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Pattern recognition of estradiol, testosterone and dihydrotestosterone in children's saliva samples using stochastic microsensors

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Stochastic microsensors based on diamond paste and three types of electroactive materials (maltodextrin (MD), α -cyclodextrin (α -CD) and 5,10,15,20-tetraphenyl-21H,23H porphyrin (P)) were developed for the assay of estradiol (E₂), testosterone (T₂) and dihydrotestosterone (DHT) in children's saliva. The main advantage of utilization of such tools is the possibility to identify and quantify all three hormones within minutes in small volumes of childen's saliva. The limits of quantification obtained for DHT, T₂, and E₂ (1 fmol/L for DHT, 1 pmol/L for T₂, and 66 fmol/L for E₂) determined using the proposed tools allows the utilization of these new methods with high reliability for the screening of saliva samples from children. This new method proposed for the assay of the three hormones overcomes the limitations (regarding limits of determination) of ELISA method which is the standard method used in clinical laboratories for the assay of DHT, T₂, and E₂ in saliva samples. The main feature of its utilization for children's saliva is to identify earlier problems related to early puberty and obesity.

ormones are natural substances formed in the body, with the role of chemical messengers released by endocrine glands, which can act on target cells from a distance. Besides cell communication, hormones are indispensable for complete and harmonious human growth. Three of the most important sex steroid hormones are 17β -estradiol (E_2), testosterone (T_2) and 5α -dihydrotestosterone (DHT). These derivates of cholesterol are responsible for the proper development of sexual characteristics and numerous essential processes in human development and reproduction. Clegg et al found a correlation between the steroid hormones and obesity¹⁻⁴.

The accurate measurement of the sex hormones in biological samples is important in evaluating ovarian, prostate and testicular function^{5,6}. E_2 is checked in clinical laboratories for female infertility and ovarian tumor diagnosis, therefore E_2 is a clinically important analyte⁷. DHT is a biomarker for benign prostatic hyperplasia (BPH) and for prostatic cancer (PCa). DHT formation is inhibited by the 5α -reductase inhibitors, so it is essential to measure the T_2 levels in the body.

In children, measurement of serum E_2 and T_2 is used in the clinical diagnosis of precocious or delayed puberty in girls and boys, respectively^{8,9}. In recent years, the use of saliva as a non-invasive alternative matrix to measure steroid hormones has gained attention¹⁰, though there are concerns about specificity and sensitivity of classical immunological (including ELISA) or liquid chromatography tandem mass spectrometry based methods for measuring the low androgen and estrogen levels in children's saliva¹¹. Therefore it is a real need to develop high sensitive and selective methods that can be used for reliable assay of steroid hormones in children's saliva.

Stochastic microsensors represent a modern tool for qualitative and quantitative analysis of targeted analytes, with a better sensitivity and selectivity than classical electrochemical sensors. Their utilization in biomedical analysis represents a good alternative to chromatographic methods^{12–22}. Stochastic response is based on channel conductivity, according to the mechanism described previously by Stefan-van Staden and Moldoveanu²¹. The stochastic sensors are highly selective for the assay of biological substances, because the value of t_{off} (known as signature of the analyte) depends on many parameters of the analyte such as: geometry and size of molecule,



capacity of unfolding, velocity related to the speed of passing through the channel; accordingly, there will be difficult to find two molecules that will have the same signature^{21,23,24}.

In this paper we propose three tools based on stochastic microsensors designed using diamond paste and three different electroactive materials: maltodextrin (MD), α -cyclodextrin (α -CD) and 5,10,15,20-tetraphenyl-21H,23H porphyrin (P), for the assay of steroid hormones in saliva samples from children. Diamond powder is used as a matrix in the design of stochastic microsensors, due to its particular electrochemical proprieties, such as wide potential range, low background current and the ability of reaching low detection limits^{13,22}. The monocrystalline form of diamond was preferred due to the diamond monocrystal properties proprieties, such as enhanced holes and electron mobilities ^{25,26}. Monocrystallin diamond paste sensors have been used in differential potentiometric voltammetry mode for the assay of biological compounds such as: L-fucose and Dfucose¹⁵, sildenafil citrate^{13,14}, and neurotransmitters¹⁶⁻¹⁹. To our knowledge, the approach has not been used previously to assay steroid hormones in biological matrices such as saliva. Here we show that the proposed method and tools can be reliable used for the assay of the three hormones in children's saliva, and can be employed in clinics for early detection and prevention of problems related to children such as early puberty, endocrin disorders, and obesity.

Experimental

Materials and reagents. All chemicals were of analytical grade. 17β-estradiol (E_2), testosterone (T_2) and 5α-dihydrotestosterone (DHT), maltodextrin (MD), α-cyclodextrin (α-CD) and 5,10,15,20-tetraphenyl-21H,23H porphyrin (P), natural monocrystalline diamond powder were purchased from Sigma Aldrich (Milwaukee, USA) and paraffin oil (d_4^{20} , 0,86 gxcm⁻¹) from Fluka (Buchs, Switzerland).

The hormones solutions were firstly dissolved in dimethylsulfoxide (DMSO), with a concentration of 10 mmol $L^{-1}\,T_2$ and DHT and 6.59 mmol $L^{-1}\,E_2$. For the preparation of solutions with different concentrations (10 $^{-16}$ mol $L^{-1}-10^{-4}$ mol L^{-1}), we used deionized water and the serial dillution technique.

Apparatus and methods. All measurements were performed with an AUTOLAB/PGSTAT 12 (Utrecht, The Netherlands) connected to a personal computer with a GPES software, used to record the measurements. A three electrode system electrochemical cell was employed. Ag/AgCl (0.1 mol $\,\mathrm{L}^{-1}\,$ KCl) electrode serves as a reference electrode in the cell and a platinum wire as a counter electrode in the cell, respectively.

Design of stochastic microsensors. Natural monocrystalline diamond powder was mixed with paraffin oil until a homogenous paste was formed. 25 μL of 10^{-3} mol/L electroactive material solution (maltodextrin (MD), $\alpha\text{-cyclodextrin}$ ($\alpha\text{-CD}$) and 5,10,15,20-tetraphenyl-21H,23H porphyrin (P)) were added to 100 mg of the paste to give the modified diamond pastes. Three plastic tubes (100 μm inner diameter) were filled with the three modified pastes and the electric contact was obtained by inserting a silver (0.5 mm in diameter) wire into the paste. Schematic representation of the microsensors is presented in Figure 1. Before each measurement, the microsensors were cleaned with deionized water. When not in use, they were kept at room temperature, in a dry place.

Samples. Saliva samples were obtained from children aged 4–10 years (6 boys, 6 girls) collected at the Universitary Hospital in Bucharest (ethics committee approval nr. 11/2013). Saliva collection was done in the morning around 8 am before eating or drinking for all subjects included in the study. Saliva sampling was performed following a mouth rinse with 5 ml of water to wash out any debris or exfoliated cells. From each subject around 1 ml of unstimulated whole saliva was collected. The sample was divided in two: one part was used for the assay of hormones using stochastic sensing, and the

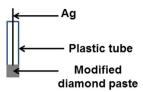


Figure 1 | Schematic representation of the stochastic microsensor.

other one was centrifuged at 2000 rpm for 10 min, and the three hormones were analysed using a standard method (see below).

Standard method. 5α -dihydrotestosterone, testosterone and estradiol were analysed using an enzyme immunoassay quantitative (ELISA) kit for DHT, E_2 and T_2 (IBL International GMBH, Hamburg, Germany) following manufacturer instructions. Briefly, 0.2 mL saliva samples and manufacturer provided standards were pipetted into wells precoated with an antibody specific for the tested hormone. After enzyme conjugate addition the wells were incubated at room temperature for one hour. Following a washing step with the provided buffer a substrate solution was added to wells. The color developed in proportion to the amount of the hormone bound in the first step. When color development was stopped, the optical density was determined using a microplate reader set to 450 nm. The limits of determination using ELISA where: 25 pg/mL for 5α -dihydrotestosterone, 6.4 pg/mL for testosterone and and 1 pg/mL for estradiol.

Results and discussion

Response characteristics of stochastic microsensors. The diagrams obtained when a potential of 125 mV was applied were specific for stochastic sensors. The response of the proposed microsensors was based on channel conductivity: the current flowing through a channel under an applied potential of 125 mV is altered when DHT, E2, and T2 are binding on the channel wall. The molecular recognition of the hormones is taking part in two stages²¹: stage 1 (molecular recognition stage) on which the hormone (DHT, E2, and T₂) extracted from the solution into the membrane-solution interface is blocking the channel, and the intensity of the current is 0 for a certain period of time named signature of the analyte (toff). The value of $t_{\rm off}$ is used for the qualitative assay of DHT, E_2 , and T_2 in the diagrams obtained for children's saliva analysis. When DHT, E2, and T₂ are interacting with the wall of the channel (Stage 2, bounding stage), the following equilibrium equations (equations (1), (2), (3)) are taking place:

$$Ch_{(i)} + DHT_{(i)} \Leftrightarrow Ch \bullet DHT_{(i)}$$
 (1)

$$Ch_{(i)} + E_{2(i)} \Leftrightarrow Ch \bullet E_{2(i)}$$
 (2)

$$Ch_{(i)} + T_{2(i)} \Leftrightarrow Ch \bullet T_{2(i)}$$
 (3)

where Ch is the channel, and i is the interface. The time of equilibrium for interaction with the channel process is defined as ton and is used for the quantitative assay of DHT, E2, and T2.

Signatures of DHT, E_2 , and T_2 (t_{off} values) are shown in Table 1. Standard solutions of each hormone, in the concentration range 0.1 fmol L^{-1} – 0.1 mmol L^{-1} , were analysed to obtain the calibration equations for all three diamond paste based microsensors. The equations of calibration with the correlation coefficients, the linear concentration ranges, the sensitivities, and the limits of determination for DHT, E_2 and T_2 are shown in Table 1.

The microsensor based on MD showed the highest sensitivity for the assay of DHT ($2.65 \times 10^{11} \text{ s mol}^{-1} \text{ L}$) and T₂ ($1.95 \times 10^8 \text{ s mol}^{-1}$ L), while for the assay of E₂ the highest sensitivity was obtained using



Microsensors based on diamond paste and	Calibration equation and correlation coefficient (r)	Linear concentration range (mol/L)	٠	Sensitivity (s/mol L-1)	Limit of detection(fmol L ⁻¹)
<u> </u>	correlation coefficient (r)	(MOI/ L)	t _{off}	(S/ MOLL -)	delection(intol L)
DHT	$1/t_{on} = 0.05 + 1.86(\pm 0.35) \times 10^{10} \times c$ r = 0.9853	$10^{-14} - 10^{-12}$	4.0	1.86×10^{10}	10
MD	$1/t_{on} = 0.08 + 2.65(\pm 0.15) \times 10^{11} \times c$ r = 0.9996	$10^{-15} - 10^{-13}$	1.8	2.65×10^{11}	1
α-CD	$1/t_{on} = 0.05 + 6.92(\pm 0.21) \times 10^8 \times c$ r = 0.9825	$10^{-13} - 10^{-11}$	5.1	6.92×10^{8}	100
T ₂					
P	$1/t_{on} = 0.065 + 1.27(\pm 0.15) \times 10^6 \times c$ r = 0.9942	$10^{-10} - 10^{-8}$	5.3	1.27×10^{6}	100
MD	$1/t_{on} = 0.061 + 1.95(\pm 0.12) \times 10^8 \times c$ r = 0.9989	$10^{-12} - 10^{-10}$	3.5	1.95×10^{8}	1
α-CD	$1/t_{on} = 0.069 + 1.27(\pm 0.09) \times 10^{7} \times c$ r = 0.9999	$10^{-11} - 10^{-9}$	3.1	1.27×10^7	10
E ₂					
P	$1/t_{on} = 0.03 + 2.51(\pm 0.21) \times 10^{9} \times c$ r = 0.9987	$6.59 \times 10^{-14} - 6.59 \times 10^{-12}$	7.4	2.51×10^{9}	66
MD	$1/t_{on} = 0.02 + 3.50(\pm 0.23) \times 10^5 \times c$ r = 0.9966	$6.59 \times 10^{-11} - 6.59 \times 10^{-8}$	8.5	3.50×10^5	66000
α-CD	$1/t_{on} = 0.06 + 1.83(\pm 0.12) \times 10^{9} \times c$ r = 0.9976	$6.59 \times 10^{-14} - 6.59 \times 10^{-12}$	5.2	1.83×10^{9}	66

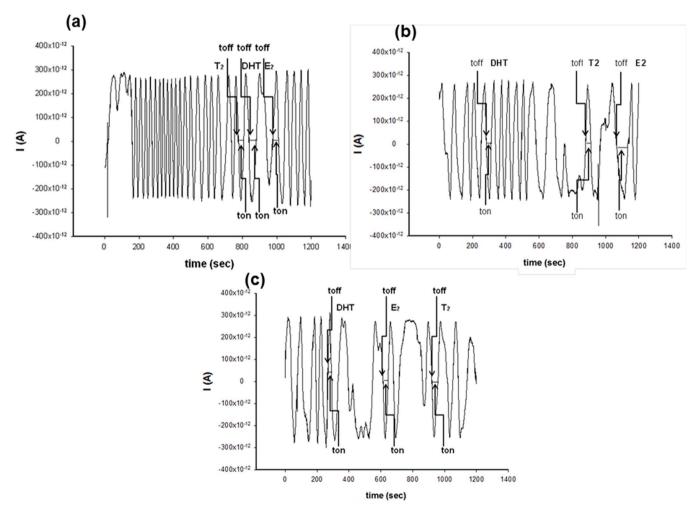


Figure 2 | Pattern recognition of the hormones in saliva sample using the stochastic microsensors based on (a) porphyrin and diamond paste; (b) maltodextrin and diamond paste; and (c) α -cyclodextrin and diamond paste.



Table 2 Determination of T2, DHT, and E2 in children's saliva using stochastic microsensors. All results are in pg/mL	ation of T2, Dł	-Tr, and E2 in	children's saliv	va using stoche	astic microsen	sors. All results	are in pg/mL					
Microsensors based	_	2	က	4	5	9	7	∞	6	10	11	12
on alamona paste and	ш	ъ	ш	ъ	ш	ш	×	٤	×	₹	\$	×
T₂ «-CD/DP	3.1 + 0.1	0.36 ± 0.09	1.2 + 0.1	1.3 + 0.1	က +I	+1	က +I	+1	+1		+1	1.1 + 0.1
P/DP	3.3 ± 0.2	0.36 ± 0.08	2.2 ± 0.1	1.7 ± 0.2	3.9 ± 0.3	1.9 ± 0.2	2.7 ± 0.2	1.9 ± 0.2	1.3 ± 0.2	1.4 ± 0.2	1.7 ± 0.1	1.3 + 0.1
MD/DP	3.2 ± 0.1	0.34 ± 0.08	1.9 ± 0.2	1.1 + 0.1	3 + 0	+1	.2 +1	+1	+1		+1	+1
ELISA	۵,	0.70 ± 0.25	1.1 ± 0.9	0.4 ± 0.2	. 4	+1) +I	+1	+1	+1	6	+1
ᇤ												
∞-CD/DP		0.30 ± 0.04	0.30 ± 0.02	$0.20 \pm 0.$	0.10 ± 0.01	± 0.02	0.30 ± 0.02	30 +	+1	10	+1	+1
	0.20 ± 0.03	0.20 ± 0.01	0.40 ± 0.02	$0.20 \pm 0.$	+1	± 0.02	+1	0.20 ± 0.02	+1	+1	+1	+1
		0.30 ± 0.02	0.30 ± 0.01	0.10 ± 0.02	0.10 ± 0.01	.02	0.30 ± 0.02	30 +	0.20 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.30 ± 0.01
	۵,	۵,	0.20 ± 0.12	0.40 ± 0.23	0.20 ± 0.12		0.40 ± 0.15	0.70 ± 0.23	+1	0.40 ± 0.13	+1	+1
F 2												
α-CD/DP	1.9 ± 0.1	2.9 ± 0.3	1.1 ± 0.1	1.1 ± 0.2	က +I	+1	+1	+1	+1	+1	+1	+1
P/DP	1.7 ± 0.2	2.3 ± 0.3	1.0 ± 0.1	1.0 ± 0.2	.2 +	+1	+1	+1	+1	+1	+1	+1
MD/DP	1.1 ± 0.2	2.7 ± 0.3	1.2 ± 0.2	1.5 ± 0.2	2.6 ± 0.2	1.5 ± 0.2	1.6 ± 0.2	1.4 ± 0.1	0.70 ± 0.02	1.0 ± 0.1	0.80 ± 0.03	1.6 ± 0.2
ELISA∘	1.8 ± 0.7	1.7 ± 0.9	1.3 ± 0.8	1.1 ± 0.8	.5	+1	+1	+1	+1	+1	1.0 ± 0.5	+1
°ELISA (standard method). bThe standard method (ELISA) could not defermine the analyte.	A) could not determir	ne the analyte.										

the microsensor based on porphyrin (2.51 \times 10° s mol⁻¹ L). The lowest determination limits were obtained using MD based microsensor for the assay of DHT (1 fmol/L), and T₂ (1 pmol/L), while for the assay of E₂ the lower limit of determination was achieved by using the porphyrin and the α -CD based microsensors (66 fmol/L).

A high reproducibility of all miscrosensors was recorded, RSD (%) values of the slopes obtained for the equations of calibration (Table 1), when the microsensors were used for 6 months dayly, were less than 1%. Also, the design was highly reproducible: three pastes of each microsensors were made and tested each for a period of three months, and in each case, the RSD (%) values were less than 0.01%.

Analytical applications. Pattern recognition of the three hormones in saliva samples from 6 boys and 6 girls (age 4-10) was done using stochastic microsensors, based on their signatures shown in Table 1. Patterns were recorded for each saliva sample, followed by the identification of signature (toff values) of each hormone, and its quantification (based on ton values) using the equation of calibration of the stochastic sensors. Examples of pattern recorded using stochastic sensors are given in Figure 2. There is a good correlation between the results obtained using the stochastic sensors, for each sample analysed (Table 2). The standard method (ELISA) was not able to determined accurately the hormones, all amounts being under its limit of determination. Therefore, the problem that the pattern recognition with stochastic microsensors solved is: the assay at very low concentration of DHT, T₂, and E₂ from childen's saliva samples. The proposed method is more accurate and reliable (RSD, % values are far lower in the case of the proposed method than those obtained for standard method) when used for very low concentrations compared with ELISA and can be reliable used for the assay of the hormones in saliva samples.

Conclusions

The paper proposed three stochastic microsensors based on modified diamond paste with porphyrin, maltodextrin and α-cyclodextrin for the assay of estradiol (E_2) , testosterone (T_2) and dihydrotestosterone (DHT) in saliva. The limits of quantification of the hormones are far lower than those recorded for the ELISA - standard method, which made them available for the assay of the hormones in the children saliva (the amounts of these hormones in children saliva are far lower than in the adults saliva, and frequently under the limit of quantification of the ELISA standard method). The pattern recognition of the three hormones in saliva samples was performed by identification of the signatures of the hormones in the patterns recorded using stochastic sensors. The quantification was reliable; very good correlations between the results were obtained by utilization of the three stochastic microsensors. The proposed pattern recognition method performed using stochastic microsensors has great future for analysis of such compounds in children saliva, making possible early detection of hormone levels and related disorders such as early onset or delayed puberty, obesity and endocrine diseases.

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Author contributions

R.I.S.vS., L.A.G., J.L. and B.C. conducted all experiments. All authors contributed to the design of the assay and discussed the results. R.I.S.vS. and L.A.G. were the primary authors of the manuscript; J.L. and B.C. commented on the manuscript at all stages. All authors reviewed the article.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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