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Competitive immunoassay by hot electron-induced electrochemiluminescence detection and using a semiautomatic electrochemiluminometer

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Abstract

A homogeneous immunoassay of T4 was developed using a semiautomatic electrochemiluminometer modified from a commercially available fluorometer. In addition, from the same analyte panel an immunometric immunoassay of TSH at similar disposable oxide-covered aluminum rake electrodes was studied using this instrument both on homogeneous and heterogeneous basis. Detection was based on hot electron-induced cathodic electrochemiluminescence utilizing a commercially available Tb(III) chelate label. The assays were reasonably sensitive and comparison was made with other older methods. Thus, it is possible to develop both non-competitive and competitive immunoassays based on detection of hot electron-induced ECL of the labels.

Keywords: Electrochemiluminescence; Bioaffinity assay; Immunoassay; Hot electron; Terbium; Labels

1. Introduction

The field of immunoassay technology is still growing, although the first results of the use of the principle of competitive inhibition of labeled antigen to antibody was published already in 1959 [1]. The next major advance in binding assay methodology was the development of two-site or sandwich-type assays [2], and then monoclonal antibody technique finally started the vast development of assay methods [3].

It was very soon realized that labels with much higher specific signal than ^{125}I were needed, and there was also a need to replace radioactive markers with non-radioactive labels. The time-resolved fluorometric immunoassay methodology

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now known as dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) was the first "ultra-sensitive" non-isotopic immunoassay methodology [4]. The same approach has subsequently been used by many other manufacturers, using enzyme, chemiluminescent and fluorescent labels. After the discovery of the polymerase chain reaction [5], different labeling methods have also been used in hybridization methods which still have a fast growing importance.

Electrochemiluminescence is a relative latecomer among the detection techniques used in the field of bioaffinity assays [6–9]. Generally, electrochemiluminescent labels have the same benefits as photoluminescent labels (long shelf life of reagents, no interference with biological compounds, wide dynamic range, high sensitivity, etc.). In addition, the excitation can be achieved by using much cheaper instrumentation, and no excitation optics are needed, which is very useful, especially in microanalytical systems of the future.

Human thyroid stimulating hormone (hTSH), secreted by the anterior pituitary gland, is a major regulating factor of thyroid hormone synthesis and secretion. It stimulates the thyroid to synthesize and release the thyroid hormones triiodothyronine (T3) and thyroxine (T4), and induces thyroglobulin production. T3 and T4 are responsible for regulating throughout the body diverse biochemical processes essential for normal development and metabolic and neural activity. Serum levels of hTSH, T3 and T4 are routinely used as indicators of thyroid function [10,11].

Labels based on $Ru(bpy)_3^{2+}$ are already in commercial use in immunoassays and DNA-probe assays [8,9], and commercial kits and instruments based on this anodic electrochemiluminescence have already been evaluated, e.g. in cases of hTSH and T4 [12]. We have also shown earlier that hTSH can be determined by manual immunometric immunoassays at oxide-coated aluminum [13] and silicon [14] electrodes, but real patient samples have not been previously measured.

The present work was carried out to develop a homogeneous electrochemiluminoimmunoassay (ECLIA) of T4 using a prototype of a semiautomatic electrochemiluminometer. In addition, a homogeneous immunoassay of TSH was studied using this instrument based on hot electron electrochemistry [6,7,15,16] and compared with a well-established reference method using real serum samples. Tb(III) chelated by N^1-(4-isothiocyanatobenzyl)diethylenetriamine-N^1,N^2,N^3,N^3-tetraacetate was used as the electrochemiluminescent label. So far, it has never been demonstrated that electrochemiluminescent Tb(III) labels can also be used in competitive immunoassays.

2. Experimental

2.1. Modifications of a fluorometer

An Arcus 1230 fluorometer (Wallac Oy, Turku, Finland) was modified to a time-resolved electrochemiluminometer by removing the excitation lamp and optics from the instrument and changing the PMT to measure light from one side of a 12-well single strip (from a detachable 96-well microtiter plate) attached in an original strip rack of Arcus (Fig. 1). A special holder for a 12-peak Al-rake electrode and a set of 12 Pt-wire counter-electrodes fitting on top of the rack was constructed (Fig. 2). Two pressing-contact springs were made for electrical contacts. One spring gave contact to a single-piece Al rake electrode that was made by cutting and forming from aluminum band with a specially made tool, and the other spring gave contact individually to each Pt

Fig. 1. Modified Arcus conveyer track also displaying the excitation electronics block added to the instrument.

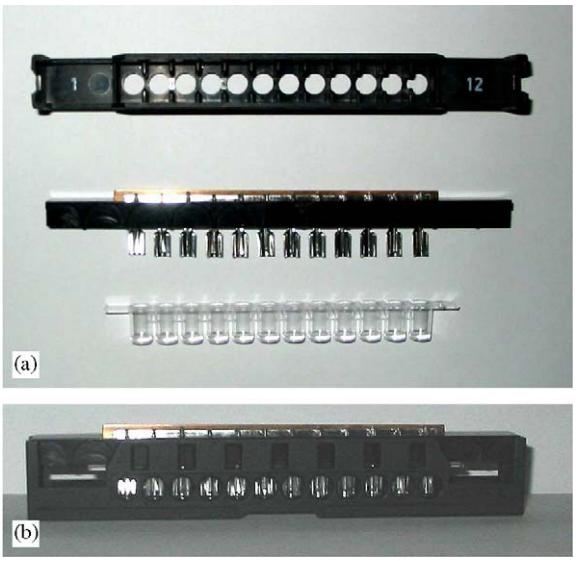

Fig. 2. Al-rake working electrode and Pt-counter electrodes. (a) Rack, rake-electrode and Pt-counter electrodes assembled in their holder, and a 12-well micro titer strip. (b) Fully assembled rack ready to be put on the conveyer track.

counterelectrode wire when it was at the measuring position in the conveyer system in its turn. The strip geometry was found to be quite beneficial for light collection and, thus, no lenses were needed for sufficiently efficient light collection to the PMT cathode situated as close as possible to the rack track.

The pressing springs were wired to a potentiostat that could give current up to 100 mA and having compliance voltage up to 10 V. The Arcus controlling program and electronics were modified to give triggering pulses to a pulse generator so that adjustable (up to 10 V) rectangular cathodic voltage pulses could be given to the working electrode with a duration of 240 μs at a frequency of 200 Hz. Also, BNC connectors for output of triggering pulses and cell current were added on the back panel of the instrument. For time-resolved ECL detection, the delay and gate time were selected to be 80 μs and 3.0 ms. The detection was based on single photon counting and integrating the gated signal over 200 excitation cycles, and the controlling program assumed that each sample was measured in duplicate and calculated also the mean and CV % for the duplicate measurements. No optical filters were used.

2.2. Reagents and electrodes

The label chelate, Tb(III) chelated by N^1-(4-isothiocyanatobenzyl)diethylenetriamine-N^1,N^2,N^3,N^3-tetraacetate (commercially available from the present Perkin-Elmer Life Sciences), was obtained from Wallac Oy (Turku, Finland) and 2,6-bis[*N,N*-bis(carboxymethyl)aminomethyl]-4-methylphenol was synthesized as described previously [7]. Electrodes were cut from an Al band (Merck Art. 1057, batch K4164557). After cutting and shaping the rake electrode, it was washed in hexane and etched in the 2.5:7:0.5 mixture of concentrated sulfuric, phosphoric and nitric acid, respectively, at room temperature for 12.0 min. After the etching (which dissolved the original oxide film and developed a new oxide film upon aluminum) the rake electrode was carefully washed with distilled water.

2.3. Homogeneous competitive immunoassay for thyroxine (T4)

2.3.1. Labeling of thyroxine

Thyroxine was bound to gelatin as follows: to 2 ml of gelatin solution (20 nmol/mL in 0.05 mol/L phosphate buffer pH 7.3) was added 200 μL (2000 nmol) of *N*-hydroxysuccinimide ester of thyroxine (Wallac Oy, Turku, Finland) in dioxane. After overnight incubation the conjugate was purified using PD10 column (Pharmacia Biotech) with 0.9% NaCl solution as eluent. T4-gelatin conjugate (1143 pmol in 200 μL of 0.9% NaCl solution) was allowed to react overnight with Tb-chelate (17 μL in water, Tb^{3+}-N^1-(4-isothiocyanatobenzyl)diethylenetriamine-N^1,N^2,N^3,N^3-tetraacetate) at a molar ratio of 1:200. The pH was adjusted to 9.5 with 0.5 mol/L Na_2CO_3. The labeled conjugate was purified from excess label by gel filtration through a 1-cm-diameter column filled with Sepharose 6B (Pharmacia Biotech) to a height of 52 cm, with an additional 5.5 cm of Sephadex G-50 (Pharmacia) on top. Tris-saline buffer (TSA) containing 0.05 mol/l Tris-HCl, pH 7.75, 0.9% NaCl, 0.05% NaN_3 was used as the eluent.

2.3.2. Coating of oxide-covered aluminum electrode with antiserum

Oxide-covered aluminum surface was used as solid phase for immunoassay. Aluminum rakes fitting to 12-well strips were coated in the micro-titer wells in the volume of 200 μL with rabbit immunoglobulins to mouse immunoglobulins (DAKOPATTS, Denmark) at the concentration 5 μg/ml in 0.05 mol/L tris-H_2SO_4 buffer, pH 7.5. After incubation overnight at room temperature, the rakes were washed by wash solution (Wallac, Turku, Finland) and saturated by standing in 300 μL of saturation solution 0.05 mol/L tris-H_2SO_4, pH 7.5, containing 0.5% BSA. After saturation, the rakes were lifted from the solution and allowed to dry at room temperature and stored dry. Dry rakes were stable for at least 6 months at room temperature.

2.3.3. Time-resolved homogeneous immunoassay of thyroxine

Monoclonal antibody to thyroxine (Medix Biotech, USA) 100 μL (0.2 nmol/L) was added to the well followed by 100 μL of gelatin-T4-Tb-conjugate (calculated to be 10 ng/well in respect to gelatin) and 20 μL of the following standards 0, 10, 50, 100, 150 and 300 nmol/L. Aluminum rakes were then moved to wells and after incubation for 1 h the electroluminescence was measured by a time-resolved electroluminometer. All components were in 0.2 mol/L borate buffer, pH 7.75, containing 0.1% NaN_3, 0.05% bovine gammaglobulin, 0.5% BSA and 0.01% Tween 40. Each set of automatic measurements gave the results for six duplicate samples.

2.4. Time-resolved ECLA of hTSH

2.4.1. Coating electrodes with antibody

Electrodes were coated with antibody by physical adsorption, by incubating the electrode in TSA-buffer containing the coating antibody (clone 8661, specific to the alpha chain of hTSH, Pharmacia, Uppsala, Sweden) 30 μg/mL for 3.0 h in a micro-titer strip. After the coating, the surface was washed with running wash solution and equilibrated overnight in TSA-buffer, pH 7.75, containing 0.1% bovine serum albumin and 5% D-sorbitol. After the equilibration, the electrodes were dried and they were stable in storage for at least 1 year.

2.4.2. Preparation of the labeled antibody

Monoclonal antibody that is specific to the β-chain of hTSH (clone 5404, Medix Biochemica, Kauniainen, Finland) was labeled with Tb^{3+}-N^1-(*p*-isothiocyanatobenzyl)-diethylene triamine-N^1,N^2,N^3,N^3 tetraacetate by allowing the antibody to react with the chelate in the molar ratio of 1:60 at pH 9.5. The pH was adjusted with 1 mol/L Na_2CO_3 solution. The labeled antibody was separated from the unreacted chelate by gel filtration (Sepharose 6B 1 × 50 cm, Sephadex G-50 1 × 5 cm) using TSA-buffer as eluent. Typically, 5–10 chelate molecules can be bound in this way to one antibody molecule. To improve the stability, 0.1% bovine serum albumin was added to the labeled antibody.

2.4.3. Homogeneous immunoassay of hTSH

The immunoassay of hTSH was carried out in the wells of micro-titer strips. First, 180 μL of assay buffer (0.2 mol/L borate–H_2SO_4 buffer, pH 7.75, 0.1% NaN_3, 0.5% bovine serum albumin, 0.05% bovine gammaglobulin and 0.01% Tween 40) and 20 μL of Wallac hTSH standard or sample (Turku, Finland) and 20 μL of label solution containing 300 ng of labeled antibody were added. Then, the strip was put in the rack and the electrodes in the strip; the rack was shaken for 1 h, transferred to the luminometer and the TR-ECL was automatically measured yielding results for six duplicate samples or standards.

2.4.4. Heterogeneous immunoassay of hTSH

The immunoassay was carried out in the wells of micro-titer strips. First, 25 μL of standard and 175 μL assay buffer (0.05 mol/L tris-HCl, pH 7.75, 0.9% NaCl, 0.5% NaN_3, 0.5% bovine serum albumin, 0.05% bovine gammaglobulin and 0.01% Tween 40) were added. Next, the electrode was added and after incubation for 1 h the electrode was washed with running wash solution and allowed to react with labeled antibody (500 ng/200 μL) for 1 h on the shaker. After the reaction, the electrode was washed with running

distilled water and the TR-ECL was measured in the measuring solution (0.2 mol/L borate buffer, pH adjusted to 7.75 with sulfuric acid, 0.01 mol/L NaN_3).

3. Results and discussion

First, the performance of the instrument was studied by measuring the dilution series of two different Tb(III) chelate labels in the measuring buffer developed for heterogeneous immunoassays. The buffer contained 0.1% NaN_3 in 0.05 M $Na_2B_4O_7$ adjusted to pH 7.75 with sulfuric acid. Fig. 3 shows that the linear calibration range covers several orders of magnitude of concentration and quite low concentration levels of the label chelates can be detected with the present instrument. The inset displays the emission spectrum of the present Tb(III) label. Because Tb(III) ion is extremely difficult to one-electron reduce or oxidize ($E^0(Tb^{3+}/Tb^{2+}) = -3.7$ V, $E^0(Tb^{4+}/Tb^{3+}) = 3.1$ V [17]), the chelate is excited by ligand-sensitized mechanism, in which the ligand is first excited by successive one-electron redox steps [18] followed by energy transfer to the central ion which finally emits by $^5D_4 \rightarrow {}^7F_J$ radiative transitions (Fig. 3) [19].

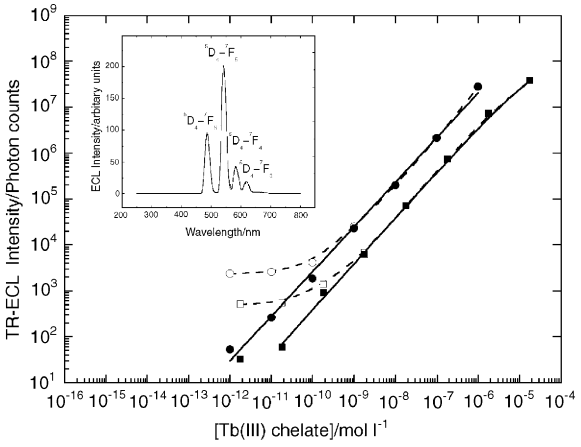

Fig. 3. Calibration curves of Tb(III) label chelates. (●) Tb^{3+}-2,6-bis[*N,N*-bis(carboxymethyl)aminomethyl]-4-methylphenol, (■) Tb^{3+}-N^1-(*p*-isothiocyanatobenzyl)-diethylene triamine-N^1,N^2,N^3,N^3 tetraacetate. Conditions: 0.2 M borate buffer, pH 7.75, 0.1% NaN_3, pulse amplitude-10 V. Open circles and squares denote the signal before subtracting the blank. The inset shows the uncorrected emission spectrum of the latter chelate (0.5 mM) measured with a Perkin-Elmer LS5 spectroluminometer in 0.05 M $Na_2B_4O_7$ buffer adjusted to pH 7.8 with sulfuric acid.

Pre-solvated hot electrons injected into aqueous electrolyte (or hydrated electrons subsequently formed) can reach reducible molecules only at a distance of up to about 200 nm from the oxide film/electrolyte interface. Hence, the washing of the unreacted labels away from the assay cell should not be necessary because the amount of label collected on the electrode surface is mainly based on the result of an immunochemical binding if the assay has reached equilibrium or is close to equilibrium.

The assay buffer was optimized for homogeneous assays by finding suitable concentrations of azide ion, Tween 40, bovine gammaglobulin and albumin. Fig. 4 shows the T4 calibration curve obtained after 1 h incubation in 200 μL volumes, with shaking during the incubation in the optimal assay buffer. The calibration curve has the same shape as that obtained by carrying out the immunoassay on the Al rake surface as described in the experimental part, except that Tb(III) was finally detected by time-resolved photoluminescence using Wallac development solution for Tb(III).

For comparison, an immunometric sandwich assay was also tested. When hTSH immunoassays

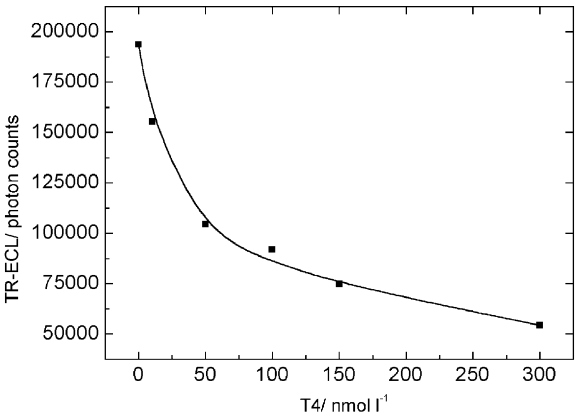

Fig. 4. Calibration curve of homogeneous T4 assay. Conditions: 0.2 M borate buffer, pH 7.75, 0.1% NaN_3, bovine gammaglobulin 0.05%, 0.5% BSA, Tween 40 0.01%.

were carried out on an immunometric basis, it was observed that in homogeneous assays the signal is considerably smaller than heterogeneous assays. This is due to the primary radical scavenging effect of proteins (bovine serum albumin, bovine gammaglobulin) [13] (Fig. 5). However, the correlation with IRMA was still reasonably good ($r = 0.989$, $n = 6$, Fig. 6). The normal range of hTSH is from 0.5 to 6 μIU/mL for healthy persons [20].

Because earlier analysis procedures used by us [13,14] were very work-demanding, the comparison with IRMA was carried out with the present semiautomatic electrochemiluminometer, also with heterogeneous assays of hTSH, using real patient samples. Fig. 5 displays the calibration curve of standards, and Fig. 7 the comparison between heterogeneous ECLIA assays and IRMA assays ($r = 0.977$, $n = 38$). Both methods give quite similar results at higher concentrations but there is more deviation at low concentrations. The RSD of two replicates was below 15%, with the present oxide-covered aluminum electrodes, but can be improved by changing the working electrodes to more pure aluminum or preferably to oxide-coated silicon electrodes having a good-quality insulating film coverage. These, however, are more expensive than the presently used relatively impure aluminum (purity only 99.9%). However, silicon electrodes can still be used on a disposable basis and provide a number of large-scale manufacturing possibilities inherited from the worlds of microelectronics manufacturing and micro-electro-mechanical systems and micro-opto-electro-mechanical systems (MEMS and MOEMS).

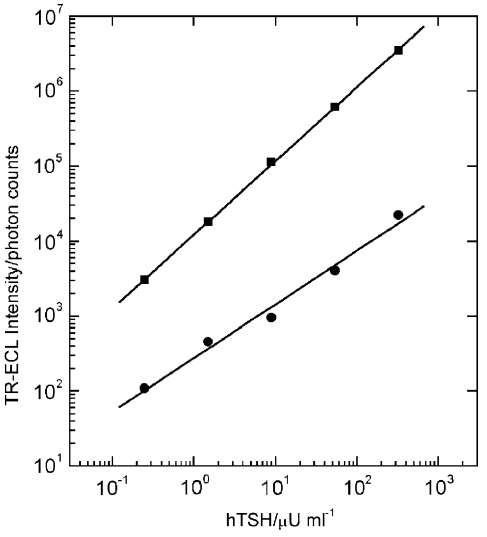

Fig. 5. Calibration curves of (a) homogeneous hTSH assay (●) and (b) heterogeneous hTSH assay (■). (a) Conditions: as in Fig. 4. (b) Conditions: as in Fig. 3.

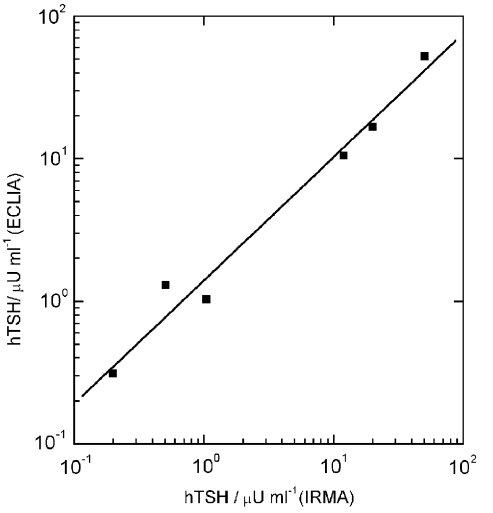

Fig. 6. Correlation between hTSH values obtained in homogeneous ECLIA assays and IRMA. Immunoradiometric assays were carried out with Orion Diagnostica IRMA kits (Turku, Finland).

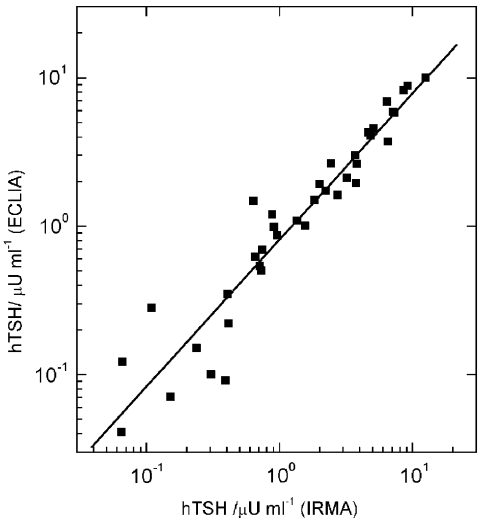

Fig. 7. Correlation between hTSH values obtained in heterogeneous ECLIA assays and IRMA.

4. Conclusions

A competitive assay type allows the use of homogeneous ECLIA assay principles in the case of hot-electron-induced ECL excitation of Tb(III) labels. However, the ECL signal is considerably higher and the calibration curve slope is better in heterogeneous ECLIA assays. The instrument prototype used here and the methods developed still need further work to get to the accuracy level of commercially available instruments and kits based on $Ru(bpy)_3^{2+}$ derivative labels and anodic ECL [12]. The incubation time can be brought down to be usable in point-of-care analysis by developing the assay cells to allow the use of very low incubation volumes and very short diffusion distances. In addition, elevating the incubation temperature to 37 °C increases the assay speed. However, due to the weaker constancy properties of thin aluminum oxide films under slightly basic conditions in aqueous solution, the working electrodes must be changed to oxide-coated silicon electrodes [14] if elevated temperatures are applied.

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