Delta H - T\ \Delta S \quad (3)$$

where R is the gas constant (8.314 J K^{-1} mol^{-1}) and T is the absolute temperature of the interaction. ΔH and K are the enthalpy of the interaction and the association constant as measured by ITC. The strong interaction between the alkyl side chains of MD and pβ-CD accounts for the high value of the association constant, and also for the negative values of ΔH (−4.96 kJ mol^{-1}) and ΔG (−25.02 kJ mol^{-1}), which indicate that the interaction is exothermic and spontaneous respectively.

The most described mechanism for the CD–guest interaction is that, in an aqueous solution, the slightly apolar CD cavity is occupied by water molecules which are energetically unfavoured (polar–apolar interaction), and therefore can be readily substituted by appropriate "guest molecules" which are less polar than water resulting in a more stable lower energy state. Complexation thermodynamics have been shown to reflect the nature of the non-covalent interactions occurring between the guest and CD molecules (Inoue et al., 1993). Indeed, many events, including desolvation of water molecules bound to the guest molecule and/or to the cyclodextrin and the formation of weak bonds (hydrogen-bonds, hydrophobic interactions) or electrostatic bonds, between the guest molecule and the cyclodextrin result in balanced enthalpic and entropic variations. Van der Waals force and hydrophobic interactions related to the size/shape matching between guest molecule and CDs cavity are those among the several possible weak non-covalent interactions which provide the most essential contributions toward the complexation of organic guests with CDs. The study of the enthalpy and the entropy leads to the differentiation between these two types of forces. Traditionally, hydrophobic interactions between two apolar molecules at room temperature have been known as entropy-driven processes, where the entropy of interaction is large and positive while the enthalpy of the process is small ($|\Delta H| < |T\Delta S|$) (Wiggins, 1997). However, van der Waals interactions are usually enthalpy-driven processes with minor favourable or unfavourable entropies of interaction $|\Delta H| > |T\Delta S|$ (Rekharsky and Inoue, 2002). In the case of MD/pβ-CD interaction, the association process was exclusively exothermic ($\Delta H < 0$) with positive and favourable entropic contribution ($\Delta S > 0$) and mostly entropy driven ($|\Delta H| < |T\Delta S|$) (Fig. 4). Large positive entropy changes usually arise from the significantly important translational and conformational freedoms of host and guest upon complexation (Rekharsky and Inoue, 2000a,b). Indeed, there is clear evidence that the cavity size of β-CD is too large to provide a significant contribution due to van der Waals-type interactions. As a result, the flexibility of the supramolecular complex formed is high resulting in a large gain in entropy. Besides, the more favourable entropy changes when the CD cavity is too large compared to the guest molecule has been reported in many previous works (Cromwell et al., 1985; Rekharsky et al., 1997; Rekharsky and Inoue, 2000a).

The formation of the supramolecular nanoassembly loaded with Gd^{3+} complex was also based upon a "lock and key" recognition process, in which the hydrophobic alkyl chains of MD and the adamantyl moieties of macrocyclic Gd^{3+} chelates are included in the cavities of the pβ-CD (Battistini et al., 2008). The large number of β-CDs contained in the pβ-CD polymer resulted in the formation of 200 nm diameter supramolecular nanoassemblies, each entrapping around 1.8×10^5 molecules of the low molecular weight Gd^{3+} complex. This system exhibited a higher relaxivity enhancement (48.4 mM^{-1} s^{-1}, at 20 MHz and 37 °C) as compared to the Gd^{3+} chelate itself (5.2 mM^{-1} s^{-1}). Adamantyl groups are known to be included and held strongly in β-CD, resulting in high association constants of their derivatives (10^3–10^5 M^{-1}) (Cromwell et al., 1985; Eftink et al., 1989; Charbonnier and Penadés, 2004; Tellini et al., 2004). Thus, they should enter in competition with the alkyl side chains for inclusion in the CDs. However, in spite of the use of an excess of adamantane-Gd^{3+} with regard to the available CDs, the nanoassemblies remained perfectly stable, i.e. the size of the nanoassemblies was constant over several days. To evaluate the strength of the different inclusion complexes formed in the adamantane-Gd^{3+} loaded nanoassemblies (i.e. CD with adamantly groups and with alkyl moieties of MD), we have studied by ITC the interaction between the synthesized Gd^{3+} complex or its hydrophobic moiety (adamantane) and β-CD or pβ-CD.

Table 1 shows that adamantyl amine has a higher affinity for β-CD (K = 17,300 M^{-1}) than for pβ-CD (K = 3650 M^{-1}). By the same way, the interaction of the Gd^{3+} complex with pβ-CD is lower than with β-CD (K = 11,000 M^{-1}). Possibly, the amphiphilic Gd^{3+} complexes have less affinity for the crosslinked matrices of pβ-CD, where their hydrophilic parts are not included inside the CDs whereas steric encumbrance effects might additionally occur. On the opposite, the interactions with free CDs in solution do not suffer from these effects.

Thus, the association constant between MD and pβ-CD was found 17 times higher than that between adamantane-Gd^{3+} complex and pβ-CD. We hypothesized that this was due, on one hand and as explained before to the "zip mechanism" of the MD:pβ-CD interaction, and on the other hand to the amphiphilic nature of the Gd^{3+} derivative. Globally, the new adamantane-Gd^{3+} contrast agent inserted well in the nanoassemblies, but did not destabilize the system.

Interestingly, the calculated stoichiometry was 1:1 and 1.6:1 for β-CD:Gd^{3+} complex and β-CD:Gd^{3+} complex, respectively. This unexpected 1.6:1 stoichiometry for Gd^{3+} complex:pβ-CD could be explained by following hypothesis:

(a) The first is that around 3 cavities of β-CD cavities interact with 2 molecules of Gd^{3+} complex. This would be the case if

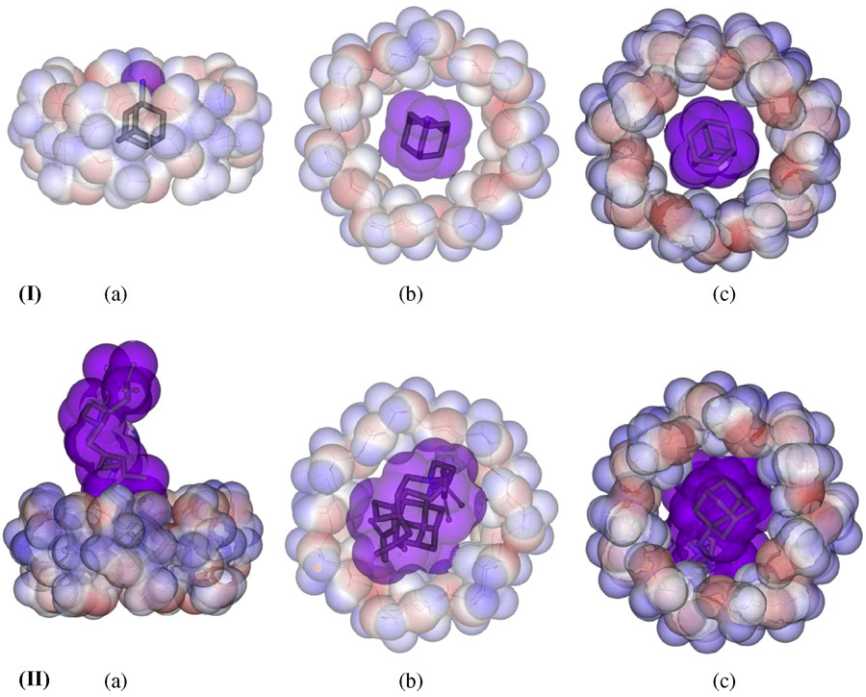

Fig. 5. Optimized structures of inclusion complexes of β-CD cavity with 1-adamantyl amine (I) and Gd^{3+} complex (II) yielded when using DS ViewerPro 6.0 software. (a) Lateral view with secondary face on top, (b) view of secondary face, and (c) view of the primary face of the inclusion complexes.

both 2:1 and 1:1 complexes between β-CD cages and Gd^{3+} complex would be formed. However, this hypothesis was not confirmed by molecular modeling since only one Gd^{3+} complex was able to interact with one β-CD cavity (Fig. 5). Indeed, Fig. 5 shows the calculated optimized structure of the inclusion complex of adamantly amine (Fig. 5.I) and Gd^{3+} complex (Fig. 5.II) with β-CD, where the un-charged adamantane moiety of the Gd^{3+} complex molecule fits very well in the β-CD cavity forming 1:1 inclusion complex. The Gd^{3+} part of the complex cannot be fitted into the β-CD cavity and should stay outside of it.

(b) The second hypothesis is that the adamantyl amine part of the Gd^{3+} complex interacts with CD cavity of the pβ-CD according to 1:1 stoichiometry and the Gd^{3+} part of the complex interacts according to weak and non-specific interactions with the β-CD cavity, or with the bridges between the β-CD cavities or/and with the external face of β-CD molecule. To evaluate the validity of this hypothesis, the fit of the titration curves was achieved according to "two-type of sites" model (Fig. 6). Two stoichiometries and association constants were thus obtained: (i) $N_1 = 1$ and $K_1 = 3000\ M^{-1}$ and (ii) $N_2 = 0.15$ and $K_2 = 640\ M^{-1}$. The first set of parameters is close to that obtained upon the interaction of adamantyl amine with pβ-CD (Table 1). These parameters should correspond to the interaction of the adamantane part of the Gd^{3+} complex with the β-CD cavity. The second set of parameters indicates that the interaction is very weak and should be attributed to non-specific interactions of the Gd^{3+} part of the complex with pβ-CD. Although this second hypothesis is partly confirmed, the nature of the non-specific interactions occurring between Gd^{3+} part of the complex and pβ-CD remains an open question.

(c) The last hypothesis is that the Gd^{3+} complexes interact according to 1:1 stoichiometry with the β-CD cages in pβ-CD, but part of these β-CD cages are inaccessible to the Gd^{3+} complexes, due to sterical encumbrance because of the crosslinks in this polymer. To study this hypothesis, we varied the percentage "*P*" of accessible cages in the mathematical model of one-site binding for ITC data treatment by imposing the concentration of pβ-CD in the stirring syringe (Table 2). The results demonstrated that 1:1 inclusion complex was formed when the concentration of pβ-CD was decreased. The calculated *K* and ΔH correspond to the interaction of Gd^{3+} complex with pβ-CD when the theoretical value of the pβ-CD concentration were closer to the ones obtained with adamantly amine (Table 1). This means that probably, only 60% of the β-CD cavities in the pβ-CD polymers might be able to interact with Gd^{3+} complexes.

Table 2
(I) Association constants (*K*) and thermodynamic parameters corresponding to inclusion complex formation of Gd^{3+} complex (0.8 mM) with pβ-CD (10 mM). (II) Predicted association constants (*K*) and thermodynamic parameters corresponding to inclusion complex formation of Gd^{3+} complex (0.8 mM) with pβ-CD. The initial concentration of pβ-CD was progressively decreased until the 1:1 stoichiometry of the interaction ($N = 1.0$), which corresponds to a percentage *P* of 60% of accessible β-CD in pβ-CD.

	[pβ-CD][a] (mM)	P^b (%)	*N*	$K\ (M^{-1})$	$\Delta H\ (kJ\ mol^{-1})$	$T\Delta S\ (kJ\ mol^{-1})$	$\Delta G\ (kJ\ mol^{-1})$
I	10	100	1.6	1460	−9.5	8.5	−18.0
	9.3	93	1.5	1700	−9.5	8.9	−18.4
	8	80	1.4	2310	−10.3	8.8	−19.1
II	7.3	73	1.2	2520	−11.3	8.1	−19.4
	6.6	66	1.1	2780	−12.4	7.2	−19.6
	6	60	1.0	3090	−13.8	6.1	−19.9

[a] Initial concentration in the stirring syringe.
[b] Supposed percentage of cyclodextrins which are able to interact with Gd^{3+} complex.

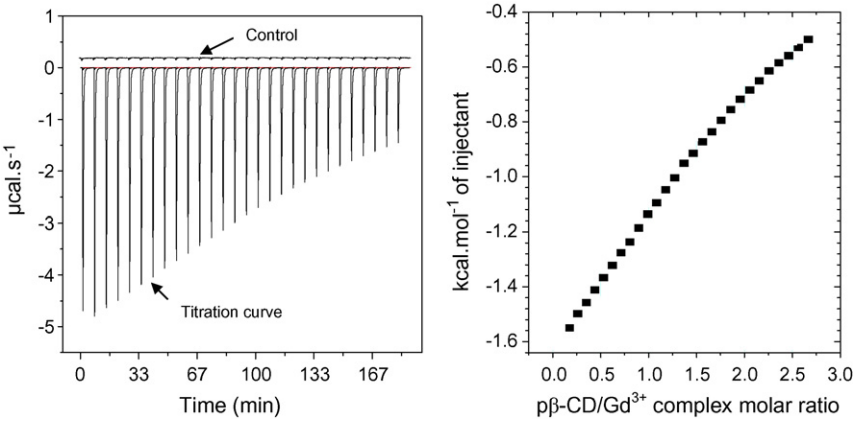

Fig. 6. Typical ITC data corresponding to the binding interaction of Gd^{3+} complex (0.8 mM) with pβ-CD (10 mM) at 25 °C (298.15 K). The integrated heat data gave a differential binding curve which was fit using a two-sites binding model, yielding the following parameters: $N_1 = 1$, $K_1 = 3000\ M^{-1}$ and $N_2 = 0.147$, $K_2 = 637\ M^{-1}$. Control consisted in successive injections of the pβ-CD solution in solely MilliQ® water. Heat flows accounting for dilution effects were further subtracted from each experimental heat flows.

Finally, as it can be seen from Table 1, the interactions of adamantly amine and Gd^{3+} complexes with both β-CD and pβ-CD were exclusively exothermic phenomena ($\Delta H < 0$) with positive entropic contribution ($\Delta S > 0$) and mostly enthalpy driven ($|\Delta H| > |T\Delta S|$). Because large enthalpic gains were observed, it is suggested that the interactions of adamantly amine and Gd^{3+} complex with both β-CD and pβ-CD are predominantly mediated by the formation of van der Waals-type bonds (Rekharsky and Inoue, 2002).

However, for the guests used (adamantyl amine or Gd^{3+} complex), variations were observed in the ΔH and ΔS changes upon their interaction with β-CD and pβ-CD, suggesting that the mechanism of binding was slightly different in the case of β-CD and pβ-CD. Indeed, for adamantly amine/β-CD and adamantly amine/pβ-CD complexes, the ΔH values were $-22.8\ kJ\ mol^{-1}$ and $-15.6\ kJ\ mol^{-1}$, respectively, while TS increased from $1.4\ kJ\ mol^{-1}$ to $4.7\ kJ\ mol^{-1}$ for adamantly amine/β-CD and adamantly amine/pβ-CD complexes, respectively. The same variation was observed for the interaction between the Gd^{3+} complex and β-CD or pβ-CD. As pointed above, large positive entropy changes arise from the important degree of freedom upon complexation. Because of the more hydrophilic environment of the CD cavity in the pβ-CD polymer (Harries et al., 2005), one could expect that the reorganization of surface/cavity neighbouring water molecules that were released upon guest inclusion is higher in the case of pβ-CD than in β-CD, resulting in more positive entropy changes. Furthermore, the desolvation upon guest inclusion and the induced dehydration of the hydroxyl groups in pβ-CD could be responsible for an entropic gain (Daoud-Mahammed et al., 2009).

4. Conclusion

ITC was experienced to be a powerful tool to better understand the interactions involved in adamantane-Gd^{3+} loaded nanoassemblies designed by using associative polymers and where alkyl side chains of MD and adamantyl moieties competed for their inclusion into the CDs cavities. It was concluded that the C12 side chains of MD interacted with the CDs of pβ-CD polymer with a 1:1 stoichiometry. All interactions were spontaneous and the association constants of MD with pβ-CD were remarkably high as compared to the ones between the Gd^{3+} complex and pβ-CD. This accounts for the stability of the Gd^{3+} loaded nanosystems, in which multiple physical crosslinks (inclusion complexes) establish between the associative polymers.

References

Aime, S., Fasano, M., Terreno, E., Botta, M., 2001a. Protein-bound metal chelates. In: Merbach, A.E., Toth, E. (Eds.), The Chemistry of Contrast Agents in Medical Magnetic Resonance Imaging. John Wiley & Sons Ltd., Chichester, pp. 193–241 (Chap. 5).

Aime, S., Botta, M., Fedeli, F., Gianolio, E., Terreno, E., Anelli, P., 2001b. High-relaxivity contrast agents for magnetic resonance imaging based on multisite interactions between a beta-cyclodextrin oligomer and suitably functionalized Gd^{III} chelates. Chem. Eur. J. 7, 5261–5269.

André, J.P., Tóth, É., Fischer, H., Seelig, A., Mäcke, H.R., Merbach, A.E., 1999. High relaxivity for monomeric Gd(DOTA)-based MRI contrast agents, thanks to micellar self-organization. Chem. Eur. J. 5, 2977–2983.

Battistini, E., Gianolio, E., Gref, R., Couvreur, P., Fuzerova, S., Othman, M., Aime, S., Badet, B., Durand, P., 2008. High-relaxivity magnetic resonance imaging (MRI) contrast agent based on supramolecular assembly between a gadolinium chelate, a modified dextran, and poly-beta-cyclodextrin. Chem. Eur. J. 14, 4551–4561.

Bellin, M.F., 2006. MR contrast agents, the old and the new. Eur. J. Radiol. (EJR) 60, 314–323.

Bouchemal, K., 2008. New challenges for pharmaceutical formulations and drug delivery systems characterization using isothermal titration calorimetry. Drug Discov. Today 13, 960–972.

Caravan, P., 2006. Strategies for increasing the sensitivity of gadolinium based MRI contrast agents. Chem. Soc. Rev. 35, 512–523, doi:10.1039/b510982p.

Caravan, P., Ellison, J.J., McMurry, T.J., Lauffer, R.B., 1999. Gadolinium (III) chelates as MRI contrast agents: structure, dynamics, and applications. Chem. Rev. 99, 2293–2352.

Chaplin, M., 2009. Cyclodextrins. In: Water Structure and Science. London South Bank University, From http://www.lsbu.ac.uk/water/cycloh.html, 28 January.

Charbonnier, F.P., Penadés, S., 2004. A straightforward synthesis of 1-adamantylmethyl glycosides, and their binding to cyclodextrins. Eur. J. Org. Chem. 17, 3650–3656.

Cromwell, W.C., Bystrom, K., Eftink, M.R., 1985. Cyclodextrin–adamantanecarboxylate inclusion complexes: studies of the variation in cavity size. J. Phys. Chem. 89, 326–332.

Daoud-Mahammed, S., Couvreur, P., Bouchemal, K., Chéron, M., Lebas, G., Amiel, C., Gref, R., 2009. Cyclodextrin and polysaccharide-based nanogels: entrapment of two hydrophobic molecules, benzophenone and tamoxifen. Biomacromolecules 10, 547–554.

Eftink, M.R., Andy, M.L., Bystrom, K., Perlmutter, H.D., Kristol, D.S., 1989. Cyclodextrin inclusion complexes: studies of the variation in the size of alicyclic guests. J. Am. Chem. Soc. 111, 6765–6772.

Erdogan, S., Roby, A., Sawant, R., Hurley, J., Torchilin, V., 2006. Gadolinium-loaded polychelating polymer-containing cancer cell-specific immunoliposomes. J. Liposome Res. 16, 45–55.

Francis, M.F., Lavoie, L., Winnik, F.M., Leroux, J.C., 2003. Solubilization of cyclosporin A in dextran-g-polyethyleneglycolalkyl ether polymeric micelles. Eur. J. Pharm. Biopharm. 56, 337–346.

Gref, R., Amiel, C., Molinard, K., Daoud-Mahammed, S., Sébille, B., Gillet, B., Beloeil, J.C., Ringard, C., Rosilio, V., Poupaert, J., Couvreur, P., 2006. New self-assembled nanogels based on host–guest interactions: characterization and drug loading. J. Control. Release 111, 316–324.

Harries, D., Rau, D.C., Parsegian, V.A., 2005. Solutes probe hydration in specific association of cyclodextrin and adamantane. J. Am. Chem. Soc. 127, 2184–2190.

Inoue, Y., Hakushi, T., Liu, Y., Tong, L., Shen, B., Jin, D., 1993. Thermodynamics of molecular recognition by cyclodextrins. 1. Calorimetric titration of inclusion complexation of naphthalenesulfonates with α-, β, γ-cyclodextrins: enthalpy–entropy compensation. J. Am. Chem. Soc. 115, 475–481.

Jasanoff, A., 2005. Functional MRI using molecular imaging agents. Trends Neurosci. 28, 120–126.
Langereis, S., Dirksen, A., Hackeng, T.M., van Genderen, M.H.P., Meijer, E.W., 2007. Dendrimers and magnetic resonance imaging. New J. Chem. 31, 1152–1160.
Mathur-de Vre, R., Lemort, M., 1995. Invited review: biophysical properties and clinical applications of magnetic resonance imaging contrast agents. Br. J. Radiol. 68, 225–247.
Mayo, S.L., Olafson, B.D., Goddard, W.A., 1990. DREIDING: a generic force field for molecular simulations. J. Phys. Chem. 94, 8897–8909.
Rekharsky, M.V., Mayhew, M.P., Goldberg, R.N., Ross, P.D., Yamashoji, Y., Inoue, Y., 1997. Thermodynamic and nuclear magnetic resonance study of the reactions of α- and β-cyclodextrin with acids, aliphatic amines, and cyclic alcohols. J. Phys. Chem. B 101, 87–100.
Rekharsky, M.V., Inoue, Y., 1998. Complexation thermodynamics of cyclodextrins. Chem. Rev. 98, 1875–1918.
Rekharsky, M.V., Inoue, Y., 2000a. Chiral recognition thermodynamics of β-cyclodextrin: the thermodynamic origin of enantioselectivity and the enthalpy–entropy compensation effect. J. Am. Chem. Soc. 122, 4418–4435.
Rekharsky, M.V., Inoue, Y., 2000b. 1:1 and 1:2 complexation thermodynamics of γ-cyclodextrin with N-carbobenzyloxy aromatic amino acids and ω-phenylalkanoic acids. J. Am. Chem. Soc. 122, 10949–10955.
Rekharsky, M.V., Inoue, Y., 2002. Complexation and chiral recognition thermodynamics of 6-amino-6-deoxy-β-cyclodextrin with anionic, cationic, and neutral chiral guests: counterbalance between Van der Waals and Coulombic interactions. J. Am. Chem. Soc. 124, 813–826.
Renard, E., Deratani, A., Volet, G., Sébille, B., 1997. Characterization of water soluble high molecular weight β-cyclodextrin–epichlorhydrin polymers. Eur. Polym. J. 33, 49–57.
Segura-Sanchez, F., Bouchemal, K., Lebas, G., Vauthier, C., Santos-Magalhaes, N.S., Ponchel, G., 2009. Elucidation of the complexation mechanism between (+)-usnic acid and cyclodextrins studied by isothermal titration calorimetry and phase-solubility diagram experiments. J. Mol. Recogn. 22, 232–241.
Sosnovik, D.E., Weissleder, R., 2007. Emerging concepts in molecular MRI. Curr. Opin. Biotechnol. 18, 4–10.
Tellini, V.H., Jover, A., Galantini, L., Meijidea, F., Tato, J.V., 2004. Crystal structure of the supramolecular linear polymer formed by the self-assembly of mono-6-deoxy-6-adamantylamide-beta-cyclodextrin. Acta Crystallogr. B 60, 204–210.
Wiggins, P.M., 1997. Hydrophobic hydration, hydrophobic forces and protein folding. Physica A 238, 113–128.
Wintgens, V., Daoud-Mahammed, S., Gref, R., Bouteiller, L., Amiel, C., 2008. Aqueous polysaccharide associations mediated by β-cyclodextrin polymers. Biomacromolecules 9, 1434–1442.
Zhang, Z., Greenfield, M.T., Spiller, M., McMurry, T.J., Lauffer, R.B., Caravan, P., 2005. Multilocus binding increases the relaxivity of protein-bound MRI contrast agents. Angew. Chem. Int. Ed. Engl. 44, 6766–6769.
Zhao, C.L., Winnik, M.A., Riess, G., Croucher, M.D., 1990. Fluorescence probe techniques used to study micelle formation in water-soluble block copolymers. Langmuir 6, 514–516.
Zhu, D., White, R.D., Hardy, P.A., Weerapreeyakul, N., Sutthanut, K., Jay, M., 2006. Biocompatible nanotemplate-engineered nanoparticles containing gadolinium: stability and relaxivity of a potential MRI contrast agent. J. Nanosci. Nanotechnol. 6, 996–1003.