Self-assembled monolayer as a pre-concentrating receptor for selective serotonin sensing

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\textbf{A B S T R A C T}

We describe a novel medium, a captopril/thiophenol (Capt/TP) mixed self-assembled monolayer (SAM) formed on gold, for selectively pre-concentrating serotonin (5-hydroxytryptamine, 5-HT). The 5-HT molecules were shown to be selectively captured via formation of a strong complex, providing anodic stripping currents upon anodic scan or drastic increases in charge-transfer resistances for a redox probe, Fe(CN)\textsubscript{6}\textsuperscript{3−/4−} pair, due to their blocking effects. The 5-HT molecules thus collected were subsequently detected by anodic stripping differential pulse voltammetry (ASDPV) or electrochemical impedance spectroscopy (EIS). The detection limit was 1.2 when detected by EIS and 28 nM with ASDPV. 5-HT was shown to be determined in physiological matrices containing ascorbic acid, dopamine, and other commonly encountered neurotransmitters.

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\textbf{1. Introduction}

Serotonin (5-hydroxytryptamine, 5-HT), a biogenic amine widely distributed in brain as well as in the intestinal mucosa and blood platelets (Nogrady, 1988), plays an important role in controlling and regulating brain functions together with other neurotransmitters. It has also been implicated in many other physiological processes and functions (Aghajanian and Sanders-Bush, 2002; Andrews, 2000; Kema et al., 2001). Hence, its sensitive and selective determination is highly desired in biological matrices containing other neurotransmitters such as dopamine (DA) for a number of reasons. A number of recent reviews have been devoted to their monitoring and analysis (Adams et al., 2008; Nichols and Nichols, 2008; Robinson et al., 2008; Kema et al., 2000).

A variety of analytical methods including fluorimetry (Panholzer et al., 1999), enzyme immunoassay (Chauveau et al., 1991), radioimmunoassay (Jeon et al., 1992), chemiluminescence (Tsunoda et al., 1999), and mass spectrometry (Cornalis et al., 1993) have been used for its analysis. Due to heavy interferences by DA, ascorbic acid (AA), uric acid, and other electroactive compounds in biological matrices, separation-based analyses with electrochemical (Chau and Patel, 2009; Patel et al., 2005; Wallingford and Ewing, 1989; Kato et al., 2007), fluorometric (Yoshitake et al., 2006), and mass spectrometric (Peterson et al., 2004) detection and molecularly imprinted polymers (Santos et al., 2009) have been used for its determination. These techniques are time-consuming and often require sample pretreatment and/or modification with fluorescent agents with high photostability and selectivity (Cao, 2007; Ishida et al., 1998).

Electrochemical methods were first developed for the detection of neurotransmitters in Adams’ laboratory (Adams, 1976) and progressed by the Wightman group (Leszczyszyn et al., 1990). While 5-HT, AA, and other neurotransmitters are oxidized at similar potentials at conventional electrodes (Robinson et al., 2008), methods such as high-speed chronoamperometry, fast-scan cyclic voltammetry (Michael and Wightman, 1999), subsecond voltammetry (Anastassiou et al., 2006), and semi-derivative voltammetry (Broderick, 2008; Broderick et al., 2008) were used for its selective analysis. Compton’s group (Kachoosangi and Compton, 2007) reported a bare edge plane pyrolytic graphite electrode for sensing low level 5-HT, and other investigators used a variety of chemically modified electrodes (Wu et al., 2003; Wang et al., 2003; Swamy and Venton, 2007; Liu et al., 2006; Li et al., 2009; Jin et al., 2004; Selvaraju and Ramaraj, 2003; Jiang and Lin, 2005; Li and Lin, 2007; Shiigi et al., 2003; Goyal et al., 2007; Ueda et al., 2006).

Modification of an electrode surface by a self-assembled monolayer (SAM) provides chemical specificity, rapid responses, high sensitivity, antifouling effects, immobilization of recognition...
agents (e.g., enzymes), and functionalization via a chemical reaction (Mirskey, 2002; Love et al., 2005). While the molecular-level control over the sensing interface of SAMs was exploited for the determination of DA with high concentration of AA present (Shervedani et al., 2006), their advantages for having a biocompatible environment have not received much attention for the analysis of neurotransmitters (Park et al., 2009).

In this study, we employ a novel captopril/thiophenol (Capt/TP) mixed SAM as a substrate for selective adsorption of 5-HT in a solution matrix containing AA and the other commonly encountered neurotransmitters. 5-HT collected by the SAM was then detected using an EG&G 273 potentiostat/galvanostat. The charge-transfer impedances undergo large changes caused by very small changes in the electrode surface due to adsorption.

2. Experimental

2.1. Reagents and apparatus

5-HT, DA (3-hydroxytyramine hydrochloride), AA, captopril (Capt), 3-mercaptop-1-propanol (MPOH), thiophenol (TP), the phosphate buffer saline (PBS) solution, and other analytical grade reagents were purchased from Sigma-Aldrich and used as received. Solutions of 5-HT, DA, and AA were prepared daily in the PBS solution of desired pH prior to use. Doubly distilled, deionized water was used for the preparation of solutions. All measurements were carried out at room temperature. Dried human blood serum was obtained from Olympus Life and Materials Science Co. (D20097 Hamburg, Germany); its solution was prepared by adding a required amount of water followed by gentle agitation and stored at −20 °C.

A Bioanalytical Systems (West Lafayette, IN) MF-2072 gold disk electrodes (0.020 cm²) modified with Capt, mixed Capt/MPOH, or mixed Capt/TP SAMs were used as a working electrode. Platinum gauze and homemade Ag/AgCl (in saturated KCl) electrodes were used as counter and reference electrodes, respectively. The cyclic voltammetric and differential pulse voltammetric experiments were performed using an EG&G 273 potentiostat/galvanostat. The EIS measurements were made with a Solartron model SI 1255 HF frequency response analyzer connected to an EG&G 273 potentiostat/galvanostat. Impedance data were obtained at the SAM modified electrodes in a 10 mM PBS (pH 7.4) solution containing AA and the other commonly encountered neurotransmitters. 5-HT collected by the SAM was then detected using the ZsimpWin® program (Princeton Applied Research). A constant phase element (CPE) model was good enough for fitting the data for the whole range of frequencies (Jurczakowski et al., 2005).

2.2. Preparation of Capt, Capt/MPOH, and Capt/TP SAM modified electrodes

Gold disk electrodes were first polished to a mirror-finish by successively polishing with 14.5, 5, 1, 0.3, and 0.05 μm alumina slurries (Buehler) followed by thorough rinsing with doubly distilled water and sonication in water, ethanol, and water, respectively, for 2 min each. These electrodes were then electrochemically polished by cycling the electrode potential between 0.0 and +1.5 V in 0.5 M H₂SO₄ until reproducible cyclic voltammograms (CVs) were obtained. After drying with nitrogen, the electrodes were dipped in a solution containing a desired concentration of Capt, or a mixture of Capt and MPOH or Capt and TP of an appropriate ratio in an ethanol/water (4:1) mixed solvent for 10 min (for EIS measurements) or 4 h (for DPV measurements) at room temperature in dark to prepare corresponding SAM modified electrodes. The electrodes were then washed thoroughly with water to remove physically adsorbed monolayers.

3. Results and discussion

3.1. Preliminary studies: interactions of analytes with SAMs

Fractional compositions (α’s) of acidic and basic forms of AA, DA, and 5-HT can be readily obtained as a function of pH using their pKₐ values of 4.1 (AA), 8.9 (DA), and 9.8 (5-HT) (Hayashi et al., 2008). A predominant form can be predicted for each conjugate acid/base system, which is important in evaluating its interaction with the surface of the SAM. Thus, electroanalysis of AA and DA has been successfully performed at gold electrodes modified with positively (Raj and Ohsaka, 2001) or negatively (Dalmia et al., 1997) charged SAMs. While many studies addressed the determination of 5-HT with AA present at SAM modified electrodes, few attempts have been made for its determination with DA present at these electrodes. Based on its structure and pKₐ value, one can judiciously select a SAM to discriminate it against interferents by adjusting pH of the solution via electrostatic interactions.

We realized that the captopril SAM acts as an effective medium for electron transfer or for 5-HT adsorption when engineered properly (see Fig. S1, Supplementary Information). To find the best SAM, two mixed SAMs of captopril were also examined: one with 3-mercaptopropanol as an aliphatic thiol (Capt/MPOH) and the other with thiophenol, an aromatic thiol (Capt/TP). We determined their surface pKₐ values to find an optimal pH for the maximum electrostatic interaction of 5-HT with the surface of the SAM. The surface pKₐ values of Capt, mixed Capt/MPOH, and mixed Capt/TP SAMs were determined to be 7.4, 6.8, and 7.5 for Capt, Capt/MPOH, and Capt/TP SAMs, respectively, by impedometric-pH titration using a series of 10 mM PBS of different pHs and a Fe(CN)$_6^{3−/4−}$ redox probe (Mozaffari et al., 2009). Based on these results, the major interaction would be electrostatic between positively charged H(5-HT)$^+$ and H(DA)$^+$ species and negatively charged carboxylate groups of these SAMs at pH ≥ 7.5. Indeed, we observed well-defined CV peaks for 5-HT and DA at the captopril SAM modified electrode, and we subsequently found that the same 5-HT redox wave obtained was observed even when the electrodes were moved to a blank PBS solution containing no 5-HT due to its incorporation into the SAM layer. We thus decided to study 5-HT at the SAM modified electrode employing: (i) stripping voltammetric detection after its pre-concentration and moving the pre-concentrated electrode to a blank PBS solution and (ii) impedometric detection by measuring the charge-transfer rate to/from the Fe(CN)$_6^{3−/4−}$ redox probe due to its blocking effect in the same blank solution.

3.2. Voltammetric studies

We obtained differential pulse voltammograms (DPVs), not CVs, for better voltammetric resolution and higher sensitivity. Fig. 1(a) shows DPVs obtained for 5-HT and DA at Capt-Au (i), Capt/MPOH-Au (ii), and Capt/TP-Au (iii) electrodes; all DPVs are well resolved with differences in peak potentials of about 180 mV. The peak currents for 5-HT and DA are the highest at the Capt/TP-Au electrode. This shows that the presence of aromatic thiol improves the capturing efficiencies for 5-HT and DA (vide infra). We thus selected...
mixed Capt/TP SAM modified electrode for further studies. Fig. 1(b) shows how DPVs are affected by pH at the Capt/TP SAM electrode in 10 mM PBS in a pH range of 6.0–9.0. The pH dependencies of peak potentials plotted in Fig. 1(c) are represented by two equations:

$$E_p(5-HT) = 0.582 \pm 0.032 \text{ pH and } E_p(DA) = 0.309 \pm 0.031 \text{ pH.}$$

The slopes of $-0.032$ and $-0.031$ mV per pH for 5-HT and DA indicate that their electrochemical oxidation is a one proton, two electron process (Wrona and Dryhurst, 1990). Peak currents also increased with an increase in pH with the highest oxidation currents observed at pH 9.0. Based on the results, we conclude that the major interaction is electrostatic between positively charged H(5-HT)$^+\text{ and }$H(DA)$^+\text{ species and fully dissociated }-\text{COO}^-\text{ (carboxylate) groups of Capt molecules on the Capt/TP SAM surface at pH 9.0.}$

3.3. Effects of mixed SAM compositions

As shown above, the Capt/TP-Au electrode generates higher signals for 5-HT and DA than Capt-Au and Capt/MPOH-Au electrodes do; this must be due to the presence of aromatic rings in the Capt/TP SAM. From a series of experiments described in Supplementary Information (Fig. S2), we found that the (10:2) Capt/TP mixed SAM is significantly more selective and sensitive to 5-HT than to other interferents and we decided to use this mixed SAM for further studies.

5-HT molecules were found to replace DA molecules present in SAM layers regardless of whether they had been pre-captured from a solution containing DA alone or both DA and 5-HT were present together; in latter cases no DA peak was detected. This indicates qualitatively that the inclusion complex of 5-HT into the mixed SAM is much stronger than that formed with DA, leading to preferential adsorption of 5-HT in the Capt/TP layers. “Caves” might have been formed between the tall Capt and short TP molecules, which would capture 5-HT molecules via: (1) electrostatic interactions between H(5-HT)$^+$ and $-\text{COO}^-$ and (2) host–guest interactions of the Capt alkyl hydrogens and TP aromatic rings with $\pi$-electrons of 5-HT (so called H–$\pi$ and $\pi$–$\pi$ interactions); similar observations have been reported on the (R)-lipo-diaza-18-crown-6 SAM (Park et al., 2009). The H–$\pi$ interaction has been shown to play important roles in protein structures and the protein–ligand recognition (Bendova et al., 2007; Nichols and Nichols, 2008). To study how strongly the 5-HT and DA molecules would interact with Capt/TP caves, a series of electrochemical experiments were performed. When an electroactive guest, i.e., 5-HT, forms a host–guest complex with a host cave on the electrode surface, the following relation is readily obtained from the Langmuir isotherm (Choi et al., 2002):

$$\frac{[5-HT]}{I_p} = \frac{1}{K_f k} + \frac{[5-HT]}{k}$$

Here $I_p$ is the voltammetric peak current at a guest concentration [5-HT], $k$ is a constant, and $K_f$ is the formation constant. We thus ran experiments, in which the SAM modified electrode was first dipped in a solution containing varied amounts of 5-HT or DA for 5 min to capture 5-HT or DA from the solution. The electrode was then taken out and washed thoroughly with water, and the amounts of 5-HT or DA captured by the SAM were determined by recording DPVs (data not shown). From [5-HT]/$I_p$ vs. [5-HT] and [DA]/$I_p$ vs. [DA] plots, $K_f$ values of 2.88 $\times$ 10$^4$ and 4.43 $\times$ 10$^4$ M$^{-1}$ were obtained for 5-HT and DA, respectively. Thus, 5-HT forms a strong enough complex with Capt/TP layers to expel pre-captured DA molecules or to prevent DA from forming complexes when both are present together.

The scan rate dependency of the 5-HT oxidation peak current indicates that the oxidation displays a mixed behavior for both diffusion- and surface-controlled processes (data not shown). A surface coverage ($\Gamma_{5-HT}^*$) of 9.5 $\times$ 10$^{-12}$ mol cm$^{-2}$ was obtained at an 5-HT concentration of 50 $\mu$M from the equation:

$$I_p = \left( \frac{n^2 F^2}{4 R T} \right) \times \nu A \Gamma_{5-HT}^*$$

where $n$ is the number of electrons, $F$ is the Faraday constant, $R$ is the gas constant, $T$ is the temperature, $\nu$ is the scan rate, $A$ is the area of the electrode, and $\Gamma_{5-HT}^*$ is the absorbed amount of the redox compound (Bard and Faulkner, 2001). The surface coverage is somewhat smaller than its monolayer coverage when the 5-HT molecules would stand vertically on the surface (Lee and Park, 1998).

3.4. Stripping DPV analysis of 5-HT

Fig. 2(a) shows DPVs for 5-HT at: (i) Capt-Au, (ii) Capt/MPOH-Au, and (iii) Capt/TP-Au electrodes after 5 min pre-concentration.
Fig. 2. (a) Anodic stripping DPVs of 5-HT at: (i) Capt-Au, (ii) Capt/MPOH-Au, and (iii) Capt/TP-Au electrodes after 5 min pre-concentration in a solution containing 50 μM 5-HT, 100 μM DA, and 1000 μM AA; (b) DPVs obtained at a (10:2) Capt/TP-Au electrode after 5 min pre-concentration in a PBS (pH 7.4) containing: (i) no; (ii) 300 μM 5-HT, 0.10 mM DA, and 1.0 mM AA; and (iii) DPV after the electrode used in (ii) was regenerated.

3.5. Effects of pre-concentration time and pH

Fig. 3(a) displays a dependence of the anodic stripping peak current on the pre-concentration time in a stirred solution of 100 μM 5-HT with both 0.10 mM DA and 1.0 mM AA present for different periods of time. The amount captured reaches an asymptote in 7 min.

An optimum pH for 5-HT pre-concentration should be between pH 7.4 and 9.5 (vide supra). In the pH range between 9.0 and 9.5, H(DA)⁺ is a minor component for DA and its competition with 5-HT would be less strong than at pH 7.4; however, we selected a physiological pH of 7.4 for its pre-concentration. Fig. 3(b) shows that the Capt/TP SAM strongly holds 5-HT at pHs lower than 9.0 due to its positively charged amine group. At pH 10, 5-HT is no longer positively charged and hence the current starts to decline. For pHs lower than 9.0, part of the H(5-HT)⁺ molecules would leave the surface before being stripped off. We therefore chose a buffer of pH 9.0 solution for all stripping measurements subsequent to pre-concentration.

3.6. Working curves for DPV currents

The calibration curve was obtained under optimized conditions for 5-HT from 0.04 to 250.0 μM in the pre-concentration solution and monitoring its ASDPV responses in a 5-HT-free solution (Fig. 4(a)). Fig. 4(b) displays a working curve shown in a linear scale \[i(nA) = 12.271(±0.999) + 0.221(±0.009) \times [5-HT] (\mu M): r = 0.997\] in a concentration range of 4.0–250 μM. Fig. 5(c) shows the plot in a log–log scale for a much wider concentration range \[log i(nA) = 0.803(±0.017) + 0.380(±0.013) \times log[5-HT] (\mu M): r = 0.994\].

3.7. Impedometric detection

EIS measurements were also studied to detect 5-HT by measuring the charge-transfer rate to/from Fe(CN)₆⁻³⁻⁻ at 5-HT pre-concentrated electrodes. Fig. 5(a) shows a series of Nyquist plots obtained for different 5-HT concentrations at the Capt/TP-Au electrode for a pre-concentration time of 5 min (curves iii–xii). The increase in the 5-HT concentration led to larger diameters of semicircles in these plots due to slower charge-transfer kinetics. The data were simulated by using a constant phase element (CPE) model shown in Fig. 5(b), from which \(R_p\) values were evaluated. The calibration curve (from 2.0 nM to 3.5 μM) was constructed using the charge-transfer resistance ratio \((R_{Sero-SAM}/R_{SAM})\) vs. [5-HT] (Fig. 5(a) inset). A wider dynamic range and a lower detection limit were obtained in relation to those obtained by DPV analysis \((R_{Sero-SAM}/R_{SAM}) = 3.80(±0.07) + 1.38(±0.04) \times log[5-HT] (\mu M): r = 0.994\).
Fig. 4. (a) DPVs obtained on the Capt/TP-Au electrode for (i) 0.0 nM, (ii) 0.04 nM, (iii) 0.08 nM, (iv) 0.16 nM, (v) 0.40 nM, (vi) 2.0 nM, (vii) 10.0 nM, (viii) 44.0 nM, (ix) 100.0 nM, and (x) 250.0 nM 5-HT in 10.0 mM PBS at pH 7.4 for 7-min pre-concentration time. The stripping DPVs were recorded from 10.0 mM PBS at pH 9.0. The calibration curve in linear (b) and log-log scales (c).

$r = 0.996$. The detection limit was remarkably low at 1.2 nM. An increased charge-transfer rate for the redox probe, Fe(CN)$_6^{3-/4-}$, might be expected due to the positive charge of the 5-HT ammonium ion, which would electrostatically attract the negatively charged redox probe (Park et al., 2007, 2009). However, the increase in charge-transfer resistance upon complexing 5-HT shows that the host–guest interactions cause the electron transfer to/from the Fe(CN)$_6^{3-/4-}$ to slow down due to the blocking effect of inserted 5-HT molecules. Depending on whether the captured analytes exert electrocatalytic or blocking effects on the electron transfer to/from probe ions, the impedometric analysis shows an enhancement or inhibition of the charge-transfer rate (Lee and Park, 1998; Park et al., 2008a,b; Yeo et al., 2009; Shervedani and Mozaffari, 2005, 2006a,b).

3.8. Effects of other interferents

As shown by the above results, AA is effectively discriminated against by the Capt/TP SAM as it is in the AA$^-$ form at pHs higher than 6.0 and is thus strongly repelled by the negative carboxylate groups of the Capt molecules. Effects of other possible interferents in biological fluids such as tryptophan, 5-hydroxytryptophan, epinephrine, norepinephrine, acetylcholine, glutamate, aspartate, glycine, tryptamine, histamine, and adenosine on the responses of the 5-HT molecules were investigated. For ASDPV experiments in PBS (pH 7.4) containing 100 µM each of aforementioned compounds and 50 µM of 5-HT, these compounds showed no interferences with 5-HT except for tryptamine and histamine, which showed $\sim$10% decreases in 5-HT peak currents. For EIS experiments in a PBS (pH 7.4) solution containing 1.0 µM each of above mentioned substances and 500 nM 5-HT, the presence of these interferents caused no changes in $R_p$ values except for DA, tryptamine, and histamine, which affected $R_p$ values for 5-HT by less than $\sim$5%. The effect of other neurotransmitters, which have a primary amine group with the $pK_a$ lower than 9.0, can be avoided by changing the pH of the pre-concentration solution to 9.5.

In order to see whether 5-HT can be determined by this method in a serum, we determined an exogenous 5-HT level in blood serum. When the serum was spiked with 50 µM for DPV and 500 nM for EIS measurements, the recovery was 97.5% for ASDPV experiments while it was 101.2% for EIS experiments. In conclusion, we have demonstrated by a series of experiments that the Capt/TP SAM captures 5-HT selectively under a variety of experimental conditions, when DPV and EIS measurements are used for its detection.

4. Conclusions

In this study, a novel procedure was developed for pre-concentrating serotonin onto a SAM layer by taking advantage of its
unique interactions with the Capt/TP mixed SAM, which was shown to selectively capture 5-HT molecules through host–guest interactions. The 5-HT molecules thus captured were detected by ASDPV to selectively capture 5-HT molecules through host–guest interactions with the Capt/TP mixed SAM, which was shown to have a detection limit of 28 and 1.2 nM in a dynamic range of two or three orders of magnitude, respectively, for DPV and EIS detection methods.

The selective recognition of 5-HT in the presence of similar amounts of DA and AA as well as other interferents indicates that its analysis can be carried out in physiological matrices. Our work demonstrates that a mixed SAM properly designed using a particular ratio of two thiolated compounds can serve for selective adsorption and recognition of biological analytes, which would have been difficult, if not impossible, by using diffusional voltammetric analysis due to their peak overlaps. This concept is very similar to the adsorptive stripping voltammetry used for analysis of heavy metal ions (Berchmans et al., 2000; Zeng et al., 2002) and organic compounds (Huang et al., 2007; Nakaniishi et al., 2002; Faull and Gupta, 2003) and opens up a possibility that specific SAMs can be designed for a strong interaction with a biological analyte.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.05.015.

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