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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 (E_2), testosterone (T_2) 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, T_2, and E_2 (1 fmol/L for DHT, 1 pmol/L for T_2, and 66 fmol/L for E_2) 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, T_2, and E_2 in saliva samples. The main feature of its utilization for children's saliva is to identify earlier problems related to early puberty and obesity.

Hormones 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 obesity[1–4].

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 analyte[7]. 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 attention[10], 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 saliva[11]. 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 Moldoveanu[21]. 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 D-fucose[15], sildenafil citrate[13,14], and neurotransmitters[16–19]. 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 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), α-cyclodextrin (α-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 other one was centrifuged at 2000 rpm for 10 min, and the three hormones were analysed using a standard method (see below).

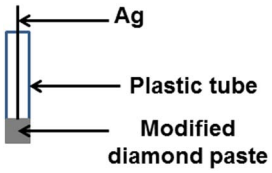

Figure 1 | Schematic representation of the stochastic microsensor.

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, E_2, and T_2 are binding on the channel wall. The molecular recognition of the hormones is taking part in two stages[21]: stage 1 (molecular recognition stage) on which the hormone (DHT, E_2, and T_2) 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 (t_{off}). The value of t_{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, E_2, and T_2 are interacting with the wall of the channel (Stage 2, bounding stage), the following equilibrium equations (equations (1), (2), (3)) are taking place:

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

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

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

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 (2.65×10^{11} s mol^{-1} L) and T_2 (1.95×10^{8} s mol^{-1} L), while for the assay of E_2 the highest sensitivity was obtained using

Table 1 | Response characteristics of stochastic microsensors used for the assay of DHT, T_2 and E_2

Microsensors based on diamond paste and	Calibration equation and correlation coefficient (r)	Linear concentration range (mol/L)	t_{off}	Sensitivity (s/mol L^{-1})	Limit of detection(fmol L^{-1})
DHT					
P	$1/t_{on} = 0.05 + 1.86(\pm0.35) \times 10^{10} \times c$ r = 0.9853	$10^{-14} - 10^{-12}$	4.0	1.86×10^{10}	10
MD	$1/t_{on} = 0.08 + 2.65(\pm0.15) \times 10^{11} \times c$ r = 0.9996	$10^{-15} - 10^{-13}$	1.8	2.65×10^{11}	1
α-CD	$1/t_{on} = 0.05 + 6.92(\pm0.21) \times 10^{8} \times c$ r = 0.9825	$10^{-13} - 10^{-11}$	5.1	6.92×10^{8}	100
T_2					
P	$1/t_{on} = 0.065 + 1.27(\pm0.15) \times 10^{6} \times c$ r = 0.9942	$10^{-10} - 10^{-8}$	5.3	1.27×10^{6}	100
MD	$1/t_{on} = 0.061 + 1.95(\pm0.12) \times 10^{8} \times c$ r = 0.9989	$10^{-12} - 10^{-10}$	3.5	1.95×10^{8}	1
α-CD	$1/t_{on} = 0.069 + 1.27(\pm0.09) \times 10^{7} \times c$ r = 0.9999	$10^{-11} - 10^{-9}$	3.1	1.27×10^{7}	10
E_2					
P	$1/t_{on} = 0.03 + 2.51(\pm0.21) \times 10^{9} \times c$ r = 0.9987	$6.59 \times 10^{-14} - 6.59 \times 10^{-12}$	7.4	2.51×10^{9}	66
MD	$1/t_{on} = 0.02 + 3.50(\pm0.23) \times 10^{5} \times c$ r = 0.9966	$6.59 \times 10^{-11} - 6.59 \times 10^{-8}$	8.5	3.50×10^{5}	66000
α-CD	$1/t_{on} = 0.06 + 1.83(\pm0.12) \times 10^{9} \times c$ r = 0.9976	$6.59 \times 10^{-14} - 6.59 \times 10^{-12}$	5.2	1.83×10^{9}	66

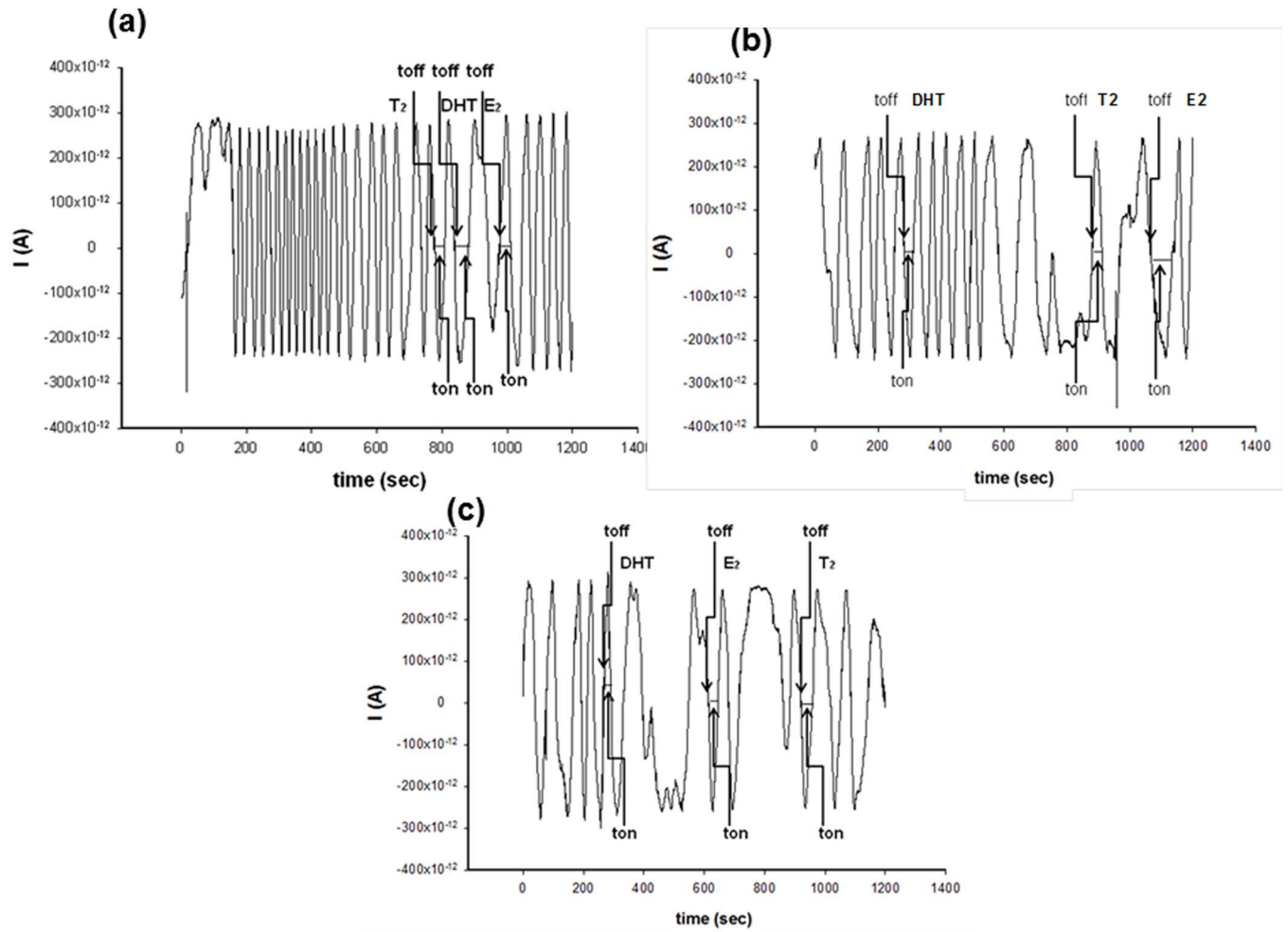

Figure 2 | Pattern recognition of the hormones in saliva sample using the stochastic microsensors based on (a) porphyrin and diamond paste; (b) maltodextrin and diamond paste; and (c) α-cyclodextrin and diamond paste.

Table 2 | Determination of T2, DHT, and E2 in children's saliva using stochastic microsensors. All results are in pg/mL

Microsensors based on diamond paste and	1	2	3	4	5	6	7	8	9	10	11	12
	F	F	F	F	F	F	M	M	M	M	M	M
T_2												
α-CD/DP	3.1 ± 0.1	0.36 ± 0.09	1.2 ± 0.1	1.3 ± 0.1	3.3 ± 0.1	1.7 ± 0.2	2.3 ± 0.1	2.1 ± 0.1	1.0 ± 0.2	1.1 ± 0.2	1.7 ± 0.2	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	3.3 ± 0.1	1.1 ± 0.1	2.2 ± 0.1	1.8 ± 0.2	1.1 ± 0.1	1.2 ± 0.1	1.6 ± 0.1	1.5 ± 0.2
ELISA[a]	[b]	0.70 ± 0.25	1.1 ± 0.9	0.4 ± 0.2	0.6 ± 0.2	0.9 ± 0.5	1.0 ± 0.4	1.4 ± 0.5	1.1 ± 0.4	0.5 ± 0.3	0.6 ± 0.3	0.3 ± 0.2
DHT												
α-CD/DP	0.30 ± 0.03	0.30 ± 0.04	0.30 ± 0.02	0.20 ± 0.02	0.10 ± 0.01	0.30 ± 0.02	0.30 ± 0.02	0.30 ± 0.02	0.30 ± 0.01	0.10 ± 0.01	0.20 ± 0.01	0.20 ± 0.02
P/DP	0.20 ± 0.03	0.20 ± 0.01	0.40 ± 0.02	0.20 ± 0.02	0.10 ± 0.01	0.40 ± 0.02	0.20 ± 0.01	0.20 ± 0.02	0.20 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.10 ± 0.02
MD/DP	0.20 ± 0.02	0.30 ± 0.02	0.30 ± 0.01	0.10 ± 0.02	0.10 ± 0.01	0.40 ± 0.02	0.30 ± 0.02	0.30 ± 0.01	0.20 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.30 ± 0.01
ELISA[a]	[b]	[b]	0.20 ± 0.12	0.40 ± 0.23	0.20 ± 0.12	0.50 ± 0.21	0.40 ± 0.15	0.70 ± 0.23	0.80 ± 0.21	0.40 ± 0.13	1.10 ± 0.21	0.30 ± 0.15
E_2												
α-CD/DP	1.9 ± 0.1	2.9 ± 0.3	1.1 ± 0.1	1.1 ± 0.2	2.3 ± 0.2	2.2 ± 0.2	1.8 ± 0.2	1.7 ± 0.2	0.20 ± 0.01	1.0 ± 0.2	0.80 ± 0.03	0.9 ± 0.1
P/DP	1.7 ± 0.2	2.3 ± 0.3	1.0 ± 0.1	1.0 ± 0.2	2.2 ± 01	2.0 ± 0.2	1.4 ± 0.1	1.5 ± 0.2	0.60 ± 0.02	1.3 ± 0.1	1.00 ± 0.03	1.0 ± 0.2
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[a]	1.8 ± 0.7	1.7 ± 0.9	1.3 ± 0.8	1.1 ± 0.8	1.5 ± 0.9	2.0 ± 0.9	1.3 ± 0.7	1.7 ± 0.8	0.50 ± 0.15	1.1 ± 0.4	1.0 ± 0.5	0.9 ± 0.4

[a]ELISA (standard method).
[b]The standard method (ELISA) could not determine the analyte.

the microsensor based on porphyrin (2.51×10^9 s mol^{-1} L). The lowest determination limits were obtained using MD based microsensor for the assay of DHT (1 fmol/L), and T_2 (1 pmol/L), while for the assay of E_2 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 (t_{off} values) of each hormone, and its quantification (based on t_{on} 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, T_2, and E_2 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.

1. Brown, L. M., Gent, L., Davis, K. & Clegg, D. J. Metabolic impact of sex hormones on obesity. *Brain Res.* **1350**, 77–85 (2010).
2. Clegg, D. J. *et al.* Sex hormones determine body fat distribution and sensitivity to adiposity signals. *Appetite* **40**, 324 (2003).
3. Clegg, D. J., Riedy, C. A., Smith, K. A., Benoit, S. C. & Woods, S. C. Differential sensitivity to central leptin and insulin in male and female rats. *Diabetes* **52**, 682–687 (2003).
4. Clegg, D. J., Brown, L. M., Woods, S. C. & Benoit, S. C. Gonadal hormones determine sensitivity to central leptin and insulin. *Diabetes* **55**, 978–987 (2006).
5. Haymond, S. & Gronowski, A. M. Reproductive related disorders. in: Burtis, C. A., Ashwood, E. R., Bruns, D. E. (Eds.), *Tietz Textbook of Clinical Chemistry and Molecular Diagnostics*, 4th Ed., Elsevier Saunders, St. Louis, 2097–2152 (2006).
6. Yamashita, K. *et al.* Development of highly sensitive quantification method for testosterone and dihydrotestosterone in human serum and prostate tissue by liquid chromatography-electrospray ionization tandem mass spectrometry. *Steroids* **74**, 920–926 (2009).

7. Zhao, L., Lin, J. M., Li, Z. & Ying, X. Development of a highly sensitive, second antibody format chemiluminescence enzyme immunoassay for the determination of 17β-estradiol in wastewater. *Anal. Chim. Acta* **558**, 290–295 (2006).
8. Cesario, S. K. & Hughes, L. A. Precocious Puberty: A Comprehensive Review of Literature. *JOGNN* **36**, 263–274 (2007).
9. Zawatski, W. & Lee, M. M. Male pubertal development: are endocrine-disrupting compounds shifting the norms? *J. Endocrinol*, **218**, R1–R12 (2013).
10. Groschl, M. Current status of salivary hormone analysis. *Clin. Chem.* **54**, 1759–1769 (2008).
11. Albrecht, L. & Styne, D. Laboratory testing of gonadal steroids in children. *Pediatr. Endocrinol. Rev.* **5** Suppl 1, 599–607 (2007).
12. Stefan-van Staden, R. I., Moldoveanu, I., Sava, D. F., Kapnissi-Christodoulou, C. & van Staden, J. F. Enantioanalysis of pipecolic acid with stochastic and potentiometric microsensors. *Chirality* **25**, 114–118 (2013).
13. Stefan-van Staden, R. I., van Staden, J. F. & Aboul-Enein, H. Y. Diamond paste-based electrodes for the determination of sildenafil citrate (Viagra). *J. Solid State Electrochem.* **14**, 997–1000 (2010).
14. Balasoiu, S. C. *et al.* Amperometric dot-sensors based on zinc porphyrins for sildenafil citrate determination. *Electrochim. Acta* **58**, 290–295 (2011).
15. Stefan-van Staden, R. I., Nejem, R. M., van Staden, J. F. & Aboul-Enein, H. Y. Determination of L- and D-fucose using amperometric electrodes based on diamond paste. *Analyst* **137**, 903–909 (2012).
16. Stefan-van Staden, R. I., Moldoveanu, I. & van Staden, J. F. Pattern recognition of neurotransmitters using multimode sensing. *J. Neurosci. Meth.* **229**, 1–7 (2014).
17. Balasoiu, S. C., Stefan-van Staden, R. I., van Staden, J. F., Pruneanu, S. & Radu, G. L. Carbon and diamond paste microelectrodes based on Mn (III) porphyrins for the determination of dopamine. *Anal. Chim. Acta* **668**, 201–207 (2010).
18. Gugoasa, L. A., Stefan-van Staden, R. I., Ciucu, A. A. & van Staden, J. F. Influence of physical immobilization of dsDNA on carbon based matrices of electrochemical sensors. *Curr. Pharm. Anal.* **10**, 20–29 (2014).
19. Stefan-van Staden, R. I., Balasoiu, S. C. & van Staden, J. F. Stochastic Dot Microsensors for the assay of dopamine in pharmaceutical samples and biological fluids. *J. Electrochem. Soc.* **159**, B839–B844 (2012).
20. Stefan-van Staden, R. I. & van Staden, J. F. New tool for screening of whole blood for early detection of breast cancer antigen(CA 153). *J. Mol. Med. Chem.* **1**, 86–91 (2013).
21. Stefan-van Staden, R. I. & Moldoveanu, I. Stochastic microsensors based on nanostructured materials used in the screening of whole blood for hepatitis B. *J. Electrochem. Soc.* **161**, B3001–B3005 (2014).
22. Stefan, R. I. & Bairu, S. G. Monocrystalline diamond paste-based electrodes and treir applications for the determination of Fe (II) in vitamins. *Anal. Chem.* **75**, 5394–5398 (2003).
23. Gu, L. Q., Bahra, O., Conlan, S., Cheley, S. & Bayley, H. Stochastic sensing of organic analytes by pore-forming protein containing a molecular adaptor. *Nature* **398**, 686–690 (1999).
24. Liu, A., Zhao, Q. & Guan, X. Stochastic nanopore sensors for the detection of terrorist agents: current status and challenges. *Anal. Chim. Acta* **675**, 106–115 (2010).
25. De Heus, P. R. The applications and properties of monocrystal. *Ind. Diam. Rev.* **57**, 15–18 (1997).
26. Nebel, C. E. *et al.* Low temperature properties of the p-type surface conductivity of diamond *Diamond. Relat. Mater.* **11**, 351–354 (2002).

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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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