AMPEROMETRIC MICROSENSORS BASED ON INULINS FOR THE ASSAY OF L-T_3 AND L-T_4

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Three different types of inulins (HD, TEX, IN) were physically immobilized on carbon nanopowder (Cn) based matrix, for the assay of thyroid hormones, L-thyroxine (3,3`,5,5`-tetraiodo-L-thyronine, L-T_4) and L-triiodothyronine (3,3`,5-triiodo-L-thyronine, L-T_3) in whole blood samples. The response characteristics of the proposed sensors were recorded using differential pulse voltammetry. The lowest detection limit for L-T_3 (8.11×10^{-10} mol L^{-1}) was reached when the microsensor based on HD was employed; while for L-T_4 (1.68×10^{-8} mol L^{-1}), it was reached using IN based microsensor. The blood samples were analyzed as collected from patients, without any pretreatment.

Keywords: thyroid hormones, blood samples, DPV, inulins

1. Introduction

Thyroid disease is known to be the second largest disease in the endocrine field. The thyroid hormones, L-thyroxin (3,3`,5,5`-tetraiodo-L-thyronine, L-T_4) and L-triiodothyronine (3,3`,5-triiodo-L-thyronine, L-T_3), which are produced by the thyroid gland, are essential for cell differentiation, cellular metabolism, normal growth and development and for the regulation of metabolic rates in each cell of the body. With normal function, the thyroid gland produces 8 μg L-T_3 and

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90 μg L-T_4 per day, and it is reported that L-T_3 is the most widely used as diagnostic marker for thyroid function [1, 2].

L-T_3 affects protein synthesis and fat metabolism. Two thirds of T_3 is secreted by the thyroid gland and one third is produced from thyroxin conversion by deiodination in peripheral tissue. This makes L-T_4 a pre-hormone which converts to the major active compound, L-T_3. In the blood, L-T_3 is binded to specific binding proteins and only a very small amount of this hormone is circulating free in the blood. High levels of L-T_3 indicate hyperthyroidism or Grave`s disease and low levels of L-T_4 indicate hypothyroidism. It is well known that obesity, metabolic syndrome, insulin resistance and atherosclerosis are consequences of hypothyroidism [3-5].

Nam and coworkers conducted a unique study on relatively young subjects, debating the relationship among thyroid hormones, visceral obesity and a surrogate marker of atherosclerosis, pulse wave velocity (PWV). Higher T3 levels had been positively associated with obesity and especially visceral obesity and increased PWV [6].

T_3 and T_4 levels were also associated with leptin levels, the product of ob gene, which plays a major role in the regulation of metabolism [7]. Leptin is mainly secreted from adipose tissue [8], and acts as an endocrine signal for fat deposition. Enhanced thyroid hormone activation and higher serum T3/T4 ratio contributes to higher levels of leptin in chickens [9] or in restricted calorie-diet rats [10].

Since T_3 and T_4 upregulate many metabolic pathways relevant for resting energy expenditure (REE), it is not unexpected that individuals with thyroid diseases reveal changes in body weight and lipolysis in adipose tissue [11]. Increased thyroid hormones are associated with obesity, while on the opposite side, *anorexia nervosa* is associated with low free L-T_4 concentrations [12].

Therefore, highly efficient and reliable analytical methods are necessary for thyroid hormones detection in biological samples. There are various methods that have been used for the assay of T_3 and T_4, such as enzyme-linked immunosorbent assay, ELISA [13], sequential injection analysis (SIA)/ immunosensor system [1], chemiluminescence immunoassay, CLIA [14], immunoradiometric assay, IRMA [15], liquid cromatography [16], electrochemiluminescence (ECL) immunosensor [17]. There is a need for a fast and simple method that can be performed with low cost and without any need for sample preparation.

In this study we proposed three amperometric microsensors based on carbon nanopowder modified with three different types of inulins for the assay of L-T_3 and L-T_4 in whole blood samples. Inulins were used to enhance the electrocatalytic property of the carbon nanopowder.

2. Experimental

2.1. Reagents and materials

All chemicals were of analytical grade. L-thyroxine (L-T_4), L-triiodothyronine (L-T_3), L-tryptophan (L-Trp), serotonin (5-HT) and carbon nanopowder (CN, <50 nm particle size (TEM), ≥99% trace metals basis) were purchased from Sigma Aldrich (Milwaukee, WI); and paraffin oil (d_4^{20}, 0,86 g x cm^{-1}) was obtained from Fluka. The inulins: Frutafit HD (HD), and Frutafit TEX (TEX) were provided by Sensus (Roosendaal, The Netherlands) and Inutec (IN) was provided by Orafti Non Food (Oreye, Belgium). KH_2PO_4 and Na_2HPO_4 (Reactivul Bucuresti) were used to prepare the phosphate buffer solution (pH=7.03).

2.2. Apparatus and methods

A multiparameter CyberScan PCD 6500 Eutech Instruments was used for measurements of pHs. Differential pulse voltammograms (DPVs) were performed with a 663 VA Stand (Metrohm, Herisau, Switzerland) connected to a PGSTAT 12 and a GPES sofware was used for recording the measurements. A platinum electrode served as counter electrode and Ag/AgCl (0.1 mol L^{-1} KCl) as reference electrode in the electrochemical cell.

The technique used for the direct assay of thyroid hormones, was differential pulse voltammetry (DPV) with step potential of 25mV, a scan rate of 50 mV/s and modulation amplitude of 100 mV. All measurements were carried out at 25^{o}C. The working, reference and auxiliary electrodes were immersed in a plastic tube containing the analyte (L-T_3 or L-T_4 of different concentrations). Calibration graphs were drawn and the unknown concentration of the analyte in the real sample was determined using the calibration graph.

2.3. Design of the microsensors based on carbon nanopowder and inulins

Three carbon nanopowder (Cnano) based microsensors were designed for the assay of L-T_3 and L-T_4. 100 mg of Cnano were mixed with 40 μL of paraffin oil to form a carbon paste. To this mixture, 3 different types of inulin solutions (10^{-3}mol*L^{-1}) were added separately: IN, HD and TEX to obtain the modified pastes. Plastic tubes (of 300 μm internal diameter) were filled with the modified pastes. The electric contact was made using a silver wire (0.5 mm in diameter). Before each use, the electrodes surfaces were washed with deionized water. When not in use, the microsensors were kept at room temperature.

2.4. Solutions and sample preparation

For all the solution preparation we used de-ionized water, and the stock solutions of L-T_3 (10^{-3}mol*L^{-1}) and L-T_4 (10^{-3}mol*L^{-1}) were used to prepare

solutions of different concentrations (10^{-12}mol*L^{-1} - 10^{-3}mol*L^{-1}) by serial dillution method. When not in use, the solutions were stored in the fridge at 2-8°C.

3. Results and discussions

3.1 Response characteristics of inulin based microsensors

Response characteristics of the proposed microsensors were obtained using differential pulse voltammetry (DPV) method. Typical DPV scans are shown in Fig. 1. Response characteristics are shown in Table 1.

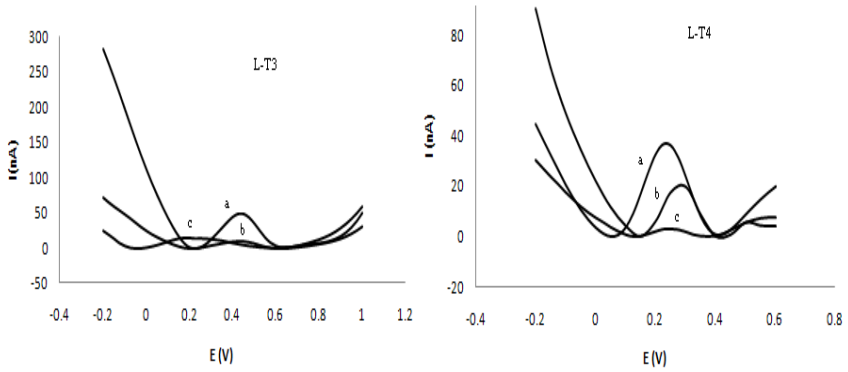

Fig.1. Typical DPV scans for L-T_3 (1.0x10^{-5}mol/L) and L-T_4 (1.0x10^{-5}mol/L) on the surfaces of carbon nanopowder electrodes based on: a) TEX, b) IN, and c) HD

Table 1

Response characteristics of the microsensors based on inulins for the assay of L-T_3 and L-T_4

Analyte	Inulin	Equation of calibration*	Sensitivity (nA/µmol/L)	Limit of detection (mol/L)	Linear concentration range (mol/L)
L-T_3	**HD**	H=10.56+0.318 x C, R=0.9999	0.318	8.11x10^{-10}	10^{-6}-10^{-4}
	IN	H=9.55+0.169 x C, R=0.9993	0.169	3.61x10^{-7}	10^{-6}-10^{-3}
	TEX	H=24.07+0.935 x C, R=0.9993	0.935	5.05x10^{-8}	10^{-6}-10^{-4}
L-T_4	**HD**	H=7.84+0.096 x C, R=0.9996	0.096	1.85x10^{-7}	10^{-6}-10^{-4}
	IN	H=35.31+0.763 x C, R=0.9999	0.763	1.63x10^{-8}	10^{-5}-10^{-3}
	TEX	H=8.54+0.576 x C, R=0.9996	0.576	6.63 x10^{-7}	10^{-6}-10^{-3}

*H = peak height <H> = nA; C – analyte concentration <C> = µmol/L

Calibration graphs of the microsensors for L-T_3 and L-T_4 are shown in Figs. 2 and 3. For the assay of L-T_4, the best response characteristics were obtained for the IN based microsensor. The highest sensitivity for the assay of L-T_3 (0.935nA/µmol*L^{-1}) was recorded when the microsensor based on TEX was used, while the lowest detection limit for L-T_3 (0.811 nmol*L^{-1}) was reached when the microsensor based on HD was employed.

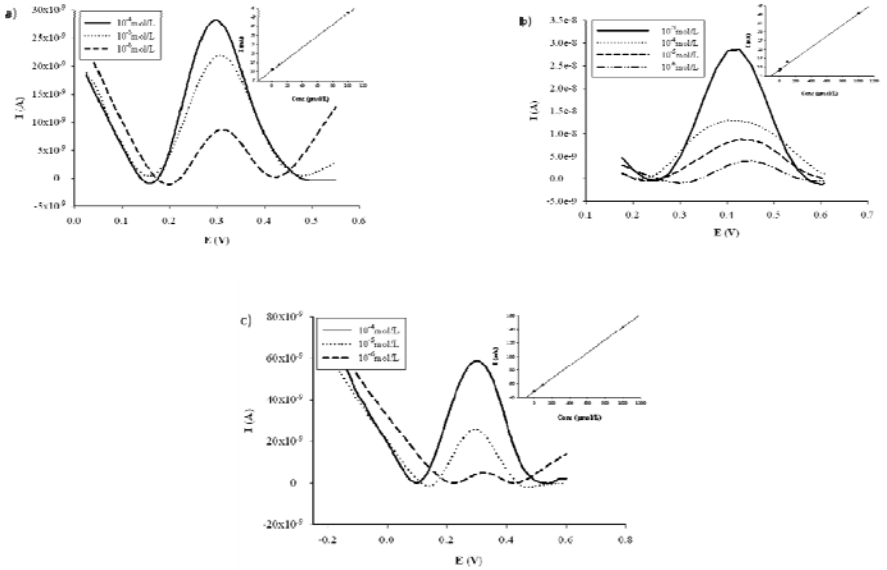

Fig. 2. Differential pulse voltammograms of a) Cnano-HD, b) Cnano-IN, and c) Cnano-TEX for L-T_3 solutions, which correspond, respectively, to the linear concentration ranges a) 10^{-6}M - 10^{-4}M, b) 10^{-6}M - 10^{-3}M and c) 10^{-6}M - 10^{-4}M. Insets: plot of the peak currents *vs* L-T_3 concentration

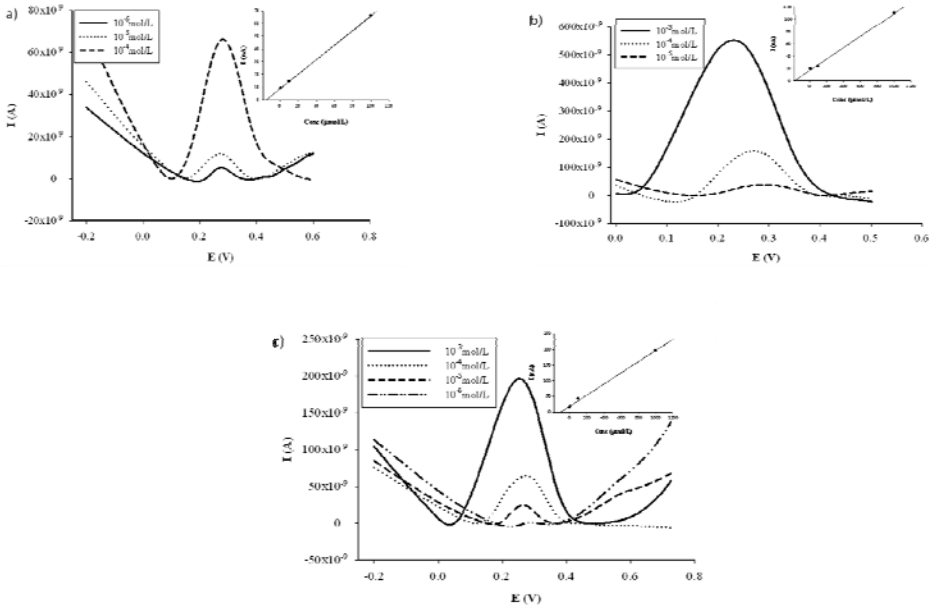

Fig. 3. Differential pulse voltammograms of a) Cnano-HD, b) Cnano-IN and c) Cnano-TEX for L-T_4 solutions, which correspond , respectively, to the linear concentration ranges a) 10^{-6}M - 10^{-4}M, b) 10^{-6}M -10^{-4}M and c) 10^{-6}M - 10^{-3}M. Insets: plot of peak currents *vs* L-T_4 concentration

Accordingly, the electrocatalytic activity of the microsensor was influenced by the type of inulin used.

The proposed microsensors are highly reliable, and stable; the RSD (%) value for the slope of the equation of calibration did change with less than 1% when the microsensors were used daily over a period of 6 months. Furthermore, 5 pastes for each microsensor were made over a period of one month, and the variation of slopes was less than 0.01%, when tested for the linear concentration range.

3.2 Selectivity

L-Tryptophan (L-Trp) and serotonin (5-HT) have been chosen as possible interfering species in the detection of thyroid hormones (L-T_3 and L-T_4). Mixed solution method was employed to determine the amperometric selectivity coefficients. The results are shown in Table 2.

Table 2

Amperometric selectivity coefficients for the amperometric microsensors

Inulin	K_{sel}^{amp}	
	L-T_3	
	L-Tryptophan	**Serotonin**
HD	2.16×10^{-2}	1.59
IN	7.47×10^{-1}	1.10
TEX	5.30×10^{-2}	8.85×10^{-1}
	L-T_4	
	L-Tryptophan	**Serotonin**
HD	3.90×10^{-2}	2.00×10^{-2}
IN	2.60×10^{-2}	3.40×10^{-2}
TEX	5.10×10^{-2}	$<< 10^{-4}$

The selectivity of the assay of one thyroid hormone in the presence of the other was determined using recovery tests. Therefore, synthetic mixtures between the hormones were prepared in different ratios. According to the results presented in the Tables 3 and 4, the best sensor for the assay of both hormones is based on carbon nanopowder and TEX; also for this sensor the recovery is dependent on the ratio between the hormones. TEX based microsensors are also selective *vs* L-tryptophan and serotonin.

Table 3

Recovery of L-T_3 in the presence of L-T_4

Inulin\ L-T_3: L-T_4 (mol/mol)	**2:1**	**1:1**	**1:2**	**1:4**	**1:9**	**1:99**
HD	98.73	161.78	159.87	112.10	105.54	139.49
IN	159.55	93.56	102.73	104.10	121.36	156.36
TEX	77.25	88.76	86.67	91.71	95.50	91.57

*N=3

Table 4

Recovery of L-T_4 in the presence of L-T_3

Inulin\ L-T_4: L-T_3 (mol/mol)	2:1	1:1	1:2	1:4	1:9	1:99
HD	99.34	127.54	76.63	80.41	121.99	86.25
IN	122.71	136.59	129.65	118.29	155.83	123.34
TEX	101.66	120.90	75.29	93.35	92.40	92.64

3.3. Analytical applications

The main analytical application is the assay of L-T_3 and L-T_4 in whole blood samples. Four blood samples were provided from the hospital, and used as collected, for the assay of the thyroid hormones using the three microsensors based on inulins and carbon nanopowder. The results are shown in Table 5.

The values of results obtained are comparable between the microsensors, proving that they can be used with a fair reliability for the assay of L-T_3 and L-T_4 in whole blood samples.

Table 5

Recovery of L-T_3 and L-T_4 in whole blood samples using the microsensors based on carbon nanopowder and inulins

Sample nr.	Inulin	Recovery, nmol/L	
		L-T_3	L-T_4
1	**HD**	-[a]	-[a]
	IN	191.10	62.73
	TEX	-[a]	-[a]
2	**HD**	-[a]	-[a]
	IN	55.80	142.08
	TEX	56.10	143.76
3	**HD**	15.90	84.50
	IN	19.58	90.62
	TEX	15.56	90.92
4	**HD**	-	4.82
	IN	153.56	8.26
	TEX	155.51	6.38

[a]No signal was recorded for these hormones in the real samples.

4. Conclusions

Three microsensors based on carbon nanopowder and inulins: Frutafit HD (HD), Frutafit TEX (TEX), and Inutec (IN) were used for the assay of the thyroid hormones (L-T_3 and L-T_4) from whole blood samples. For the assay of L-T_4, the best response characteristic were obtained for the IN based microsensor, while the highest sensitivity for the assay of L-T_3 (0.935nA/μmol*L^{-1}) was recorded when the microsensor was based on TEX. The best selectivity was obtained when TEX

was used in the design of the microsensors, although the accuracy of the assay of the hormones is dependent on their ratio. The microsensors were used for the assay of thyroid hormones in whole blood samples with fair accuracy.

Acknowledgments

The authors are grateful for the financial support obtained from the program IDEAS Contract Nr. 123/05.10.2011, UEFISCDI.

The work has been funded by the Sectoral Operational Programme Human Resources Development 2007-2013 of the Ministry of European Funds through the Financial Agreement POSDRU/159/1.5/S/134398.

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