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A Cyclodextrin Self-Assembled Monolayer (SAM) Based Surface Plasmon Resonance (SPR) Sensor for Enantioselective Analysis of Thyroxine

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Abstract

Heptakis{6-deoxy-6-[12-(thiododecyl) undecanamido-β-cyclodextrin has been produced by reaction of Heptakis(6-deoxy-6-amino)-β-cyclodextrin and 12-(thiododecyl)undecanoic acid using *O*-Benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) as activating agent. Self-assembled monolayers of this macrocycle have been used in a surface plasmon resonance (SPR) sensor; it has been shown that this system is suitable to discriminate between D and L enantiomers of thyroxine, with a greater affinity for the D-enantiomer.

Introduction

Molecular recognition [1–3] is one of the most important concepts in biological/biochemical sciences. It implies a set of weak forces (non-covalent) interactions ranging from hydrogen–bonds to van der Waals interactions through ionic and dipole–dipole interactions [4]. In biological systems, because of the asymmetry of a large majority of molecules (proteins, carbohydrates, nucleic acids, etc.) these interactions occur in an asymmetric fashion. Keeping this in mind, it seems obvious that artificial drugs have to be chiral to be integrated correctly in the vast and complex game of molecular interactions of living systems [5]. In fact, it has been demonstrated that in many cases, while they behave similarly in a symmetric environment, two enantiomers of the same molecule have different effects in biological systems [6]. Thus, it is of great importance to develop, in parallel to asymmetric synthetic methodologies, separation and sensing systems able to discriminate between two optical isomers. In the past few decades, many chiral selectors have been designed for chiral separation by high performance liquid chromatography (HPLC) [7], gas chromatography (GC) [8] and capillary electrophoresis (CE) [9] and are used in the pharmaceutical industry for enantio-analysis. In contrast, enantiospecific sensing systems are much less well developed. Nevertheless, there are some reports on the use of quartz crystal microbalance (QCM) [10–14] and surface plasmon resonance (SPR) [15, 16] based sensors for enantio-analysis of mixtures. For example, it has been shown that imprinted polymer films could be efficient QCM functional layers for enantioselective detection of serine [10], terpenes [11], dansyl-phenylalanine [12] and propanolol [13]. Paolese *et al.* [14] have shown that a self assembled monolayer (SAM) of a porphyrin diad on a QCM electrode is able to enantio-discriminate analytes in the gas-phase. Films prepared by the spreader-bar approach have also been used as sensitive layers in a SPR sensor in order to discriminate between different binaphthyl diols [15].

α, β and γ-Cyclodextrins are macrocyclic molecules composed of 6, 7 or 8 D-glucopyranose units respectively, linked by α-(1→4) glycosidic bonds [17]. They have been widely studied in supramolecular/host-guest chemistry [1–3] due to their ability to include lipophilic molecules in their hydrophobic cavity. They have also been extensively used as chiral selectors in separation technology [18] and it has been shown that cyclodextrin based polymers could be used as sensitive layers in chiral sensing by SPR [16]. Reinhoudt has extensively studied the ability of these macrocycle to form well packed SAMs on gold surfaces [19–24].

L-Thyroxine (3,3′,5,5′-tetraiodothyronine or T_4) is a derivative of tyrosine (Figure 1), it is the major hormone secreted by thyroid follicular cells [25] and is used as a biochemical indicator of thyroid function. Synthetically prepared thyroxine is used in the treatment of thyroid gland deficiency diseases.

In this paper, we report on enantioselective sensing of thyroxine by SPR using as a sensitive functional layer a SAM of Heptakis{6-deoxy-6-[12-(thiododecyl) undecanamido]}-β-cyclodextrin (**3**) produced *via* an

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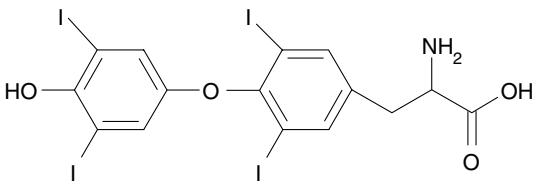

Figure 1. Molecular formula of thyroxine (3,3′,5,5′-tetraiodothyronine).

improved synthetic approach (Figure 2) compared to that described in literature [24].

Experimental

Synthesis

General

^{13}C and ^{1}H NMR spectra were recorded on a Varian 300 MHz. Electro-Spray Mass Spectrometry (ES-MS) was performed using a Perkin Elmer Sciex API 165 in positive mode. Solvents (ACS grade for synthesis and HPLC grade for ES-MS) and chemicals were purchased from Fluka (Switzerland) and used without further purification.

Heptakis(6-deoxy-6-amino)-β-cyclodextrin, **1** [26], and 12-(thiododecyl)undecanoic acid, **2** [27], have been synthesized according to literature procedures.

Synthesis of Heptakis{6-deoxy-6-[12-(thiododecyl)undecanamido]}-β-cyclodextrin (3)

Under anhydrous condition, in dry DMF, at 0 °C, 2 (625 mg, 1.56 mmol), *O*-Benzotriazol-1-yl-*N*,*N*,*N*′,N′-tetramethyluronium tetrafluoroborate (TBTU) (495 mg, 1.56 mmol) and 4-ethylmorpholine (400 μl, 3.02 mmol) were dissolved and maintained under stirring during 10 min. After the addition of **1** (250 mg, 0.22 mmol) the mixture was allowed to warm at room temperature and stirred for additional 16 h. All DMF was evaporated under reduced pressure and the yellow residue obtained dissolved in dichloromethane (100 ml), washed with HCl (1 M, 100 ml), water (100 ml), dried over magnesium sulphate and evaporated. The pale yellow residue produced was dissolved in dichlormethane (5 ml), the addition of acetone (100 ml) yielded a white solid, which was filtered and copiously washed with acetone to afford **3** (505 mg) in 60% yield. All analytical values (TLC, ES-MS, ^{1}H and ^{13}C NMR) are in perfect agreement with those published [24].

Sensor chip preparation and SAM formation

All glassware used to prepare monolayers was cleaned in *piraña* solution (H_2SO_4:H_2O_2; 70:30, v/v) and abundantly rinsed with de-ionized water (purified with a Millipore MiliQ water system, resistivity ≥ 18 MΩ cm) (Warning: *piraña* solution is highly oxidative and corrosive; it could explode unexpectedly and must be handled with extreme care). SIA Kit Au® (glass substrates (10 × 12 mm) covered on one side with a 50 nm thick gold layer) obtained from BIAcore® (Uppsala, Sweden) were cleaned as follows. Gold substrates were immersed in acetone and submitted to ultrasonic treatment during 20 min. This treatment was repeated with methanol. They were subsequently immersed in a *piraña* solution during 1 h, washed abundantly with de-ionized water, acetone and dried under argon. Self-assembled monolayers of **3** were prepared by immersing freshly cleaned gold substrates in a 0.1 M solution of **3** (EtOH:$CHCl_3$, 1:2, v/v) during 16 h. Subsequently, the substrates were removed from the solution, thoroughly rinsed with chloroform, dried and assembled in the chip holder.

Surface plasmon resonance (SPR) experiments

Binding assays were performed using a BIAcore® X apparatus. Sensor chips were equilibrated using a BIAcore HBS-N® buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, filtered and degassed) using the continuous flow procedure overnight (5 μl min^{-1}). Thyroxine solutions were prepared as follows: D- or L- thyroxine (7.7 mg) were dissolved in 1 ml NaOH 1 N solution and

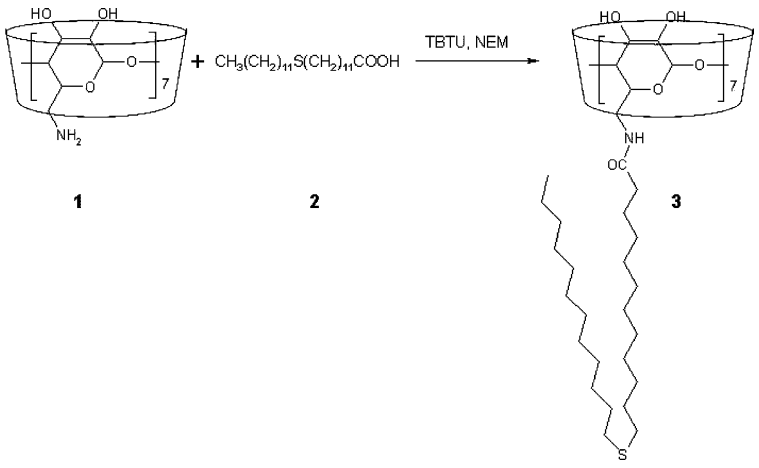

Figure 2. Synthesis of Heptakis{6-deoxy-6-[12-(thiododecyl)undecanamido]}-β-cyclodextrin, TBTU: *O*-Benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate and NEM: *N*-ethylmorpholine.

then diluted in the running buffer (1/100). Mixtures of enantiomers were prepared by mixing these solutions and degassed prior to use. Experiments were carried out using a flow rate of 5 μl/min and 35 μl of thyroxine solution for each binding assay.

The BIAevaluation® software was utilized for evaluation of binding constants using the 1:1 Langmuir adsorption model. The model given by the manufacturer for a 1:1 binding experiment is explained by the following equations:

$$\frac{d[B]}{dt} = -(k_a \times [A] \times [B] - k_d \times [AB]$$

$$\frac{d[AB]}{dt} = -(k_a \times [A] \times [B] - k_d \times [AB]$$

$$K_a = \frac{k_a}{k_d}$$

where k_a is the association rate constant, k_d the dissociation rate constant, K_a the association constant, $[A]$ the concentration of the analyte, $[B]$ the concentration of the ligand, $[AB]$ the concentration of the complex formed by binding A to B. Values for $d[AB]/dt$ and $d[B]/dt$ are obtained from measurements of the slope at multiple time points during the real-time binding–unbinding experiment.

Regeneration of the sensor chip was achieved by competitive elution (injection of 35 μl of a 0.1 M β-cyclodextrin solution in NaOH (0.1 M) and flushed with the running buffer until the value measured came back to the baseline). Each experiment was repeated at least twice to ensure reproducibility of the results.

Results and discussion

Synthesis

The synthetic route to heptakis{6-deoxy-6-[12-(thiodecyl)undecanamido-β-cyclodextrin, **3**, is presented in Figure 2. It has been carried out by reacting Heptakis(6-deoxy-6-amino)-β-cyclodextrin (**1**) and 12-(thiodecyl) undecanoic (**2**) using *O*-Benzotriazol-1-yl-*N*,*N*,*N*′,*N*′ -tetramethyluronium tetrafluoroborate (TBTU) as activating agent in freshly dried dichloromethane. This reaction affords **3** in 60% yield. The synthesis of **3** has been previously described by Beulen *et al.* [24]. In this paper, the authors report on the use of *N*,*N*′-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazol (HoBT) as coupling agents. This synthetic procedure requires extra purification of the crude product twice by flash chromatography and results in a loss of material (yield: 45%). In the procedure proposed here, all reagents: **2**, TBTU and its degradation product (HoBT) are soluble in acetone and no column purification is necessary, the yield of the reaction is therefore improved.

Thyroxine binding assays

In Figure 3 are presented binding sensograms measured for D- and L-thyroxine. After the equilibration stage, during all the experiment (including binding–unbinding and regeneration steps) a stable baseline was obtained (not shown) suggesting that no degradation of the SAM occurs. During the short initial phase of the injection of analytes, a clear uptake is observed for both sensograms, this initial increase could be mainly attributed to the change of refractive index in the measurement cell due to the change of buffer. After this initial variation, the sensor response measured for the L-enantiomer shows a slight increase up to 400 arbitrary unit (au) while those for the D-enantiomer is much more higher, almost linear and reach a 37% higher value, at the end of the injection, of 550 au. During the unbinding (washing) step, the sensor response for the L-enantiomer decreases rapidly and asymptotically approaches the baseline while the value for D-enantiomer decreases more slowly and levels off at a value of 220 au. From this, we conclude that there is a differential selective binding of the D over L-enantiomers of thyroxine on a

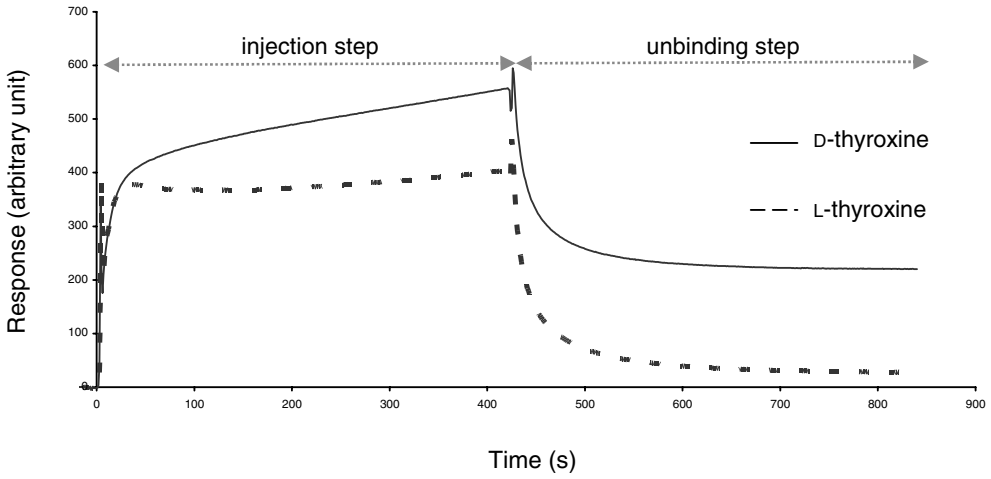

Figure 3. Binding sensograms of D- and L-thyroxine, values are expressed in arbitrary units and normalized to 0 at the beginning of the injection.

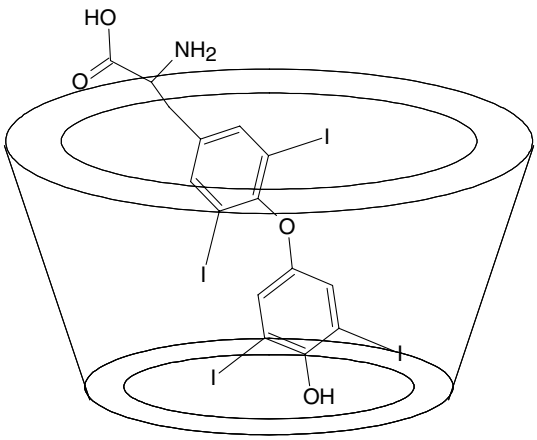

Figure 4. Schematic representation of the inclusion complex of thyroxine in the cyclodextrin cavity.

sensor chip where a chiral SAM of **3** is immobilized and exposed as a sensor layer.

The affinity constant (K_{aff}) for the D-enantiomer (4.7×10^8 M^{-1}) is much more important than those for L-enantiomer (3.8×10^5 M^{-1}). As evocated in the introduction, cyclodextrins have been widely used for chiral separation of small molecules; this ability to enantio-discriminate analytes arises from a differential inclusion of guest molecules in the chiral cavity of the cyclodextrins. We can expect that chiral sensing properties of cyclodextrin based SAMs measured in the presented experiments are due to the same inclusion phenomenon. A schematic representation of a possible inclusion complex between thyroxine and the cyclodextrin skeleton is presented in Figure 4. It could be supposed that the interaction occurs by a deep inclusion of the hydrophobic part of thyroxine (D or L- enantiomers) in the lipophilic cavity of the cyclodextrin skeleton. Thus, the chiral center and the polar functions of thyroxine are segregated outside the macrocycle and interact, in an enantioselective fashion, with secondary alcohol functions of the cyclodextrin molecule through hydrogen bonding.

The ability of **3**-based SAMs to discriminate between the two enantiomers of thyroxine has been evaluated by monitoring the apparent affinity constant with varying proportions of each enantiomer, these results are presented in Figure 5. There is a linear increase of the apparent affinity constant ranging from the value measured for the L-enantiomer to that measured for the D-enantiomer. It means that this sensor could be used for the evaluation of the enantiomeric composition of a mixture of D- and L-thyroxine. It must be noted that problems of reproducibility should arise from differences in the quality of the gold layers which cause variations in the surface coverage of the SAM. Nevertheless this problem could be circumvented by measuring for each chip a 'calibration curve' as done in Figure 4.

Conclusion

In this paper, a new synthetic route to heptakis{6-deoxy-6-[12-(thiododecyl)undecanamido-β-cyclodextrin using *O*-Benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate is described. SAMs of these cyclodextrins have been prepared and used in a SPR sensor. It has been shown that these chiral SAMs possess enantioselective binding properties and present a greater affinity for the D-enantiomer of thyroxine with regard to the L-enantiomer. In view of the widespread use of cyclodextrins and principally β-cyclodextrin in chiral separation, it seems obvious that the use of **3**-based SAMs as sensitive layers for chiral sensors could be expanded to a large range of analytes. Actually, the main limitation for

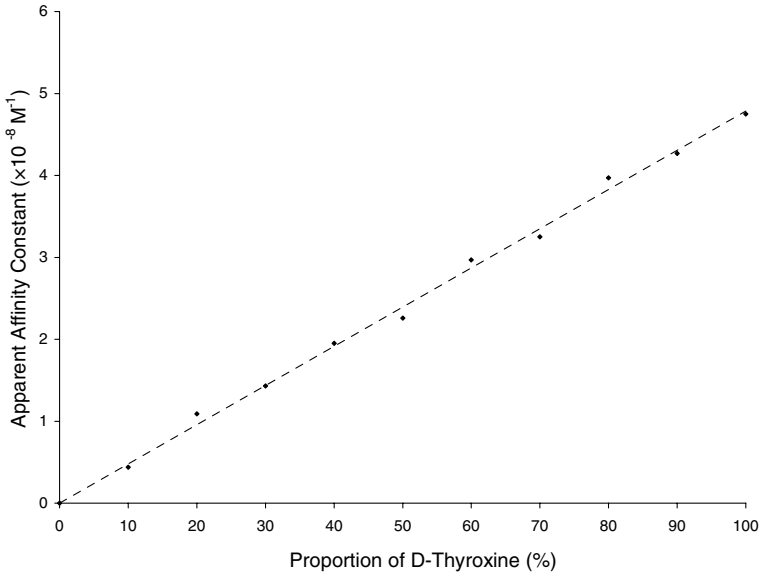

Figure 5. apparent affinity constants measured for varying proportions of D- and L-thyroxine.

the design of a highly versatile chiral sensor using SPR detection system is the limitation of the technique to water soluble molecules with relative high molecular weight (typically higher than 500). Investigations are currently underway to transfer **3**-based SAMs on more sensitive systems (microcantilever arrays) in order to expand the number of analytes which could be analyzed.

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