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ORIGINAL ARTICLE

New CD derivatives as self-assembling contrast agents for magnetic resonance imaging (MRI)

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Abstract A way to improve Magnetic Resonance Imaging is to deliver a larger number of Imaging Probe units to the target site. Aiming at this objective, we prepared a self-assembling system consisting of: 1) a β-cyclodextrin (β-CD) bearing a covalently bonded Gd complex (DTPA-Lys); 2) a polypeptide containing a high percentage of tyrosine residues (PLT); 3) a second β-CD derivative bearing a covalently bonded peptide vector (CCK8) that can recognize a specific cell-membrane receptor. Both β-CD derivatives can form stable inclusion complexes with the aromatic moieties of the polypeptide. The formation of a supramolecular adduct having a long reorientational correlation time entailed a marked relaxivity increase (per Gd^{3+} ion), which recommends it as a promising model for detail enhancement procedures at the target site. Out of three different synthetic pathways that could be used for binding a CD to DTPA, the most convenient one involved a micro-wave(MW)-assisted Mannich aminomethylation of a monopropargyl β-CD by the primary amino group of *t*-butyl-DTPA-Lys.

Keywords Cyclodextrin · Self-assembling system · Contrast agents · Magnetic resonance imaging · Microwaves · Ultrasound

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Introduction

Owing to its excellent resolution, magnetic resonance imaging (MRI) is considered the technique of choice in modern diagnostic investigations [1]. More than 35% of clinical scans are currently performed with the administration of contrast agents (CAs), affording a better discrimination between pathological and normal tissues. Since contrast in a MR image mostly arises from differences in relaxation times of tissue water protons, contrast agents used in clinical practice are mainly paramagnetic chelates of Gd(III) ion [2–4] that increase the longitudinal relaxation rate of water protons ($1/T_1$). All gadolinium compounds approved to date share an aspecific extracellular distribution. The next step to improve the technique should rely on systems that are able to recognize specific epitopes on the cellular membrane such as may be singled out as early reporters of disease. The targeting of over-expressed membrane receptors with specific radiopharmaceuticals [5–7] is already a well-established diagnostic method for several types of tumor. However, in spite of its high sensitivity, this approach suffers from poor image resolution.

In the case of MRI, this targeting strategy is hampered by the exceedingly low concentration of such receptors [8, 9] and by the relatively low sensitivity of gadolinium complexes in comparison with tracers used in others diagnostic techniques (i.e. SPECT, PET, optical imaging). To compensate for this drawback it is necessary to accumulate at the target site a large number of CA units that can recognize specific cell-membrane markers of a given disease. One of the possible strategies is the formation of an aggregate of Gd(III) complexes at the target site. This goal can be

achieved by different means, namely: (i) a macromolecule carrying many paramagnetic complexes and a specific vector that can recognize a disease marker on the cell membrane; (ii) a targeting polymeric ''tree carrier'' containing many binding centres that can form a supramolecular adduct with suitably modified Gd complexes. Such a supramolecular adduct could be formed at the target site by a three-step procedure consisting in the sequential administration of (a) the targeting molecule (TM, a small peptide), (b) a polymer that can bind both the TM and a large number of CA molecules and (c) the CA itself. This procedure should be preferred because the single components, having a smaller molecular size than the overall adduct, could more easily cross the vessel barrier to reach the target site.

In this context we developed a self-assembling system consisting of: (1) a β-cyclodextrin (β-CD) bearing a covalently bonded CA; (2) a polypeptide containing a large number of tyrosine residues; (3) a second β-CD derivative bearing a covalently bonded peptide vector that can recognize a specific cell-membrane receptor. Both β-CD derivatives can form stable inclusion complexes with the aromatic moieties of the polypeptide. Fig. 1 shows the self-assembled adduct that should form on the cell membrane. As a vector we used CCK8, a peptide that can recognize the colecistokinine receptors on the cell surface, whereas the polypeptide which is to constitute the ''trunk of the CA tree'' was PLT, a poly-lysine-tyrosine with a molecular weigh of 100 kDa, formed by 47 lysine and 53 tyrosine residues. The CA chosen for this study was a Gd-DTPA (DTPA = diethylentriaminopentaacetic acid) that had been modified to carry a pendant CD arm. The most critical synthetic steps in the present work were carried out using non-conventional methods such as sonochemical protocols and microwave (MW)-promoted reactions.

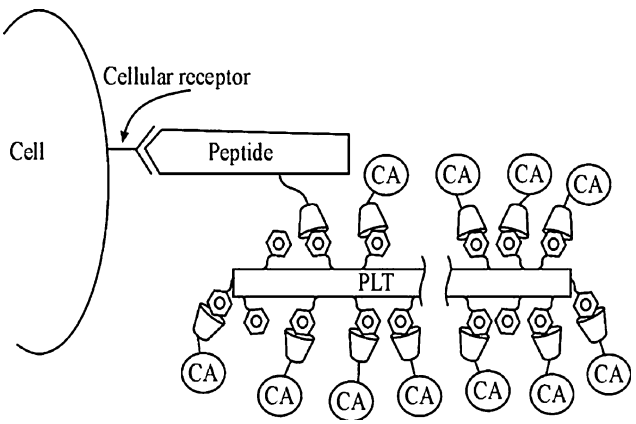


Fig. 1 Schematic representation of a self-assembling second-generation MRI contrast agent

Experimental

All chemicals were purchased either from Sigma-Aldrich Co.; native CDs were kindly donated by Wacker Chemie (Germany). Chemicals were used without purification unless otherwise stated. NMR spectra were recorded on a Bruker Avance 300 (operating at 7 Tesla) using as internal reference the deuterated solvent (when this was D_2O a small amount of tert-butyl alcohol was added as internal standard). The IR spectra were recorded on a Shimadzu FT-IR 8001 spectrophotometer; MALDI mass spectra were acquired in the positive reflectron ion mode with delayed extraction on a Reflex III time of flight instrument (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm nitrogen laser. HPLC analyses and separations were carried out on Amersham AKTA purifier 10/100 and on Waters instruments (pump Waters 1525EF, diode array detector 2996 and auto-sampler 717 plus). Column chromatography (CC) was performed on silica gel 60 (Merck). Analytical TLC (Thin Layer Chromatography) was carried out using plates from Alugram Sil-Macherey Nagel (F_{254}, 0.25 mm); spots were visualized either with phosphomolybdic acid or by heating after the plates had been sprayed with 5% sulphuric acid in ethanol. The sonochemical apparatus used in the present work was developed in the laboratory of one of the authors [12]. MW-promoted reactions were carried out in a MicroSYNTH Milestone oven.

Longitudinal water proton relaxation rates were measured on a Stelar Spinmaster (Mede, Pavia, Italy) spectrometer operating at 20 MHz, by means of the standard inversion-recovery technique (16 experiments, 2 scans). A typical 90° pulse width was 4 μs and the reproducibility of the T_1 data was ±0.5%. The temperature was kept at 25 ± 0.1 °C with a Stelar VTC-91 air-flow heater equipped with a copper-constantan thermocouple.

6-*O*-TBDMS-β-CD [13] and *t*-butyl-DTPA-Lys [14] were prepared following published procedures.

6′-deoxy-6′-formyl-β-CD (5)

Conventional procedure [15]

β-CD (500 mg, 0.44 mmol) was dissolved in DMSO (10 ml) and Dess-Martin periodinane reagent was added (374 mg, 0.88 mmol). After 2 h stirring at RT, the

product was recovered as a white precipitate on adding acetone. The solid was filtered off, washed with acetone and dried under vacuum. 150 mg of the desired product were collected (yield 28%).

Ultrasound (US)-assisted reaction

In the PTFE vessel of the sonochemical reactor [12] 30 ml DMSO, *β*-CD (3g, 2.64 mmol) and Dess-Martin periodinane reagent (1.68g, 3.96 mmol) were added. The mixture was irradiated (60W, 19.2 kHz) for 1 h under nitrogen atmosphere. The product was recovered as a white solid by addition of acetone. The precipitate was filtered off, washed with acetone and dried under vacuum. 1.8 g of the desired product were collected (yield 60%). R_f 0.35 (CH_3CN/H_2O 2:1); IR: *v* 3420; 1730; 1472; 1245; 1036; 835; ^{1}H-NMR (DMSO-d_6)? *δ*: 9.65 (s, 1H); 5.76–5.70 (m, OH); 4.89 (brs, 1H); 4.78 (brs, 6H); 4.53 (m, OH); 4.17–4.13 (m, 1H); 3.58–3.20 (m, 39H); MALDI-TOF MS: *m/z* calcd. for $[MNa]^+$ 1155.4; found 1156.

t-butyl-DTPA-Lys-*β*-*CD (6), (reductive amination)*

6′-deoxy-6′-formyl-*β*-CD (5) (235 mg, 0.21 mmol) was dissolved in 30 ml methanol and the solution was poured in the sonochemical reactor [12]. pH was adjusted to 5 with acetic acid before *t*-butyl-DTPA-Lys (185 mg, 0.25 mmol was added and the mixture was irradiated with US (140W , 19.2 kHz) for 3 h at 40 °C. Complete conversion was then achieved by heating under reflux for 5 h. Without the preliminary sonication the oxidation gave a much lower yield. After it was allowed to cool down to room temperature, $NaBH_3CN$ (85%, 15.5 mg, 0.21 mmol) was added and the mixture was heated at reflux temperature under nitrogen atmosphere for 10 h. The solvent was then removed and the crude product was purified by CC, (CH_3CN/water 2:1) yielding 50 mg of *t*-butyl-DTPA-Lys-*β*-CD (Yield = 18%) as a pale yellow powder.

R_f = 0.64 (CH_3CN/H_2O 2:1). ^{1}H-NMR (D_2O) *δ*: 5.38–5.28 (m, 7H); 3.83–3.53 (m, 40H); 3.39 (m, 9 H); 2.95–2.83 (m, 12H); 1.65 (m, 2H); 1.41–1.37 (brs, 45; overlapped 4H); MALDI-TOF MS: *m/z* calcd. for[-$MNa]^+$ 1883.8; found 1884.5.

DTPA-Lys-*β*-CD (7)

The *t*-Butyl ester groups of 6 were cleaved off by treatment with TFA under stirring at room temperature for 6 h. After solvent evaporation under vacuum DTPA-Lys-*β*-CD was quantitatively recovered as a white powder. R_f: 0.43 (CH_3CN/H_2O 2:1);^{1}H-NMR (D_2O) *δ*: 5.38 (brs, 6H); 5.19 (d, J = 3.9 Hz, 1H) 4.08–3.35 (m, 40H, overlapped 9H); 3.26–3.15 (m, 12H); 1.66 (m, 2H); 1.48 (m, 4H); 1.24 (m, 2H); ^{13}C-NMR ($CDCl_3$): *δ*175.9, 169.5, 99.9, 77.2, 73.5, 71.8, 69.8, 62.9, 60.9, 55.9, 53.3, 53.0, 46.4, 29.4, 28.0, 23.3; MALDI-TOF MS: *m/z* calcd. for $[MNa]^+$ 1603.6; found 1603.9.

2^I-O-propargyl-$6^{I–VII}$-O-tBDMS-*β*-CD (10)

Following the procedure described in a previous paper of ours [16], we obtained *2^I-O-propargyl-6^{I-VII}-O-tBDMS-β-CD* in 40% yield as a white powder. R_f: 0.26 ($CHCl_3/CH_3OH$ 4:1); IR: *v* 3420, 3325, 1473, 1254, 1086, 1040, 835 cm^{-1}; ^{1}H-NMR ($CDCl_3$) *δ*: 4.9 (br.s, 7 H), 4.5 (br.qd, 2H), 4.1–3.9 (m, 14H), 3.8–3.5 (m, 28H), 2.4 (t, 1H), 0.88 (s, 63H), 0.05 (s, 42 H); ^{13}C-NMR($CDCl_3$) *δ*: 102.7, 82.1, 75.7, 73.8, 73.4, 73.2, 72.6, 62.1, 60.1, 25.8, 18.2, -5.0, -5.2; MALDI-TOF MS: *m/z* calcd. for $[MNa]^+$ 1993.9; found 1994.0.

t-butyl-DTPA-Lys-2^I-O-propargyl-6^{I-VII}-O-tBDMS-*β*-CD (11)

Paraformaldheyde (9 mg, 0.3 mmol) and *t*-butyl-DTPA-Lys (75 mg, 0.1 mmol) were dissolved in acetonitrile (10 ml). The mixture was irradiated with MW at 300 W for 15 min (the temperature rose to 84 °C in 2 min, then stayed constant). Compound 10 (200 mg, 0.1 mmol), and a catalytic amount of CuI were poured in to THF (5 ml) and added to the reaction mixture previously cooled down to room temperature. The ensuing reaction was carried out under MW (300 W) for 15 min (84 °C). The reacted mixture was diluted with EtOAc, washed with H_2O and brine, and finally dried (Na_2SO_4). The crude residue was purified by CC (silica, $CHCl_3/CH_3OH$ 9:1, 4:1) giving 82 mg of (11) as a pale yellow powder (yield 30%). R_f 0.51 ($CHCl_3$/CH_3OH = 4:1); ^{1}H-NMR ($CDCl_3$) *δ*: 4.9 (brs, 7H), 4.5 (s, 2H), 4.2–3.8 (m, 14H), 3.8–3.2 (m, 39H), 2.8 (m, 10H), 1.7 (m, 2H) 1.5 (s, 45H, overlapped 2H), 1.3 (m, 2H) 0.88 (s, 63H), 0.05 (s, 42H); MALDI-TOF MS: *m/z* calcd. for $[MNa]^+$ 2751.5; found 2751.9.

DTPA-Lys-2^I-O-propargyl-*β*-CD (12)

The *t*-Butyl ester groups on DTPA-Lys were cleaved off by treatment with TFA under stirring at room temperature for 6 hours. After solvent evaporation under vacuum, the product 12 was quantitatively recovered as a white powder. ^{1}H-NMR (D_2O) *δ*: 5.1 (brs, 7H), 4.7 (s, 2H), 4.2–3.3 (m, 65H), 2.2 (m, 2H), 1.9

(s, 2H), 1.3 (m, 2H); MALDI-TOF MS: *m*/*z* calcd. for $[MNa]^+$ 1671.6; found 1672.3

General procedure for the synthesis of Gd complexes

Gd(III) complexes were synthesized in water by adding stoichiometric amounts of $GdCl_3$ to a 40 mM ligand solution at pH = 6.7 (pH was adjusted with 0.5 M NaOH). After stirring at RT for 24 h, the pH likewise readjusted to 8.5. Excess Gd(III) was then removed by centrifugation (7000 g/min) as $Gd(OH)_3$. Residual Gd(III) free ion was estimated by UV-Vis spectroscopy using the Xylenol Orange method [17]. All complexes used in the following were shown to contain less than 0.3% (mol/mol) residual free Gd(III) ion.

CCK8-CD (β-CD-NH-GDYMGWMDF-$CONH_2$)

Solid-phase peptide synthesis was carried out with commercially available Fmoc aminoacids in an automatic peptide synthesizer. Rink amide resin was activated by removing the Fmoc protection with piperidine, then the first aminoacid was attached with the aid of HBTU, HOBT and DIPEA. By the automatic procedure the required aminoacids were linked together in the sequence GDYMGWMDF; finally β-CD was conjugated to the peptide by reductive amination between the mono aldehyde CD and the peptide *N*-terminus as described in the literature [18]. The peptide was then cleaved from the resin, deprotected and purified by HPLC (Analytical method: column Waters Atlantis RPd C18, 5 μm, 4.6 × 150, elution with 0.1% TFA in acetinitrile, flow rate of 1 ml/min. Preparative separation was performed on Waters Atlantis RPd C18, 5 μm, 19 × 100, flow rate of 20 ml/min, scaling up the analitycal run).

MS – MALDI-TOF: *m*/*z* calcd. for $[MNa]^+$ 2277.4; found 2278.0

Results

Syntheses of β-CD derivatives coupled to CA, were carried out by three different routes, to yield compounds differing in the nature of the spacer linking DTPA-Lys and CD. Interestingly, these differences did not affect the relaxometric properties of the Gd complexes nor their interaction with PLT.

The first approach is represented in Scheme 1. It shows the conjugation of 6-monoamino-β-CD [10], through a maleyldiamide spacer, to the lysine arm of DTPA-Lys, which was subsequently complexed with Gd(III) [11].

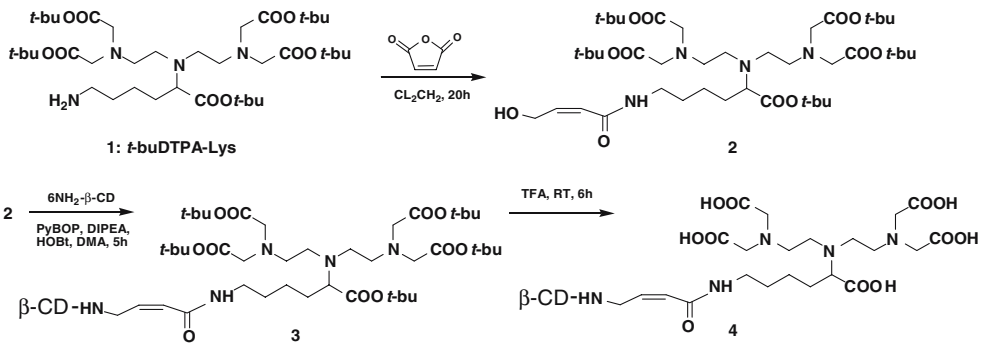
Although the three reaction steps were optimized, the overall yields of compound 4 were not satisfactory. In order to improve the preparation of Gd complexes we undertook a detailed study of the reaction conditions with the aim of minimizing the number of synthetic steps, shortening the overall reaction time and obtaining better yield). We found that the best route for binding a CD to the DTPA ligand involved the use of propargyl CD in a MW-assisted Mannich reaction with *t*-butyl-DTPA-Lys. The use of microwaves in the Mannich reaction and of ultrasound in the oxidation of CD and its successive reductive amination afforded high yields and cut down reaction times. With the help of US and MW different routes were explored to conjugate an appropriately modified CD with DTPA-Lys. One pathway involved the formation of a 6-monoformyl CD derivative by oxidation of native CD with Dess Martin periodinane. This reaction was strongly accelerated by US irradiation that increased the solubility of native CD in the medium and also promoted the reaction at the solid-liquid interface [19]. The following imine formation by reaction of formyl-CD with *t*-butyl-DTPA-Lys only occurred under US. This activated the surface of solid formyl-CD, which is poorly soluble in the chosen solvent; the reaction was completed by successive heating under reflux. Both activating energies appeared to be required, as the reaction failed when one of them was omittedScheme 2.

An alternative synthetic route exploited the Mannich aminomethylation of propargyl-CD with *t*-butyl-DTPA-Lys. The Schiff base was formed by *t*-butyl-DTPA-Lys and paraformaldheyde in acetonitrile under MW irradiation for 15 minutes. Cu(I) catalysed its reaction with propargyl-CD (4) under MW irradiation (15 min); successive deprotection in TFA gave the final ligand (6).

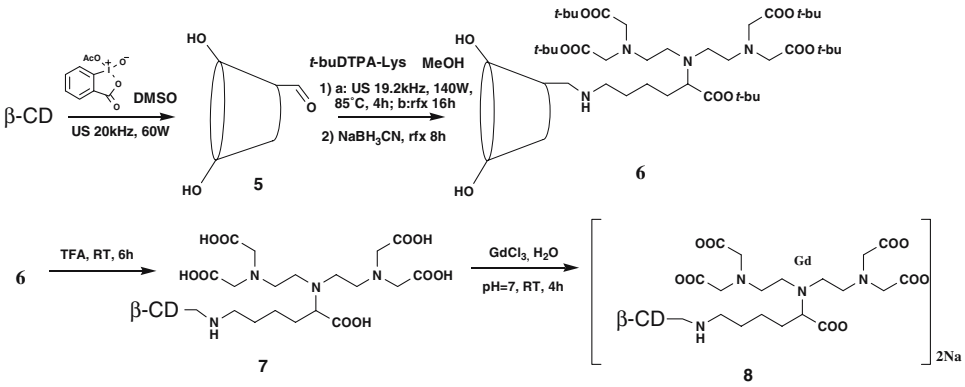
In this procedure MW irradiation dramatically reduced the reaction time and avoided the product loss caused by CD degradation. In fact heating the reaction mixture under reflux for several days generated a great deal of degradation by-products, and the yield was markedly lower than that obtained by MW irradiation (Scheme 3).

The peptide vector CCK8-CD was synthesized in solid phase using an automatic peptide synthesizer and Fmoc aminoacids. The carboxylic group of amino acids was activated with HBTU and HOBT, and the coupling reactions were performed in presence of DIPEA. Deprotection of amino groups was carried out in presence of piperidine. CD was finally conjugated to

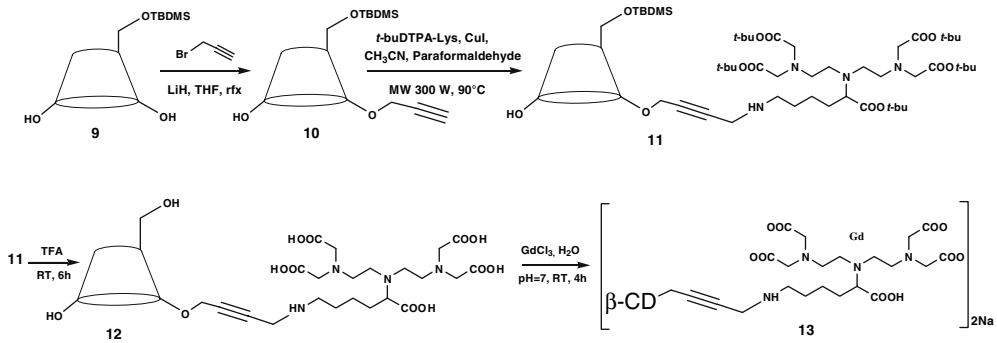
Scheme 1 Synthesis of DTPA-Lys-maleyldiamido-β-CD



Scheme 2 Synthesis of DTPA-Lys-6-amino-β-CD



Scheme 3 Synthesis of DTPA-Lys-2^{I}-*O*-propargyl-β-CD



the N-terminus of the peptide (GDYMGWMDF) by reductive amination with the monoformyl CD [18].

NMR relaxometric characterization of Gd(III) complexes with ligands (7) and (12) was carried out by measuring the increase in the relaxation rate of water protons in a 1 mM solution of complex (relaxivity, r_{1p}). The measured relaxivity at 20 MHz and 25 °C was for all complexes 11.6 $mM^{-1}s^{-1}$, a value fully consistent with a Gd complex having a molecular weight of about 2 kDa and one water molecule directly bonded to the metal [20]. When a complex interacted with PLT (mol. wt. = 100 kDa) its reorientational correlation time (τ_R) changed to equal that of the macromolecule, with a consequent relaxivity increase up to 32 $mM^{-1}s^{-1}$; this phenomenon is called Proton Relaxation Enhancement (PRE) [21]. The experimental plot shown in Fig. 2, when analysed by the PRE equation, enabled us to determine the affinity constants for the binding of complexes (Gd–8 and Gd-13) to PLT (Ka = 1.6×10^4 M^{-1}), as well as the number of interaction sites (40). PLT is made up of 53 aromatic tyrosine residues and 47 hydrophilic lysine residues, but only 40 tyrosine side chains were involved in the formation of inclusion complexes with Gd-3 or Gd-6. This finding may be attributed to steric hindrance between CD moieties when crowded on the polypeptide backbone.

Although the equilibrium constant for the binding of CCK8-CD to PLT was not measured, on the base of

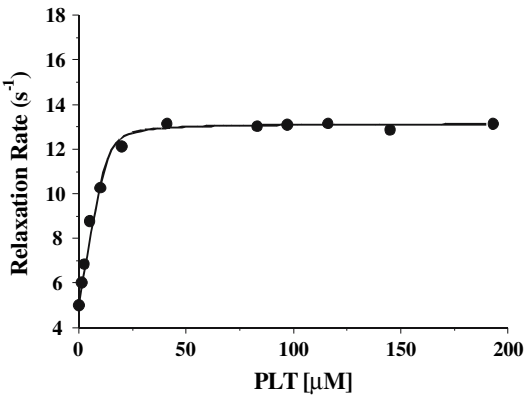


Fig. 2 Direct relaxometric titration of 0.2 mM Gd-DTPA-Lys-2^{I}-*O*-propargyl-*β*-CD (13) with PLT peptide at 20 MHz and 25 °C

our previous experience on similar models we can assume that its value should not significantly differ from the values reported above for the *β*-CD bearing Gd complexes.

Discussion

The aim of the present work was to investigate in vitro the use of *β*-CD derivatives in designing amplification procedures for MRI applications. We exploited the multi-valency approach to obtain highly stable supramolecular adducts. In this regard PLT bearing 53% tyrosine residues proved to be an excellent substrate for complexing *β*-CD-containing ligands, as the formation constants for Gd complexes 8 and 13 were of the order of 1×10^4. The constants for binary adducts between monomeric *β*-CD and Gd complexes bearing aromatic residues are ca. two orders of magnitude lower [22]. Thus PLT behaved as an efficient ''collector'' of *β*-CD bearing substrates. The 47 lysine residues contained in our PLT likely contributed to the stability of the supramolecular complexes with Gd-containing ligands by the electrostatic interactions between the negatively charged metal chelate moiety and the positively charged *ε* amino groups. Moreover the presence of the latter was also responsible for the excellent water solubility of PLT and its supramolecular adducts.

The CCK8 octapeptide was selected for this study because of its outstanding ability to bind to somatostatin receptors [23–25]. Molecular graphic modelling suggests that conjugation of a *β*-CD residue to CCK8 should not entail any intramolecular interaction between the hydrophobic tryptophan residue and the *β*-CD cavity.

Finally, the present work highlighted once more the importance of MW and US as tools in organic synthesis.

Our self-assembling system, comprising the CD-peptide vector (the root of the CA tree), the PLT polypeptide (the trunk of the tree) and the CD-CAs (the branches of the tree), looks indeed like a promising model for the task of accumulating a large number of CAs onto each target site (in our model system the expected number would be 40 for each membrane receptor).

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