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ORIGINAL ARTICLE

Nanodimer cyclodextrin ligands with high affinity to steroids

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Abstract Molecular-imprinting by cross-linking of ligands of β-cyclodextrin (CD) complex with steroids has been developed for the synthesis of tailor-made CD dimer. Steroids of androstane (9α-hydroxy-androst-4-en-3,17-dione, androst-4-en-3,17-dione, androsta-1,4-dien-3,17-dione (ADD)) and pregnane (hydrocortisone, 6-methyl-hydrocortisone, 20-hydroxymethylpregna-1,4-diene-3-one (HMPD)) series were used as template molecules. For imprinting procedure, crystalline β-CD complexes of exact stoichiometry (β-CD: steroid template = 2:1) were synthesized following by toluene 2,4-diisocyanate (TDI) cross-linking**.** The attempts to produce CD dimer for steroid without hydrophobic side chain failed, while tailor-made CD dimer has been obtained using HMPD as a template. The dimer was characterized by ^{1}H NMR and mass-spectrometry. The complex stability constant (K_S) towards HMPD template exceeded 10^7 M^{-1}. The K_S of CD dimer with ADD exceeded the corresponded value of TDI-modified CD monomer by more than an order of magnitude. The dimer was applied for quantitative extraction of ADD from aqueous solution using dialysis membranes impermeable for CD. The value of K_S for ADD estimated from balanced concentrations of dialysis data corresponded to that calculated by nonlinear spectrometric method.

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Introduction

Cyclodextrins (CD) are cyclic oligosaccharides capable to form guest–host inclusion complexes with hydrophobic compounds. The CDs, which contain seven glucose units—β-CDs, are widely used in medicine, biotechnology, cosmetics, food and textile industry due to their commercial availability [1]. Chemical modifications of β-CDs are aimed at the increase of affinity to potential guests, solubilizing properties, and biological compatibility. Method of molecular-imprinting is a promising approach for oriented chemical modifications of CDs.

The method is based on a covalent cross-linking of CD complex ligands followed by elimination of the guest molecule from the CD cavity. For the complexes with several CD molecules, the formation of such tailor-made structures results in di-, tri-, and higher structural modifications of CD. Such ordered nanostructures were complementary to the template guest molecule and often selective to it [2–4]. Polymeric CD obtained by molecular-imprinting on cholesterol and stigmasterol via diisocyanate cross-linking demonstrated selectivity to steroids [5, 6]. Similar polymeric CDs were used as selective stationary phase on HPLC column for sterol separation [5]. The molecular-imprinting of CD by cholesterol and stigmasterol promoted the formation of dimers and trimers of CD [7].

In this paper, we developed the molecular-imprinting procedure for steroid templates without long alkyl chain in order to obtain water-soluble dimer CD with high affinity to steroid molecules.

Experimental

Reagents and materials

Native β-CD, randomly methylated β-CD (DS 1.69, RAMEB) were obtained from Wacker Chemie GmbH (Germany), androsta-1,4-dien-3,17-dione (ADD), 4-pregnene-11β,17α,21-triol-3,20-dione (F), 6α-methyl-4-pregnene-11β,17α,21-triol-3,20-dione (6-methyl-F), methyl orange (MetOr), benzoylated cellulose tube membranes impermeable for compounds of molecular weight over 2,000 Da were purchased from Sigma (USA), 9α-hydroxyandrost-4-en-3,17-dione (9-OH-AD), 20-hydroxymethylpregna-1,4-diene-3-one (HMPD) were obtained from G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms (Russia), toluene-2,4-diisocyanate (TDI)—from Fluka, Inc. (Buchs, Switzerland). Dimethyl sulfoxide (DMSO) was dried with molecular sieve 4Å and distilled under reduced pressure. Other materials and solvents were of analytical grade and purchased from domestic companies (Russia).

^{1}H-NMR, mass-, and UV-measurements

^{1}H NMR spectra of modified β-CD and inclusion CD complexes with steroids were measured on a Bruker Avance 400 spectrometer at 400 MHz. The spectra were recorded in deuterated DMSO (16 scans) at a temperature of 293 K.

Mass-spectra of CDs were recorded on LCQ DecaXP spectrometer (Thermo Finnigan, USA) equipped with electrospray ionization source (ESI).

Aliquots of steroids ethyl acetate extracts were applied to Kieselgel 254 (Merck) plates and developed in a mixture of benzene:chloroform (1:1, v/v). Spots on the plates were visualized under UV light (254 nm) and quantified at 240 nm on spectrometer UV-1700 (Shimadzu, Japan) after elution with ethyl alcohol.

Determination of complex stability constant

For determination of stability constant (K_S) of CD complex with steroids, the nonlinear spectrometric method was used [8]. Parameters of competitive CD complexation were calculated by simultaneous fitting of two types of curves. Those of the first type were the dependencies of absorbance of MetOr solution on CD concentration. The curves of the second type reflected the displacement of the dye from CD complex by steroids. Host CD solutions (1×10^{-5}–5×10^{-3} M) and those of guest MetOr (2×10^{-5} M) were prepared using 0.1 M phosphate buffer solution (PBS) (pH 2.67). Guest steroid solutions (1×10^{-6}–5×10^{-3} M) were prepared in 2.5×10^{-4} and 5.0×10^{-4} M solutions of CDs in 0.1 M PBS (pH 2.67) and included MetOr (2×10^{-5} M).

Absorbance measurements were performed on spectrometer UV-1700 (Shimadzu, Japan) with a thermostated cell holder at 505 nm (pH 2.67, PBS 0.1 M, 30 °C).

Synthesis of dimer CD by molecular-imprinting method

General procedure for the obtaining of crystalline CD–steroid inclusion complex

The procedure was applied for all steroids used. Steroid (2.5 mmol) was added as a fine powder to 50 mL water solution of RAMEB (6.5 g, 5.0 mmol). The suspension was stirred at 40 °C until full dissolution and heated to 70 °C. Then, β-CD 6.8 g (6 mmol) was added and stirred for 3 h under room temperature. The precipitate obtained was collected by filtration, carefully washed with icy water, and dried under vacuum at 80 °C till constant weight.

To verify steroid content, the steroid molecule was extracted from the complex with simultaneous disconnection of complex structure.

Fine suspension of CD complex was heated in isopropanol under stirring at 80 °C for 2 h. The precipitate was filtered, alcoholic solution was evaporated and the residue was re-dissolved in acetonitrile for analysis. Steroid content in CD complex was in accordance with stoichiometry of 2:1 (β-CD:steroid). Yield of CD complex per steroid was 93–96%.

In addition, the complex stoichiometry was examined by ^{1}H NMR spectroscopy analyses. In ^{1}H NMR spectrum (DMSO d6) of the complexes isolated the ratio of signals of 7 protons of the CD at C1 of the glucose element δ_H 4.81 (s, 7H, H-1) and 3 methyl protons at C18 of steroid δ_H 0.69–0.85 (s, 3H, CH_3) corresponded to 2 within the error of integration that pointed to the stoichiometry CD:steroid = 2:1.

Cross-linking of ligands in CD complex

Inclusion complex containing 2.0 mmol steroid was dissolved in 40 mL of dry DMSO, and 5 mL of TDI solution (696 mg, 4 mmol) in dry DMSO was added drop by drop. Reaction mixture was incubated at 65 °C for 3 h under stirring. Then the mixture was poured into vigorously stirring acetone (450 mL). The resultant white powder was carefully washed by acetone, filtered, and dried under vacuum. To remove TDI modified β-CD monomer the aqueous solution of CD obtained (4.6 g) was placed into dialysis bag against 1,500 mL of deionized water for 24 h under room temperature. Then solution was freeze dried and solid white substance was obtained. In the case of HMPD using as a template, 3.0 g of the substance was obtained.

In control, non-imprinting procedure was carried out in a similar way. β-CD (4.54 g, 4.0 mmol) was used for TDI modification without steroid template.

Equilibrium dialysis

CD dimer (210 mg, 0.09 mmol) in 10 mL aqueous solution was placed in cellulose tube (1.27 in.) with CD impermeable pores and immersed into ADD solution (200 mL) at 30 °C under stirring. ADD solution was sampled during 16 h until the equilibrium was achieved. The initial and appropriate equilibrium concentrations of ADD were used to determine the K_S of ADD with CD dimer.

Results and discussion

Two-step synthesis of CD dimer by molecular-imprinting method

General scheme of two-step molecular-imprinting method is presented in Fig. 1. As a first step, crystalline CD-steroid complex of stoichiometry of 2:1 was obtained and isolated. Subsequently the complex was treated with TDI for cross-linking of CD ligands and then the steroid guest was eliminated by the treatment with organic solvent.

In order to obtain steroid CD inclusion complex of exact stoichiometry, the original method based on competitive complex formation of steroids with various CDs was developed. The preparation of soluble complexes of steroids with RAMEB (A_L-type) has been carried out. Then native β-CD was added into solution, the insoluble complexes of B_I type followed were precipitated.

The content of steroid in crystal CD complex was estimated by two methods: (a) by complex destruction with steroid extraction; (b) by ^{1}H NMR spectroscopy of crystalline CD complex. Both methods confirmed the CD to steroid ratio of two to one.

In order to estimate the impact of hydrophobic side chain in molecular-imprinting, steroids with and without side chain were applied as templates. The formers were steroids of pregnane series (F and its 6-methyl derivative), and HMPD which contains short hydrophobic chain. The latters were AD and its derivatives which do not have any side chain. The results showed that only HMPD was suitable as a template for synthesis of CD dimer. The attempts to synthesize CD dimer for steroids either without hydrophobic side chain (i.e. androstane steroids) or with pregnane side chain containing three oxygen functions (i.e. F, 6-methyl-F) failed. Mass spectrum of TDI modified CD obtained by imprinting method was almost the same as that for the nonprinted one. Monomeric CDs (M = 1,283–1,579) were major products. The formation of dimers and trimers was observed in trace quantities. Probably, the binding force in inclusion complexes in DMSO reaction medium was insufficient for these steroid templates.

Unlike, the complex of HMPD with native β-CD was stable in DMSO that may be explained by participation of the side chain of the steroid molecule in complex formation. Tailor-made CD obtained by imprinting on HMPD was isolated and characterized by ^{1}H NMR and ESI mass-spectrometry. ^{1}H NMR spectroscopy confirmed that the template molecules were completely removed from CD dimer. Signals with chemical shifts in area δ_H 8.21; 8.47; 8.21 ppm (s, 3H, Ar–H) indicated the presence of benzene protons of the cross-link in dimeric CD. The share of

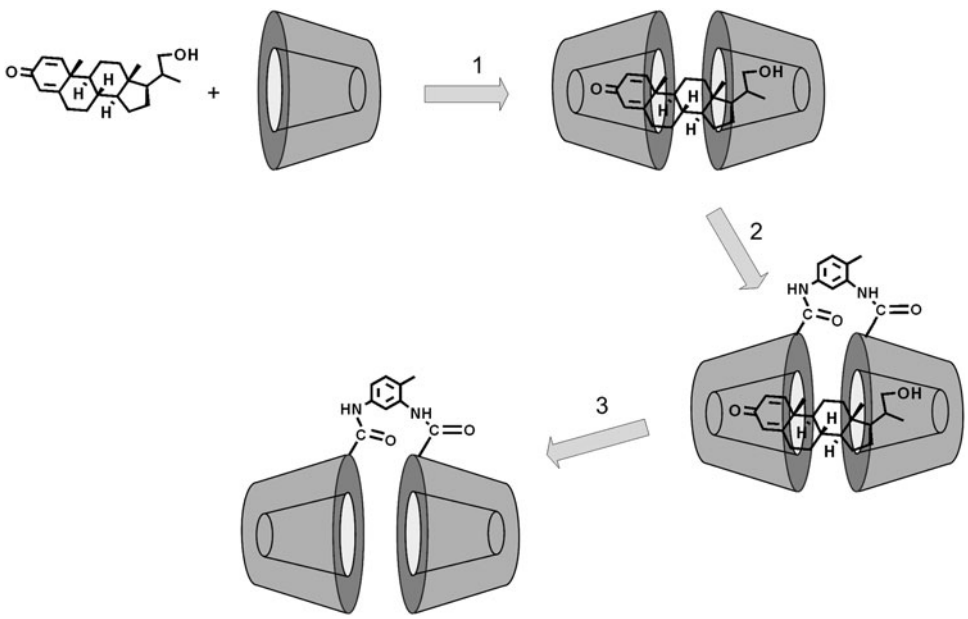

Fig. 1 The scheme of synthesis of CD dimer by two-step molecular-imprinting method: *1* the formation of crystalline CD complex with HMPD; *2* TDI cross-linking in DMSO; *3* elimination of steroid guest

isocyanate cross-link in the dimer was estimated by the ratio of signals of methyl group within benzene ring of the cross-link δ_H 3.53 ppm (s, 3H, Ar–CH_3) to 14H dimer-CD at C-1 δ_H 4.81 ppm. The share of TDI of the cross-link was 1.3 to the linked dimer. Used as a template in the molecular-imprinting, HMPD promoted the formation of dimer structures. Signals corresponded to CD dimers, in which two CD rings were connected by one TDI linkage (mass number M = 2,444), were recorded as major ones in the ESI mass-spectra.

Estimation of the affinity of CD dimers to steroid templates revealed extremely high magnitude of K_S. Values of K_S with steroids were determined by non-linear competitive spectrometric method (Table 1). For ADD the error of evaluation of K_S did not exceed 10%. For the template steroid (HMPD) the precise estimation of K_S required a special method due to abnormally high affinity to dimer (over 10^7 M^{-1}). The value of K_S of CD dimer with ADD increased more than one order in comparison with control (CD that was TDI modified in the absence of HMPD template). In the same time the affinity of TDI modified CD monomer to ADD was comparable to that of commercial RAMEB (Table 1). The imprinting effect on the pregnane template with hydrophobic side chain led to the formation of dimer with high affinity to ADD and extraordinary high affinity to the template HMPD.

Therefore, the two-step procedure for molecular-imprinting may be used for obtaining tailor-made CD dimers. It is of importance that the procedure suggested here excludes the formation of tailor-made CD trimer as it was reported earlier for imprinting method based on cholesterol template [7].

TDI modified CD dimer in model experiment on equilibrium dialysis of ADD

CD dimer was used for quantitative extraction of steroids from aqueous solutions. The solution of CD dimer was placed into dialysis tube (Fig. 2b, V_1), the tube was placed into ADD solution (Fig. 2b, V_2). Steroid transfer from V_2 to V_1 through the membrane was accompanied by the decrease of ADD concentration in V_2 (Fig. 2a, V_1). In V_1 chamber the equilibrium (1) was strongly shifted towards complex formation due to high affinity of dimers to ADD. Therefore, in both chambers (V_1 and V_2) concentrations of free ADD form (C^{ex}_{ADD}) equalized. In the model system two types of equilibrium are realized:

(i) slow equilibrium transfer of ADD through the membrane (Fig. 2b);
(ii) controlled by diffusion fast equilibrium between free and CD complex form of steroid (1).

$$[\mathrm{ADD}] + [\mathrm{CD}] \overset{K_{\mathrm{ADD}}}{\leftrightarrow} [\mathrm{ADD}-\mathrm{CD}] \quad K_{\mathrm{ADD}} = \frac{[\mathrm{ADD}-\mathrm{CD}]}{[\mathrm{ADD}][\mathrm{CD}]} \tag{1}$$

The dynamics of ADD extraction from aqueous solutions with various initial concentrations of $C^0_{ADD,1} = 140\,\mu M$ and $C^0_{ADD,2} = 100\,\mu M$ is shown in Fig. 2a. The equilibrium concentrations of ADD ($C^{ex}_{ADD,1} = 5.5\,\mu M$ and $C^{ex}_{ADD,2} = 3.0\,\mu M$), which correspond to two initial concentrations of $C^0_{ADD,1}$ and $C^0_{ADD,2}$ respectively, were used for the calculation of K_{ADD1}. The value of [ADD] at the dialysis equilibrium is C^{ex}_{ADD} in V_2.

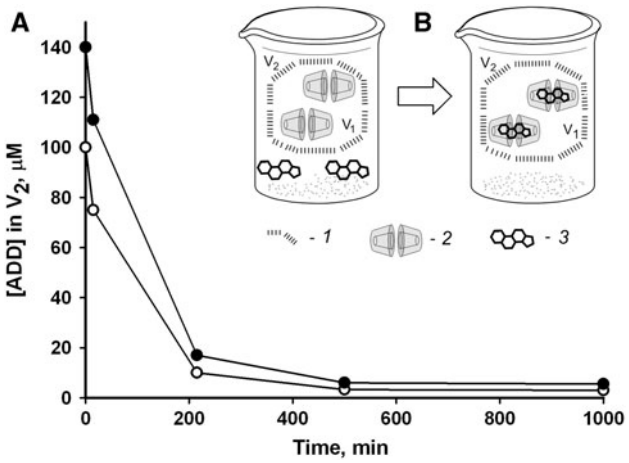

Fig. 2 The dynamics of extraction of ADD from aqueous solutions with initial steroid concentrations $C^0_{ADD,1} = 140\,\mu M$ (*filled circles*) and $C^0_{ADD,2} = 100\,\mu M$ (*empty circles*) at 30 °C by dimer in CD impermeable membrane (**a**) and the scheme of model experiment on equilibrium dialysis of ADD (**b**) (*1* membrane, *2* CD dimer, *3* steroid)

Table 1 Effect of CD imprinting on K_S determined by nonlinear spectrometric method

CD	K_S (M^{-1})	
	HMPD	ADD
Tailor-made CD dimer (template-HMPD)	$>10^7$	84000 ± 7000 84400[a]
TDI modified CD (without matrix)	12500 ± 1000	5550 ± 400
RAMEB	14900 ± 900	5800 ± 400

[a] Overage value calculated from dialysis data

The concentration [ADD − CD] in V_1 may be defined as:

$$\begin{aligned}[\text{ADD}-\text{CD}] &= m_{\text{ADD}-\text{CD}}/V_1 = (m^0_{\text{ADD}} - m^{\text{ex}}_{\text{ADD}})/V_1 \\ &= (C^0_{\text{ADD}} \times V_2 - C^{\text{ex}}_{\text{ADD}}(V_2 + V_1))/V_1 \\ &= C^0_{\text{ADD}}(V_2/V_1) - C^{\text{ex}}_{\text{ADD}}(1 + V_2/V_1) \qquad (2)\end{aligned}$$

From the equation of material balance for the CD comes the following:

$$\begin{aligned}[\text{CD}] &= C_{\text{CD}} - [\text{ADD}-\text{CD}] \\ &= C_{\text{CD}} - C^0_{\text{ADD}}(V_2/V_1) + C^{\text{ex}}_{\text{ADD}}(1 + V_2/V_1), \qquad (3)\end{aligned}$$

where C_{CD} is molar concentration of CD in V_1. Hence, the expression for K_{ADD} (1) transforms into the following:

$$\begin{aligned}K_{\text{ADD}} = (C^0_{\text{ADD}}(V_2/V_1) - C^{\text{ex}}_{\text{ADD}}(1 + V_2/V_1))/(C^{\text{ex}}_{\text{ADD}} \\ \times (C_{\text{CD}} - C^0_{\text{ADD}}(V_2/V_1) + C^{\text{ex}}_{\text{ADD}}(1 + V_2/V_1))). \qquad (4)\end{aligned}$$

Estimated from the data of balanced steroid concentrations the value of K_{ADD} (Table 1) was in good accordance with that obtained by nonlinear spectrometric method. It should be concluded that model experiment confirmed high affinity of dimer CD to steroid.

Thus, the linked CD dimer obtained forms water-soluble inclusion complexes with steroids and their complex stability constants are substantially higher than those obtained on the basis of monomeric CDs. This material can be potentially applied for specific recognition of steroids as target molecules, as well as drug delivery system with adversely affect drug absorption.

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