Nanostructured sensors containing immobilized nuclear receptors for thyroid hormone detection

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Nanostructured Sensors Containing Immobilized Nuclear Receptors for Thyroid Hormone Detection

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INTRODUCTION

Nuclear receptors (NR) are important for metazoan intercellular signals to initiate and regulate gene expression. They are cellular signal transducers that respond directly through physical associations with a specific hormone.¹,² The thyroid hormone receptors (TRs) are one member of this receptor group of transcriptional regulatory compounds that include other ligand-induced transcription factors, such as receptors for steroid hormones, vitamin D, retinoids, fatty acids and prostaglandins. The TRs are receptors proteins encoded by two genes, TRα and TRβ, that are capable of binding to specific regions of DNA to trigger vital functions. This activation cascade depends on the interaction with thyroid hormones (THs) to initiate the mechanisms of gene expression regulation.

Many human diseases may occur as a consequence of alterations of hormone levels. Thyroid hormones, for example, influence various metabolic processes in humans, such as heart beat rate and other cardiac functions, serum cholesterol levels, connective tissue metabolism, and basal metabolic rate.¹,² Two of the hormones secreted from the thyroid gland are the 3,5,3′-triiodothyronine (thyroxine or T4) and 3,5,3′-triiodothyronine (triiodothyronine or T3). The use of these hormones, in addition to synthetic analogues such as 3,5,3′-triiodothyroacetic acid (TRIAC) and 3,5-dimethyl-4-(4′-hydroxy-3′-isopropylbenzyl phenoxy) acetic acid (GC-1) may be interesting for the development of new therapeutic approaches.¹,² GC-1 is selective for binding and activating both TRβ1 and TRα1 and exhibits selective actions in vitro and in vivo. This ligand can be used to study the central nervous system, brown adipose tissue and tadpole metamorphosis. Furthermore, the GC-1 analogue ligand has been proven to decrease the serum levels of cholesterol and triglycerides while not affecting heart rate.⁵

The methods currently employed for the detection and quantification of TH levels are usually based on radioimmunoassays, fluorimetric techniques or amperometric measurements.⁶ Radioimmunoassay is a technique that employs commercially available kits, presenting a good sensitivity and very low limits of detection.⁷,⁸ Using
radioimmunoassay methods, Oliveira et al. reported the detection of T3 and T4 molecules at concentrations down to 0.02 and 0.5 ng/mL, respectively.9 Fluorescence spectroscopy, on the other hand, has been widely exploited for the quantification of THs. This technique may exhibit sensitivity and detection limits comparable to the radioimmunoassay methods, with the possibility of multianalyte detection and a better cost-effectiveness.10–12 As a variation of the technique, time-resolved fluororesoimmunoassay (TR-FIA) has been used in the simultaneous quantification of thyroid-stimulating hormone (TSH) and T4.11 The method allowed the assays to be performed in ranges of 20–300 nmol/L with a limit of detection of 4.1 nmol/L for T4.11

Electrochemical immunoensors have also been proven to be capable of the simultaneous detection of L-T4, D-T4 and L-T3 molecules.13 Murata et al.14 have reported an affinity-based biosensor for the detection of hormones as estrogen and THs, in which TR-LBD was immobilized on a thiol-modified gold electrode; the detection response was evaluated by cyclic and differential pulse voltammetries. These techniques showed the specific binding of T3 to the bioreceptor layer, i.e., the receptor immobilized on a gold electrode. A disadvantage of the electrochemical techniques, in these cases, is the relatively low selectivity (in the presence of possible interferent molecules) and low sensitivity.

The present study was aimed at the development of an alternative biosensor system based on the use of nanostructured bioreceptor platforms in which the LBD region of TRβ1 was immobilized on interdigitated electrodes using self-assembled monolayers (SAMs). The detection of specific THs was performed via capacitance measurements using electrical impedance spectroscopy. This strategy allowed the detection and separation of different THs at nanomolar concentrations. The main advantages of the strategy employed here are the cost-effectiveness of the systems (including the bioreceptor platform and the detection apparatus) and the increased sensitivity that results from the nanometric nature of the immobilized bioreceptor film.

**EXPERIMENTAL DETAILS**

**Materials**

The reagents employed in the functionalization of the interdigitated electrodes via SAMs were 11-mercaptoundecanoic acid (MUA) and octadecylsilane, which were acquired from Aldrich and used without further purification. Carbon tetrachloride 99.9%, hexadecane 99%, chloroform 99%, ethanol 95% and acetone 99% were also purchased from Aldrich. The ligands T3, T4, TRIAC and GC-1 were purchased from SIGMA. TRβ1-LBD was expressed and purified according to previously established protocols15 and fused with His-tag to the N-terminal residue. All the solutions were prepared in 20 mM Heps pH 8, 50 mM NaCl and 5% glycerol buffer, following the procedures described by Figueira et al.16 Potassium ferrocyanide trihydrate, K4[Fe(CN)6]·3H2O, and P. A. sulfurous acid were purchased from Mallinckrodt and Reagen, respectively.

**Immobilization and Electrode Preparation**

TRβ1-LBD was immobilized on interdigitated electrodes for the detection measurements. The electrodes comprised 80 pairs of Cr/Au tracks (20 μm wide and 100 nm thick) deposited on glass substrates with a track separation of 20 μm. After being cleaned with acetone, the electrodes had their gold tracks and the glassy regions—between tracks—functionalized with SAMs of MUA and octadecylsilane monolayers,17 respectively, according to the previously described method.18 Briefly, a solution of 8% chloroform, 12% carbon tetrachloride and 80% n-hexadecane (%v/v) was rapidly mixed with silane to avoid air exposure and the formation of polymerized material. After being immersed in this mixture, the electrodes were sonicated in chloroform three times to remove excess of silane. The substrates were dried under mild nitrogen flow and stored for 24 h to complete the functionalization process. The MUA layer was obtained by dropping a 1 mM ethanolic solution on the electrode surface at room temperature and leaving the electrode to dry in air at room temperature. The substrates were subsequently rinsed 3 times with ethanol to remove any poorly adsorbed material and dried under flowing nitrogen.

After the electrodes were functionalized, the receptor layer was adsorbed by drop-casting the electrodes in an 8.6 μM TRβ1-LBD solution for 1 h, followed by rinsing with buffer. The adsorption of TRβ1-LBD on a MUA-modified gold surface was monitored using surface Plasmon resonance (SPR) measurements. SPR measurements were carried out in a SensiQ analyzer, Nomadics Inc.

The electrodes containing MUA-SAMs were subjected to cyclic voltammetry (CV) to verify the efficiency of deposition of the SAMs. CV was performed using a conventional three-electrode cell, in which the interdigitated electrode was the working electrode, an Ag/AgCl system was used as the reference, and platinum foil (1 cm² of area) served as the counter electrode. Voltammograms were collected upon the application of a potential that was varied from -100 mV to 700 mV in an acidic solution of 4 mM K4[Fe(CN)6]·3H2O, which served as the electrolyte.

**Morphologic and Spectroscopic Characterization**

For morphological and spectroscopic studies, the same SAM/TRβ1-LBD architecture was deposited on flat gold-covered glass substrates. The ligands T3, T4, TRIAC and GC-1 were deposited over the TRβ1-LBD layer from 100 μM solutions using the same immobilization and washing steps described previously. AFM images were obtained for films containing isolated TRβ1-LBD, and the receptor-ligand pairs with T3, T4 and TRIAC as analytes.
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Reflection-mode Fourier Transform Infrared (FTIR) spectroscopy was performed using a Nicolet 470 Nexus instrument; data were collected from 3500 to 800 cm\(^{-1}\).

Data Acquisition

The detection of T3, T4, TRIAC and GC-1 was achieved via impedance spectroscopy, in which the capacitance of the modified electrode with TRβ1-LBD was monitored in the presence of different concentrations of the analytes (THs and analogues). Figure 1 depicts the experimental apparatus for the detection of the thyroid hormones and analogues. Capacitance measurements in the range 50 Hz to 1 MHz were performed with a Solartron 1260A impedance/gain phase analyzer upon the application of a 50 mV AC signal. The rationale behind using electrical capacitance measurements was related to the monitoring of the capacitance changes induced by adsorption of the hormones and analogues molecules on the bioreceptor electrode. As it has been reported, such changes in the capacitance depend on several factors, and may arise from changes in factors including the electrode geometry and/or the organic film and its interaction with the solution, which ultimately results in changes in the electrical double-layer formed at the interface.\(^{19-21}\) Here, the latter effect is more likely to occur due to frequencies employed in the detection analyses (100 Hz and 1 kHz), it has been suggested by Taylor et al.\(^{19}\) Also worthwhile to mention is that because of the nanoscale architecture of the bioreceptor layer on the electrode, the electrical response is almost immediate when in contact with an analyte solution. Three electrodes were employed to improve the efficiency of the detection technique: 21, 22

(1) a bare electrode;
(2) an electrode containing an organic thin film comprised of thiol and silane SAMs (silatiol); and
(3) an electrode containing the immobilized TRβ1-LBD (biosensor electrode).

After the electrical measurements, the capacitance values for the 3 electrodes were statistically correlated using principal component analysis (PCA).\(^{20-23}\) The PCA technique has been largely employed as an efficient statistical tool capable of revealing the relevant information of a given set of input data in a lower dimension system.\(^{20}\) PCA analysis was used here to investigate the influence of the specific electrode (biosensor electrode) on the separation of the analyte samples, which was conducted by performing binary combinations of the electrodes using the capacitance data at 1 kHz from all analytes (T3, T4, TRIAC and GC-1) separately at a concentration of 50 nM in Hepes buffer. In both cases, PCA plots were built from capacitance values collected at 100 Hz and 1 kHz.

The ability of a given electrodes combination to distinguish among the analyte samples was also investigated using PCA.

RESULTS

CV was performed to analyze the adsorption of the MUA monolayer on the electrodes. The voltammograms collected before and after the functionalization of the electrodes are shown in Figure 2. The decrease in the oxidation current is evidence that the metallic surface of the electrode was blocked by the SAMs. The voltammetric profile for a bare interdigitated electrode is typical of an electron-transfer mechanism.\(^{24}\) However, after the SAM adsorption formed by 11-AMU thiol, the oxidation current decreases, which suggests that the thiol blocking monolayer is adsorbed. The remaining oxidation current may be related to defects in the thiol monolayer adsorption.

Immobilization of TRβ1-LBD receptor on the functionalized electrodes may occur via non-specific binding, through electrostatic interactions between carboxylic groups from MUA and amino groups from TRβ1-LBD. Interactions via H-bonding are also expected. Adsorption of TRβ1-LBD on functionalized electrodes was monitored

![Figure 1](image1.png)

**Figure 1.** Illustration of the electrical measurements for the detection of THs and analogues: (1) Interdigitated electrode immersed in analyte solution; (2) Zoomed area showing the electrode tracks functionalized with “silatiol” SAMs that contain immobilized TRβ1-LBD; (3) Solartron equipment and (4) Data acquisition computer.

![Figure 2](image2.png)

**Figure 2.** Cyclic voltammograms of the interdigitated electrode in the absence of SAM (bare electrode) (—) and after functionalization with 1.0 mM 11-AMU (–).
Figure 3. Increase in the effective refractive index ($\eta_{\text{eff}}$) as a function of time upon adsorption of TR$\beta$1-LBD on gold-functionalized electrodes.

via SPR measurements (Fig. 3). Upon injection of 8.6 $\mu$M of TR$\beta$1-LBD on the COOH (MUA) functionalized surface, an increase in the effective refractive index ($\eta_{\text{eff}}$) is observed, indicating adsorption of the TR$\beta$1-LBD. Saturation is reached after ca. 8 min. After washing, a constant signal response is still observed in the SPR curve, indicating the effective adsorption of the receptor molecule.

Figure 4 shows the FTIR spectra for a thiol monolayer adsorbed on glass substrate covered with gold (AuSH), a TR$\beta$1-LBD layer directly adsorbed on a gold surface (AuTR) and the monolayer TR$\beta$1-LBD adsorbed on the thiol monolayer (AuSHTR). No high-intensity bands are apparent in the AuSH spectrum, which is consistent with results in the literature. For samples that contained the SH group, a low intensity band appeared in the range of 2590–2540 cm$^{-1}$. Binding of the methylene group to the sulfur atom (CH$_2$−S) results in low-intensity bands due to symmetric (2878–2846 cm$^{-1}$) and non-symmetric (2948–2922 cm$^{-1}$) stretches, as well as angular deformations (1435–1410 cm$^{-1}$). In the AuTR spectrum, the characteristic bands of amino-acid residues from TR$\beta$1-LBD are observed. Amides usually exhibit large bands from C=O at 1640–1620 cm$^{-1}$, whereas N−H angular deformations can cause band overlap at approximately 1800 cm$^{-1}$. Stretches attributable to N-H and C−N are observed at approximately 3475 cm$^{-1}$ and 1400–1500 cm$^{-1}$, respectively. The spectra for AuTR and AuSHTR revealed a significant difference in the intensities of the C−H stretching bands at 3050–2600 cm$^{-1}$. This difference is a consequence of the amount of material that interacted with the substrate surface.

The morphology of the electrodes was revealed by AFM. The image collected in tapping mode of a gold substrate with a 11-mercaptoundecanoic acid (MUA) monolayer is shown in Figure 5(A). Figures 5(B) to (E) depict the AFM images of the multilayers that contain immobilized TR$\beta$1-LBD, and TR$\beta$1-LBD after interaction with...
the hormones T3, T4 and TRIAC, respectively. The white globular spots in Figure 5(B) are related to protein aggregates and indicate the adsorption of the TRβ1-LBD.

Detection measurements were performed using electrical impedance spectroscopy. Capacitance measurements were performed after the electrodes were immersed for 20 min in solutions that contained the ligands (hormones and analogues) at different concentrations in 20 mM Hepes pH 8, 50 mM NaCl and 5% glycerol buffer. Principal component analysis (PCA) was used to statistically correlate the samples, similar to the method we have described in previous papers. The statistical analysis was first employed to investigate the influence of the specific electrode (biosensor electrode) on the separation of the analyte samples, which was conducted by performing binary combinations of the electrodes using the capacitance data at 1 kHz from all analytes (T3, T4, TRIAC and GC-1) separately at a concentration of 50 nM in Hepes buffer. Figures 6(A)–(C) depict the outputs of the PCA analyses. As evident from the data in Figure 6(A), the data remain grouped when PCA is carried out using electrodes (1) and (2), and a good separation was not observed. However, if data from the specific electrode that contains the immobilized TRβ1-LBD (electrode (1)) is combined, for example, with the data for the bare electrode, a good

![Figure 6](image_url)

**Figure 6.** Binary combinations of (A) bare and silatiol electrodes, (B) bare and biosensor electrodes and (C) silatiol and biosensor electrodes, at 1 kHz and a concentration of 50 nM in 20 mM Hepes pH 8, 50 mM NaCl and 5% glycerol buffer. Each factor corresponds to a linear combination of the independent variables. The contribution a variable to a given factor is obtained by the projection of an arbitrary point on that factor axis onto that original variable axis. Each loading represents the relative contribution of every original variable to a factor axis.

![Figure 7](image_url)

**Figure 7.** PCA plots combining the capacitance values from the 3 electrodes collected in 50 nM solutions of the ligands T3, T4, TRIAC and GC-1 at (A) 100 Hz and (B) 1 kHz. All measurements were carried out in 20 mM Hepes pH 8, 50 mM NaCl and 5% glycerol buffer.
Figure 8. PCA plot obtained using the capacitance data (for a frequency of 100 Hz) from the 3 electrodes employed, immersed in solutions at different concentrations of T3, T4 and TRIAC.

separation is observed between the two electrodes, as shown in Figure 6(B).

PCA analyses were further employed to correlate the capacitance data from the 3 electrodes collected at frequencies of 100 Hz and 1 kHz in a physiological-level concentration of 50 nM for all the analytes in Hepes buffer. As shown in Figures 7(A) and (B), although a good separation was observed for both frequencies, data appear less dispersed and better separated at a frequency of 100 Hz (Fig. 7(A)). This frequency was further used to investigate the ability of the sensors to distinguish between different concentrations of the analytes.

The output of the PCA analyses that combined the capacitance values of the 3 electrodes collected after the electrodes were immersed in solutions that contained T3, T4 and TRIAC at different concentrations is shown in Figure 8. The ability of the system to distinguish between T3, T4 and TRIAC is evident for the concentration range of 2.5 to 500 nM.

DISCUSSION

The efficiency of the electrode surface modification using MUA monolayers has been proven via CV, as shown in Figure 2, where a decrease in the oxidation current indicated that the platforms (interdigitated electrodes) were satisfactorily covered by the organized thiol monolayers. AFM analyses carried out after each adsorption step confirmed the modification of the electrodes, as will be discussed below. An efficient surface coverage is important in this case because it may improve the signal-to-noise ratio during the detection experiments by avoiding non-specific interactions between analytes and the bare, uncovered gold sites. The adsorption of TRβ1-LBD was further confirmed via SPR analyses, through which an increase in SPR signal followed by saturation was observed, as depicted in Figure 3.

The immobilization of TRβ1-LBD on the electrodes, as analyzed via FTIR spectroscopy (Fig. 4) revealed that receptor adsorption is larger in the absence of the thiol layer because more gold atoms are available to interact non-specifically with the protein. These interactions result from the attraction between amino-acid residues that contain sulfur atoms and gold atoms from the substrate. Upon surface functionalization, the TRβ1-LBD hystidine tail may interact with the thiol carboxylic groups. The latter architecture possibly induces an orientation to the protein, which optimizes the access of the ligand to the active site localized next to the C-terminal residue. The functionalization minimizes the non-specific interactions and thereby optimizes the selectivity in the detection measurements of the THs, as will be discussed later. Also, the significant C—N band shifted from 1500 cm$^{-1}$ for AuTR to 1400 cm$^{-1}$ for AuSHTR, which indicates that the thiol SAM affects the orientation of the adsorbed protein.

The adsorption of TRβ1-LBD was further confirmed via AFM analyses, through which an increase in aggregate diameter could be observed after receptor immobilization, as shown in Figure 5(B). An explanation for the increase in the average roughness values observed after adsorption of the ligands T3, T4 and TRIAC (as summarized on Table I) is not straightforward. The increase may be due to the greater amount of T3, T4 and TRIAC adsorbed on the electrode, or even to the conformational changes that occurred in the TRβ1-LBD upon adsorption of the ligands.

The feasibility of the nanostructured sensors to separate and distinguish between different thyroid hormones was clearly demonstrated by impedance measurements and PCA analyses, as shown in Figures 6–8. The sensors were able to distinguish between different concentrations at physiological levels of T3 and T4 and the analogues TRIAC and GC-1, which suggests the applicability of the films in the detection and possibly to the quantification of THs. The choice of a specific value of frequency makes possible the evaluation of both THs and the synthetic analogues. This distinction is related to ligand affinities for TRβ1 because, at the same concentration (50 nM), these molecules interact with the receptor differently according to their structure and functions. A good distinction between the signals from the buffer and the ligands was also observed in all cases. In general, the separation of

<table>
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<th>Sample</th>
<th>Roughness (nm)</th>
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<tbody>
<tr>
<td>Au-MUA</td>
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</tr>
<tr>
<td>Au-MUA-TRβ1 LBD</td>
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</tr>
<tr>
<td>Au-MUA-TRβ1 LBD T3</td>
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<tr>
<td>Au-MUA-TRβ1 LBD T4</td>
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<tr>
<td>Au-MUA-TRβ1 LBD TRIAC</td>
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THs and analogues is related to the different interactions of the ligands with the receptor, which results in capacitance changes in the multilayer. Because the detection using interdigitated electrodes and the capacitance measurements at a fixed frequency are relatively inexpensive, as well as the materials employed for electrodes modification (due to the very small amounts needed), the systems presented here represent an attractive alternative for the detection and identification of thyroid hormones, especially for hormone replacement therapies.

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REFERENCES


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