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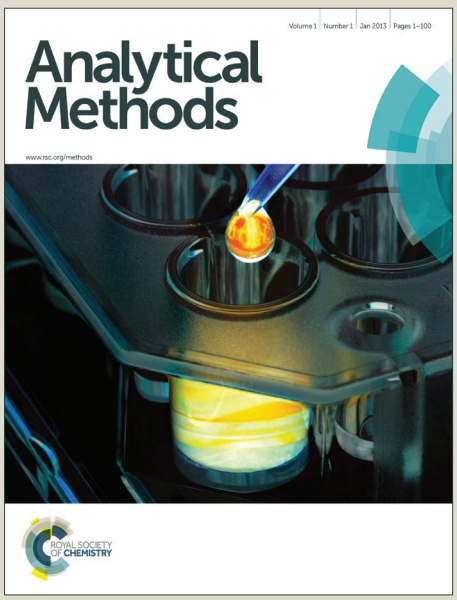

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Electrochemical determination of selected neurotransmitters at electrodes modified with oppositely charged carbon nanoparticles

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Abstract

The electrocatalytic oxidation of neurotransmitters on the electrodes modified with oppositely charged carbon nanoparticles have been investigated. These nanoparticles were deposited at the electrode from the aqueous suspensions *via* a layer-by-layer method. The electrocatalytic response was evaluated with cyclic voltammetry, differential pulse voltammetry, and chronoamperometry. The modified electrode exhibited good electrocatalytic properties towards, not only dopamine oxidation, but also for epinephrine and serotonin oxidation. This allows to separate their voltammetric signals from the signals of interfering substances such as ascorbic acid or uric acid. The obtained calibration curves are in the range 0.4-350 μM, 1–49 μM and 0.8–100 μM with a detection limit of 0.4 μM, 1.0 μM and 0.8 μM for dopamine, epinephrine and serotonin, respectively. In addition these carbon nanoparticulate electrodes show excellent sample-to-sample reproducibility (the relative standard deviations for n = 7 equal 0.7 %) and, maintained 94 % of electrochemical signal corresponding to dopamine oxidation after 18 months storage.

***Keywords**:* Neurotransmitters, Carbon film electrode; Carbon nanoparticles; Layer-by-layer; Sensor;

1. Introduction

Neurotransmitters are chemical substances which are responsible for communication between nerve cells. Dopamine (DA), epinephrine (EP) and serotonin (5-HT) are among the most abundant neurotransmitters. Although their concentration in most of the body is very low (normal levels in serum are $< 8.9\times10^{-10}$ mol/dm^3, 1.1-1.4 $\times10^{-8}$ mol/dm^3, and 4.5-12 $\times10^{-7}$ mol/dm^3 for DA, EP and 5-HT, respectively[1]) they have a significant impact on human endocrine and immune systems. Lack of balance between them may increase the risk of developing diseases such as Parkinson's, Alzheimer's, Schizophrenia, various neuroblastoma, adrenocortical carcinoma, pituitary adenoma or depression [2, 3]. In order to diagnose these diseases an assay of neurotransmitters is highly desirable. Currently, a wide range of techniques such as chromatographic methods [4], electrophoresis [5], electrochemical methods [6], fluorimetry [7], and mass spectroscopy [8] are applied for detection of DA, EP and 5-HT (or their metabolites [9]) in real samples (serum or urine) [10-13]. The electrochemical methods are simple, highly selective and sensitive, cheap and the electrochemical devices are easy to miniaturize. These advantages make them suitable for clinical analysis. However, the main drawback of the electrochemical methods is the overlap of the electrochemical signal of neurotransmitters with the signal of some interfering substances such as ascorbic acid (AA) or uric acid (UA) which are present in high concentrations: AA (0.1-0.6 mM) in the extracellular fluid of the brain [14], UA in the blood (0.15-0.45 mM) or excreted in urine (1.19-2.98 mmol/day) [15]. This problem can be solved by electrode modification with enzymes from the oxidoreductase group e.g laccase [16], and/or nanomaterials [11, 17-20] - which exhibit electrocatalytic activity towards oxidation of phenolic compounds (DA, EP) or aromatic amines (5-HT). This significantly improves the selectivity of electrochemical sensors for neurotransmitters. Although successful application of unmodified edge plane pyrolytic graphite electrodes for neurotransmitters' sensing [10] in the presence of interfering compounds was reported, but this material is expensive and not suitable for thin film preparation.

Among nanoparticulate materials successfully applied for DA, EP and 5-HT electrochemical sensing one can find mainly metal [17, 21], metal oxide [18] and carbon-based nanostructures [22-24]. The latter group has gained attention due to its remarkable electrochemical properties (electrocatalytic ability, superb electrical conductivity and high surface area). Carbon-based materials like carbon nanotubes [19, 25-27] or graphene [20, 28, 29] have been widely applied for selective electrochemical determination of DA, EP and 5-HT. Quite recently hydrophilic carbon nanoparticles (CNPs) with phenyl sulfonate functionalities as one of the

nanoparticulate forms of carbon were introduced for electrodes modification [30-33]. This material is commercially available from Cabot Corporation (Emperor 2000) and its production is based on diazonium [34] chemistry or controlled vapour-phase pyrolysis of hydrocarbons [35]. Unlike carbon nanotubes or graphene, it has been known for many years and it is widely used in industry, for example as a filler or a pigment. It offers most of the advantages of nanocarbons like extremely high surface area, high level of interfacial edge sites, reactive surface sites and good electrical conductivity.

In contrast to other nanocarbon materials CNPs form stable suspension in water, because of their hydrophilic functionalities. The negative charge of phenylsulphonate functional groups make them suitable for electrode surface modification with layer-by-layer procedure [33].

Their deposition at the electrode surface has been achieved for example by encapsulation in polymer [36], using electrostatic interactions with polyelectrolytes [30, 37, 38] or with positively charged objects (gold nanoparticles [39] and functionalized silicate submicrometer particle [40, 41]), sol-gel processed silicate film [31], or just by drop-coating the CNPs suspension on the electrode surface [42, 43]. Commercially available CNPs can be further functionalized to replace the sulfonate functionalities with positively charged ammonium groups. This modification allows the production of three dimensional nanoparticulate film electrodes [44], built entirely from carbon.

The CNPs based electrodes [45] were already applied for electrochemical sensing of biologically important substances: simultaneously acetaminophen and tramadol [42], naltrexone [43], azathioprine [46], piroxicam [47], dopamine in the presence of ascorbate [30, 41], and benzophenone or triclosan [48]. These electrodes also provides favorable conditions for efficient electron exchange between the electrode substrate and a wide range of redox enzymes [32, 33, 49, 50]. Recently one of these electrodes has been successfully applied as an anode in self-powered sensor for ascorbic acid detection [51].

Although films consisting CNPs linked with ionomer [30] or functionalized silicate submicroparticles [41] were earlier employed for DA detection, here we propose to apply a film electrode entirely composed of oppositely charged carbon nanoparticles for electrochemical determination of selected neurotransmitters: DA, EP and 5-HT in the presence of interfering substances. Although the detection limit of the obtained electrodes is too high for analysis of real human (serum) samples these electrodes can still, thanks to the wide analytical window, be applied for monitoring of neurotransmitter release from cells in the near field [52, 53]. In fact there

are no reported instances of DA detection using electrochemical methods that can measure DA on the level found in serum, with the exception of flow injection analysis. But this is a method that is not discriminating and requires prior separation of analytes using e.g. HPLC.

2. Experimental section

2.1. Chemicals and materials

DA, EP, UA, 5-HT were purchased from Sigma–Aldrich, AA was from Riedel-de Haën. H_3PO_4 and NaOH were from Chempur. Negatively charged CNPs (ca. 7.8 nm mean diameter, with a typical bulk density of 320 g dm^{-3}, Emperor 2000) were supplied by Cabot Corporation (Dukinfield, United Kingdom). These nanoparticles were used for preparation of positively charged CNPs following a procedure described earlier [44]. Indium tin dioxide (ITO) coated glass plates (resistivity 8-12 Ω·cm) were obtained from Delta Technologies Ltd., USA. Water was filtered and demineralized with an ELIX system (Millipore). All reagents were used as received.

2.2. Electrode modification

The carbon nanoparticles were immobilized onto indium tin oxide (ITO) covered glass sheets *via* layer-by-layer assembly [33]. Before the preparation the substrate was cleaned with ethanol, then with deionized water and finally heated for 30 minutes in a tube furnace (Barnstead International) at 500 °C in air to remove organic impurities. Suspensions of both types of particles were obtained by mixing 3 mg of particles with 1 ml of deionized water followed by sonication of the mixture for 1 hour. ITO slides was immersed alternately into the positively and negatively charged CNPs suspension for 1 minute. Every such step was followed by drying and immersion in pure water for 2 s to remove weakly bonded particles. The above procedure will be called one immersion and withdrawal step in this paper. The electrodes prepared by 1, 3, 5 and 10 alternative immersion and withdrawal steps will be marked CNP(+/-), CNP(3+/-), CNP(5+/-) and CNP(10+/-). The electrode surface was defined by masking the electrode with scotch tape so as to expose a circular area of 0.2 cm^2. Electric contact was assured by a piece of copper tape between a crocodile clip and the conducting side of the ITO glass.

2.3. Instrumentation and cell

Electrochemical experiments were done with an Autolab PGSTAT 30 (Metrohm Autolab) electrochemical system with GPES software in a conventional three electrode cell. Modified ITO, platinum wire (d = 0.5 mm) and Ag|AgCl|$KCl_{sat.}$ were used as the working, counter and reference electrode, respectively. All experiments were carried out at ambient temperature (22 ± 2 °C).

3. Results and Discussion

3.1 Electrochemical behaviour of dopamine at the electrode modified with carbon nanoparticles.

Fig. 1 shows cyclic voltammograms of dopamine at the electrodes modified with different number of layers of carbon nanoparticles (curve (b-e)) and at a bare ITO electrode (curve (a)) in 0.1 M phosphate buffer solution (pH 5.0) in the presence of interfering substances (AA and UA). The concentration of dopamine in this case is very high therefore these experiments were performed at pH 5.0 in order to avoid effect of DA polymerization. At bare the ITO electrode, only two poorly defined anodic peaks are visible indicating that the signals of DA and AA overlap. In contrast, three well defined oxidation peaks at about 0.2 V, 0.48 V and 0.58 V - corresponding to the oxidation of AA, DA, and UA, respectively are seen on voltammograms recorded at the carbon nanoparticulate electrodes. Obviously this material catalyzes the oxidation of AA and DA as can be judged by the negative shift of their peak potentials. This leads to better separation of their electrochemical signals. Reversible voltammatry is recorded for DA (see Supplementary Material Fig. F1, Fig. F2(A)). Similar results were observed earlier on other CNP modified electrodes where the protonated form of DA probably interacts via electrostatic interactions with negatively charged CNP, promoting accumulation of dopamine [14, 41]. Moreover, the value of the current density, at the CNP modified electrodes, is significantly higher than on bare ITO electrode. This result together with voltammetric signals separation indicates that CNPs create well developed electrode surface suitable for (bio)sensing [33, 49].

For number of deposition steps larger than three the magnitude of the anodic peaks current density increases only slightly and the peak positions stay basically the same (Fig. 1).

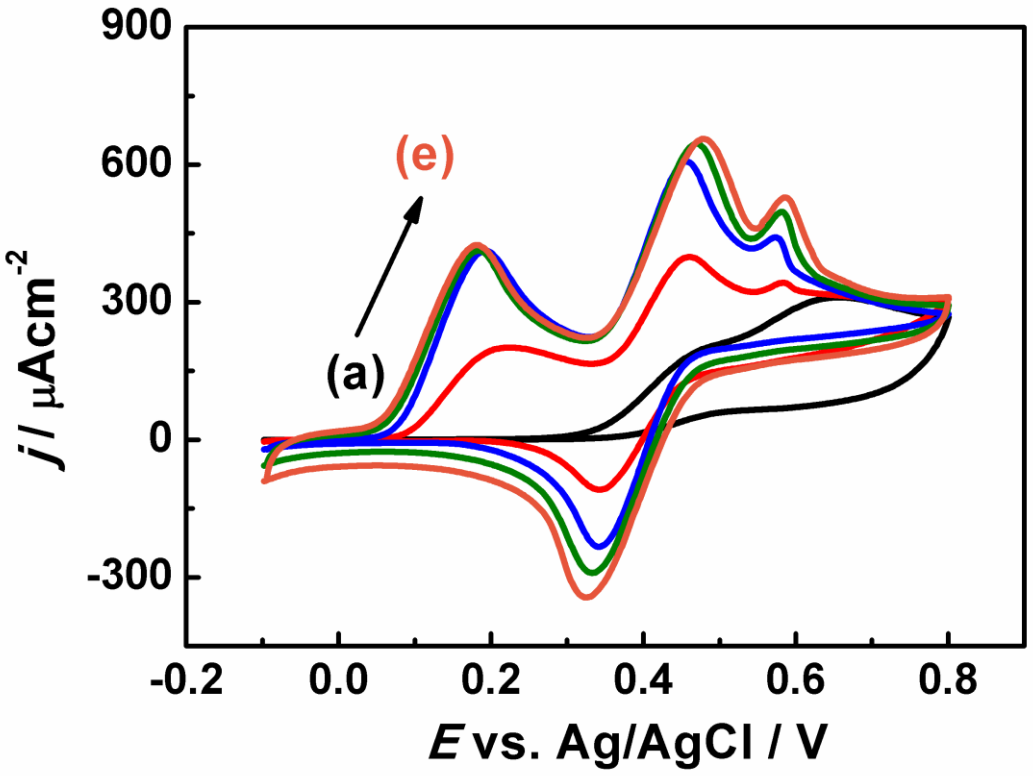

Fig. 1 Cyclic voltammograms obtained with (a) a bare ITO and (b) CNPs(+/-) (c) CNPs(3+/-) (d) CNPs(5+/-) and (e) CNPs(10+/-) electrodes in 2 mM AA, 2 mM DA, 1 mM UA in 0.1 M phosphate buffer pH 5.0. Scan rate 20 mV s^{-1}.

This confirms that already the CNPs(3+/-) electrode exhibits strong electrocatalytic effect offering high current density and signal separation and therefore it was selected for further experiments. From SEM images it was noted that this was the smallest number of steps that covers the whole electrode surface without leaving bare ITO [33] (Fig. 2).

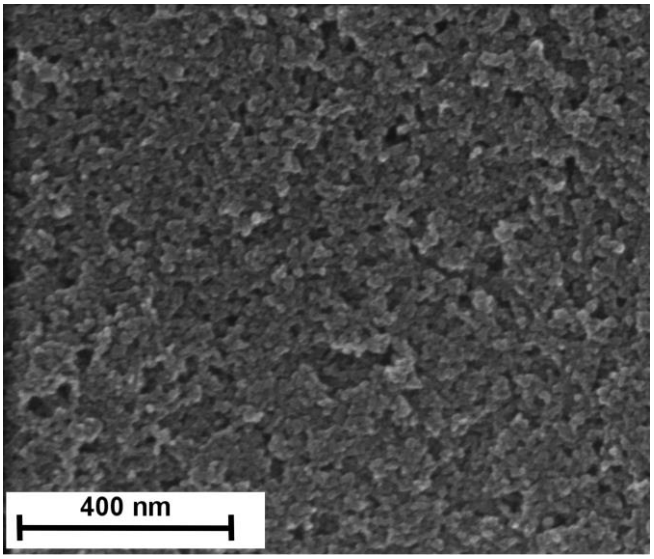

Fig. 2 SEM image of the ITO electrode coated by three immersion and withdrawal steps to positively and negatively charged carbon particles aqueous suspensions alternatively.

Additionally, voltammetric experiments which were performed in the presence of a simple redox probe such as $Fe(CN)_6^{3-}$ does not indicate any accumulation effect (see Supplementary Material Fig. F3). This indicates strong electrostatic interactions between both components of the film contributing to its stability. Also, as can be observed from the cyclic voltammograms obtained for the electrode modified by CNP(3+/-) and bare ITO electrode that the presence of carbon nanoparticulate material increases the capacitive current demonstrating a well developed electroactive surface area (see Supplementary Material Fig. F3). However the increase of the faradaic current is not observed because the electrochemical reaction occurs only at the outer layer of carbon nanoparticles.

The electrochemical oxidation of dopamine and the studied interfering compounds are two-electron coupled with two-proton reactions [10, 30, 54]. Therefore, the behaviour of the CNP(3+/-) electrode was additionally studied in a wider pH range (Fig. 3). Indeed, in pH 5.0 the peak potentials are shifted towards more positive values as compared with pH 8.0, showing that protons are participating in the electrochemical reaction. As can be seen in the inset of Fig. 3 the peak potential for DA (E_p= -0.082pH + 0.84, R^2=0.91) oxidation varies linearly with pH with a slope value diverge from theoretical value of -0.059 V per pH unit. This is probably due to adsorption of polymerised dopamine formed during electrooxidation reaction at the pH higher than 5 that blocks the electrode surface [55, 56]. As the result, the studied electrode reaction at pH above 5 might be rather quasi- than reversible.

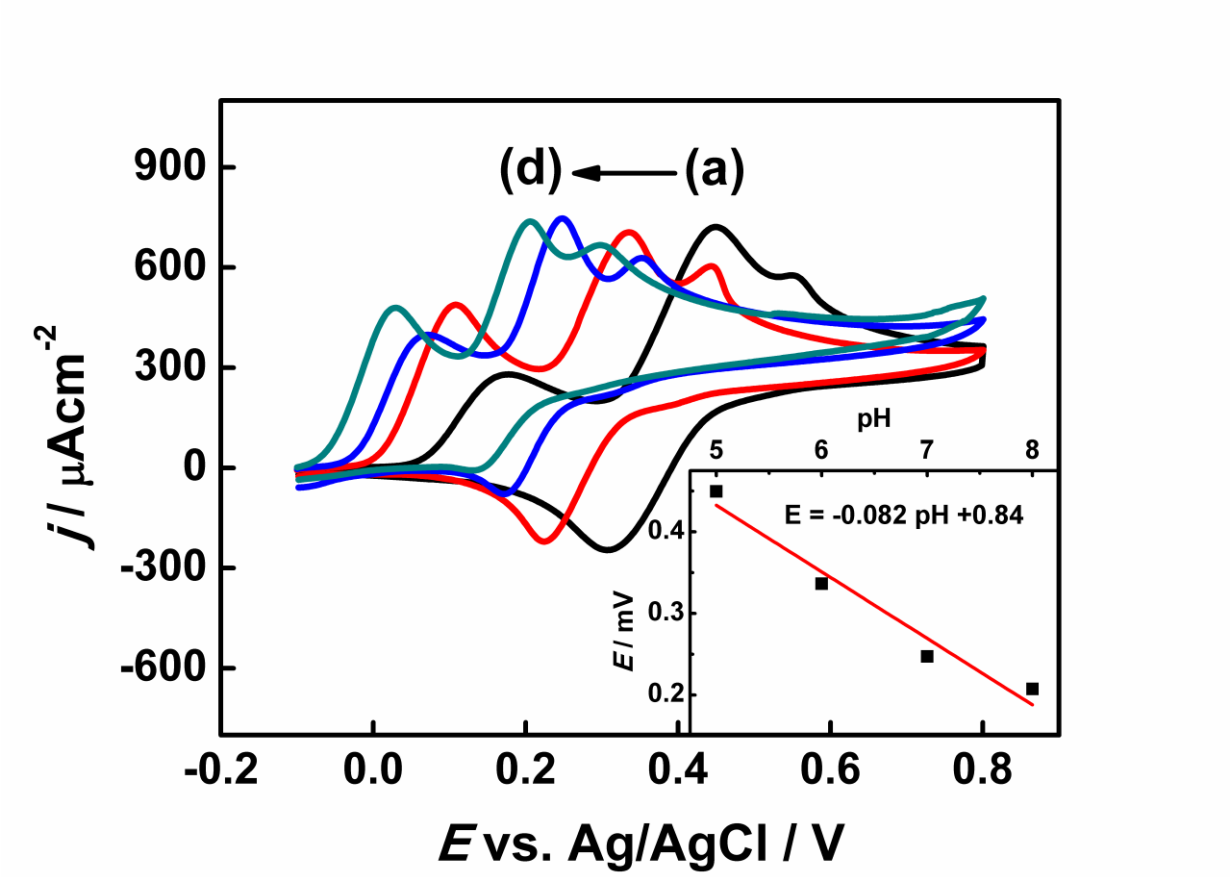

Fig. 3 Cyclic voltammograms obtained with CNPs(3+/-) electrode in 2 mM AA, 2 mM DA, 1 mM UA in 0.1 M phosphate buffer pH (a) 5.0, (b) 6.0, (c) 7.0, (d) 8.0. Scan rate 20 mV s^{-1}.

The stability of the voltammetric response of the CNPs(3+/-) electrodes was also evaluated (Fig. 4). This experiment was carried out in pH 7.0 in order to simulate physiological conditions. The magnitude of the peak current density corresponding to DA (and also UA) oxidation decreased only by ca 6 % after 18 months on shelf in air (778.89 µA cm^{-2} ± 29.14 µA cm^{-2} for n = 6) . The signal corresponding to AA oxidation is more affected by storage, but this is less important if DA is an analyte.

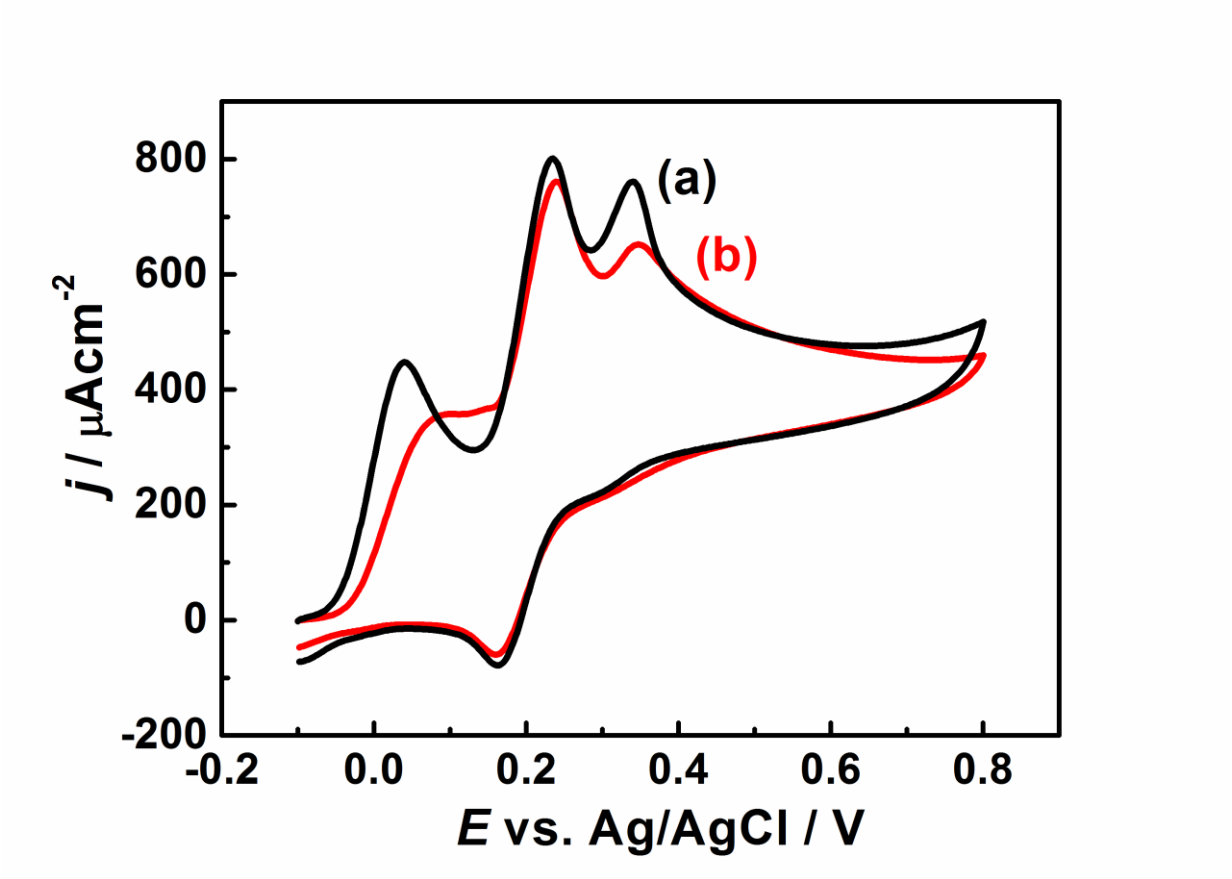

Fig. 4 Cyclic voltammograms obtained with (a) fresh and (b) stored for 18 months CNPs(3+/-) electrode in 2 mM AA, 2 mM DA, 1 mM UA in 0.1 M photosphate buffer pH 7.0. Scan rate 20 mV s^{-1}.

3.2 DA, EP and 5-HT sensing

The DPV method offers improved sensitivity in both the electrochemical signal and the detection limit as compared to cyclic voltammetry [57], therefore the response of the DA in phosphate buffer solution with pH 5.0 and 7.0 in the presence of the interfering substances was investigated *via* application of that technique (Fig. 5). Additionally, CNPs(3+/-) electrode was utilized for detecting other neurotransmitters such as EP and 5-HT in the presence of UA and AA (Fig. 6). DA, EP and 5-HT are implicated in several neurological diseases, and at the same time they coexist in biological systems influencing each other. Therefore, in terms of better diagnostics and studies under chromaffin cells, it is useful to detect them separately in their mixture [10, 12, 13]. Unfortunately, the simultaneous determination of DA, EP and 5-HT with the CNPs(3+/-) electrode was not possible (see Supplementary Material Fig. F4) because of the similar oxidation potential of DA and EP (see Supplementary Material Fig. F1, F2) leading to

overlap of those peaks (see Supplementary Material Fig. F4, F5). Therefore they have been studied separately.

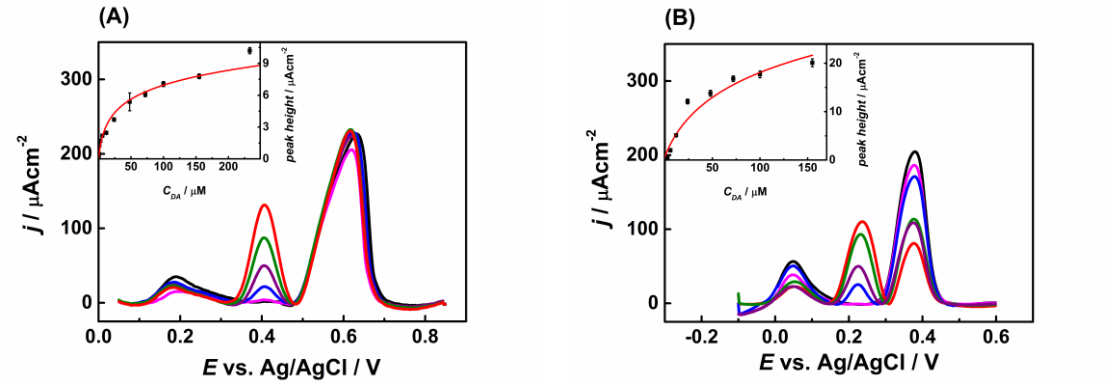

Fig. 5 DPV voltammograms obtained with CNPs(3+/-) electrode immersed in (A) 2 mM AA, 0.4–350 μM DA and 1 mM UA in 0.1 M phosphate buffer pH 5.0 (B) 2 mM AA, 0.3–160 μM DA and 1 mM UA in 0.1 M phosphate buffer, pH 7.0. DPV parameters: scan rate 20 mV s^{-1}, pulse interval 100 ms, pulse amplitude 50 mV, pulse width 50 ms.

As it is seen in Fig. 5, the peak current corresponding to electrooxidation of DA increases with the increase of the target biomolecule concentration. However the peak current vs. DA concentration (c_{DA}) dependence is clearly not linear. This is most likely due to adsorption of polymerised dopamine formed during electrooxidation reaction that blocks the electrode surface [55, 56] suggesting that maintaining pH at 5 is not enough to prevent the polymerization of DA. By modeling the adsorption using a Hill isoterm [14, 58-60] the peak current vs. c_{DA} dependence can be fitted to the function:

$$I_{DA} = S \times c_{DA}\left(1 - \frac{c_{DA}^{m}}{K_A + c_{DA}^{m}}\right) + B, \qquad (1)$$

where B is an offset, S is the sensitivity, K_A is related to the adsorption strength and m the Hill cooperativity coefficient. In this case m = 0.75; a value less than unity, which means that the adsorption is negatively cooperative. This model was recently used to model the response curve of dopamine determination in a microfluidic system . At low concentrations the adsorption has a minor effect, and the response is simply given as $I_{DA} = S \times c_{DA} + B$. For pH 5.0 and 7.0 respectively, the detection limits are estimated to be 0.4 μM and 0.3 μM at S/N= 3, and the relative standard deviations (RSD%) for n = 7 equal 0.7 % and 1.1 %. The effect of DA

polymerization is more pronounced at higher pH, which is clearly seen by the blocking effect on the peak currents of UA and AA in Fig. 5B.

The detection limit of CNPs(3+/-) electrode is slightly higher than earlier reported for other CNPs films obtained by the layer-by-layer method [30, 41]. However, the proposed carbon nanoparticulate electrode showed significant advantage over above mentioned electrodes [30, 41] and others nanocarbon based electrodes in terms of reproducibility, and stability (Table 1). Additionally its preparation is fast and straightforward.

Table 1 Comparisons of analytical parameters of different nanocarbon based electrodes applied for determination of dopamine.

Electrode material	**Interferences**	**Calibration range (μM)**	**Detection limit (μM)**	**RSD (%)**	**Stability**	**Method**	**Ref.**
ITO/functionalized silicate particles/CNP	AA, UA, AC, citric acid, NADH, tryptophan	0.3-18	0.1	1.64	50 % after 10 days	DPV	41
ITO/CNP/PDDA	AA	0.1-10	0.05	-	-	DPV	30
ITO/ polyaminoamine-MWCNT/Ni tetrasulfonated phtalocyanines	AA	2.5-240	0.54	-	-	CV	61
GC/SWCNT/ cetylpyridinum bromide	AA, UA, citric acid, glucose, cysteine, hippuric acid	4-120	0.6	-	-	DPV	19
GC/OMC/Nafione	AA, UA	1-90	0.5	-	-	DPV	23
CFE/ GEF	AA, UA	1.36-125.69	1.36	1.8	96.3 % after 20 days	DPV	20
GC/CNO/PDDA	AA, UA	50-4000	10	1.5	-	DPV	22
GC/β-cyclodextrin-MWCNT/chitosan	-	0.1-25	0.06	4.6	85 % after 30 days	DPV	62
GC/functionalized-OMC/IL	AA	0.1-500	0.0041*	6.4	97.1 % after 1 week	DPV	54
graphite/PDDA/ MWCNT-polystyrene sulfonate	AA, UA	50-350	0.15	2.5	90 % after 3 weeks	AC	63
ITO/CNPs	AA, UA	0.4-350	0.4	0.7	94 % after 18 months	DPV	**This work**

OMC ordered mesoporous carbon, *GC* glassy carbon, *MWNT* multiwalled carbon nanotube, *SWNT* single-walled carbon nanotube, *PDDA* poly(diallyldimethylammonium chloride), *IL* ionic liquid, *CFE* carbon fibre electrode, *GEF* graphene flowers, *CNO* carbon nanoanion.
* The lowest concentration actually measured is 0.1 μM.

Further we explored the possibility of determination of the neurotransmitters EP and 5-HT in the presence of UA and AA separately with the CNPs(3+/-) electrode (Fig. 6). Figure 6A and 6B demonstrate results of DPV measurements in micromolar solutions of epinephrine or serotonin in the presence of 2 mM ascorbic acid and 1 mM uric acid. Under these conditions the EP oxidation peak is seen at ca. 0.40 V whereas the signal of 5-HT is split into two oxidation peaks at ca. 0.05 V and 0.25 V. Such results were earlier reported by Yao *et. al* [64] and explained by the formation of electro-active intermediate products of 5-HT oxidation [64].

From differential pulse voltammetry experiments (Fig. 6A) it is clearly visible that the EP peak current is proportional to the epinephrine concentration, and the CNPs(3+/-) electrode shows a linear range from 1 to 49 μM with the detection limit (S/N = 3) equal to 1 μM (Table 2). In the case of serotonin the similar effect like for DA is observed where the dependence of peak current of DA on concentration is not linear (Fig. 6B). The obtained calibration curve for serotonin is in the range 0.8-100 μM with the detection limit (S/N = 3) of 0.8 μM for (Table 2). Also similarly as for DA the intensities of the signals related to AA and UA oxidation evolve with the increase of EP and 5-HT concentration (Fig. 6A,B). This might result from adsorption of EP and 5-HT during their oxidation.

The proposed electrochemical sensor exhibits higher detection limit of epinephrine in comparison with the other CNTs [25, 27, 65, 66] and graphene [28] based sensors, but wider linear dynamic range than that of CNTs modified basal plane pyrolytic graphite [25]. In the case of serotonin, the detection limit of the CNPs based sensor is higher than at carbon ionic liquid electrode modified with $Co(OH)_2$ nanoparticles and multi-walled carbon nanotubes [26] and graphene modified glassy carbon electrode [67] but lower than at multimembrane carbon fiber microelectrodes [24].

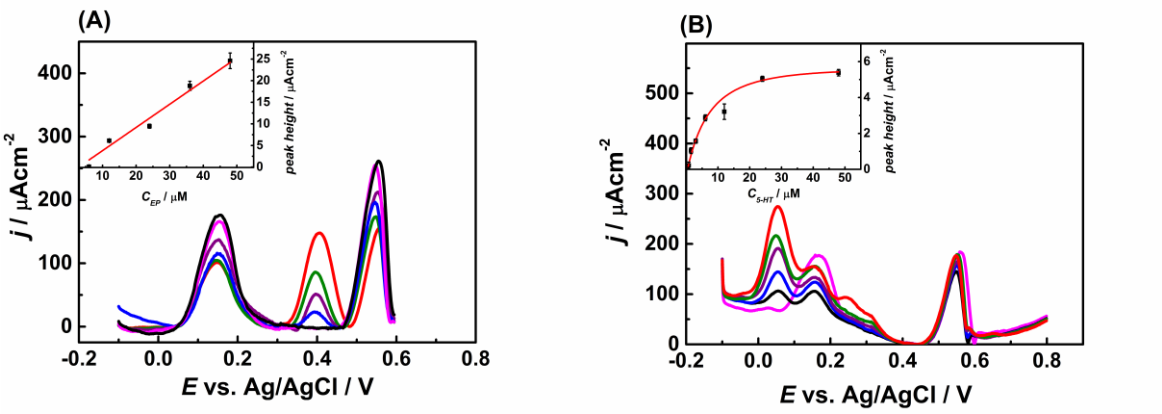

Fig. 6 DPV voltammograms obtained with CNPs(3+/-) electrode immersed in (A) 2 mM AA, 1–49 μM EP and 1 mM UA in 0.1 M phosphate buffer, pH 5.0 (B) 2 mM AA, 0.8–100 μM 5-HT and 1 mM UA in 0.1 M phosphate buffer, pH 5.0. DPV parameters: scan rate 20 mV s^{-1}, pulse interval 100 ms, pulse amplitude 50 mV, pulse width 50 ms.

Table 2 Calibration curve parameters for the determination of DA, EP and 5-HT at CNPs(3+/-) modified ITO electrode in 2 mM AA, 1 mM UA in 0.1 M photosphate buffer pH 5.0.

Biomolecule	Oxidation potential (V vs. Ag/AgCl)	Calibration range (μM)	Detection limit (μM)	Sensitivity (μA $μM^{-1}$)	*r*	RSD (%) for n = 5
DA	0.40	0.4-350	0.4	0.038	0.974	0.7
EP	0.40	1-49	1.0	0.063	0.930	1.8
5-HT	0.25	0.8-100	0.8	0.055	0.985	2.1

In order to evaluate response time and stability of the obtained CNPs-based sensor chronamperometry was performed. These experiments were carried out in stirred solution at 0.45 V, 0.40 V and 0.25 V in dopamine, epinephrine and serotonin solution, respectively. In order to avoid the impact of the signals from interfering substances on the detection of 5-HT the 0.25 V potential was chosen instead of 0.05 V. After successive addition of 182 μl of 1mM neurotransmitters solutions to 6.5 ml of phosphate buffer solution, a stepwise growth of the oxidation current is observed (Fig. 7). The current stabilizes after 55 s, 55 s, and 16 s for DA,

EP and 5-HT, respectively. In the case of DA and EP the signal is quite stable during 40 minutes, however for 5-HT it disappears after 7 minutes. This may be due to that serotonin reaches saturation earlier than DA and EP, but this can also be because it breaks down and therefore the concentration does not increase between measurements. The serotonin calibration curve is also markedly non-linear, and can be fitted with the same model as used above for DPVs.

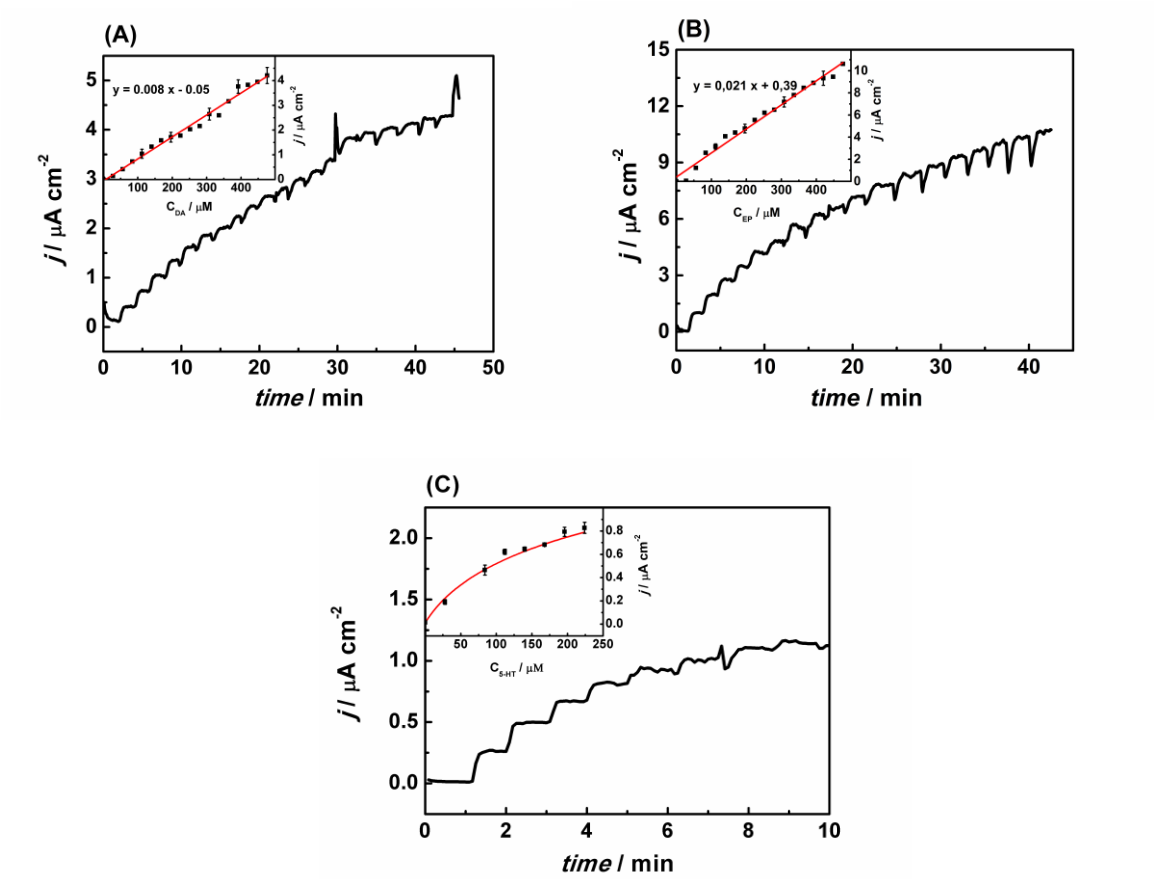

Fig. 7 Amperometric response of the CNP (3+/-) electrode in 0.1 M phosphate buffer (pH 5.0), after subsequent addition of (A) dopamine's (B) epinephrine's and (C) serotonin's samples to stirred solution. Every addition step corresponds to increase of DA, EP, 5-HT concentration by 28 µM. The potential was kept at (A) 0.45, (B) 0.40 and (C) 0.25 V.

Conclusions

In the current study film electrodes composed of oppositely charged carbon nanoparticles were utilized as electrochemical CNPs based sensor for detecting selected neurotransmitters. These electrodes exhibits electrocatalytic oxidation of DA, EP and 5-HT. The coexisting AA and UA had not interfered in detection of the above mentioned analytes, due to electrocatalytic effect. The constructed CNPs based sensor exhibits a wide calibration range with good low detection limit and stability. Even though the simultaneous determination of each neurotransmitter from their mixture was not possible with the CNP(3+/-) electrode, this electrode can be successfully applied for distinction of the two of them. Also it is worth noting that the obtained CNPs based sensor is reproducible from sample to sample and stable over 18 months storage. Additionally, its preparation is fast, straightforward, and precludes the usage of volatile organic solvents. It uses very cheap, commercially available substrates as compared to pyrolytic graphite, carbon nanotubes or graphene. Therefore, it seems promising candidate for sensing neurotransmitters and other biologically important molecules difficult to oxidize at standard electrodes.

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