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Issues related to the development and application of electrochemically modulated liquid chromatography (EMLC)

Bin Lin
Iowa State University

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Issues related to the development and application of electrochemically modulated liquid chromatography (EMLC)

by

Bin Lin

A dissertation submitted to the graduate faculty

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GENERAL INTRODUCTION

Introduction

Electrochemistry has a number of advantages as an analytical methodology. Nevertheless, it perhaps remains best known in industry only for its deficiencies in relation to spectroscopy and chromatography. By coupling electrochemistry with other techniques, it is now clear that the unique difficulties of conventional electrochemistry can often be overcome so that its strength can be applied to solving important problems. The marriage of liquid chromatography and electrochemical detection is one of the better known cases, which has been one of the most used analytical techniques in the last decade because of its high sensitivity and versatility[1-3]. Electrochemically modulated liquid chromatography (EMLC) is another case that combines electrochemistry and chromatography, and has potential broad applicability in industry. This technique exploits electrosorption and electrodesorption concepts by taking advantage of the effect of changes in applied potential (E_{appl}) on the interactions between analytes and an electrode surface, transforming a chromatographic column into a three-electrode electrochemical cell by using the stationary phase as a the working electrode. The tremendous potential of this technique lies in the ability to manipulate extensively the retention sensitivity of a the wide variety of sample mixtures.

EMLC is a subclass of high performance liquid chromatography (HPLC). HPLC continues to be one of the most widely used analytical tools in the pharmaceutical, medical, biochemical, and many other analytical laboratories [4-7]. This situation reflects the capability of HPLC in separating a wide range of complex mixtures, given the appropriate choice of stationary phase material and mobile phase composition. A large number of

columns with different stationary phases have been developed for various modes of separations, including normal-phase, reversed-phase, affinity, and ion chromatography [8-11]. Normally, it is the mobile phase, and not the stationary phase in HPLC that is manipulated to enhance the efficiency of a separation. Once the stationary phase is chosen, it remains fixed in its composition, i.e., a "passive stationary phase". It is therefore of great desire that, during the chromatographic process, we can not only manipulate the mobile phase composition, but also could use an "active" stationary phase which can hop among various states with different properties based on the user's instructions. In this way, we can build an effective communication channel between user and stationary phase, and serve the user's demand at will. To achieve this goal, many strategies have been proposed, such as transformable stationary phases and dynamic coating techniques [12, 13]. The disadvantage of these approaches is that such a stationary phase can only be changed to discrete state. EMLC is another alternative technique and combines electrochemistry and chromatography. This technique originated in early 1960's when Fujinaga et al. [14] as well as Strohl [15] and Roe [16] combined thin-layer electrochemical techniques and liquid chromatographic technology. In this method, the composition of the stationary phase is controlled electrochemically. Conductive stationary phases such as carbon [15] or amalgamated nickel [16] are used in order to manipulate their surface charge through alterations of applied potential. By doing this, continuous state changes on stationary phase surface can be realized.

A significant advance in this area was brought by Antrim et al. [17] in 1984. For the first time, the concept of "electrochromatography" was proposed. This concept came from the fact that the capacity factors of analytes could be manipulated through the application of

various constant voltages to a carbonaceous column. Their work also included the development of a column with stainless steel as the container for the stationary phase which could withstand pressures up to ~3000 psi.

More recently, Nagaoka investigated the influence of applied voltage on the retention with different kinds of stationary phases which were either microporous glassy carbon [18], or glassy carbon spheres coated with crown ethers [19], polyaniline [20, 21], and polypyrrole [21]. Lam et al. [22] found the retention of β-lactoglobulin could be manipulated by changing the redox states of heme in a heme-agarose bed with additives in the mobile phase.

Our group started research on EMLC in the early 1990's. Since that time, a great deal of progress has been achieved. Deinhammer et al. reported some improvements in the preparations of a polypyrrole coating on glassy carbon (GC), and applied these coatings to the separations of adenosine phosphates [23] and dansyl amino acids [24]. Our group demonstrated the first-ever ability to manipulate retention by changing electrochemically the composition of a stationary phase during the elution. In these early investigations, a Nafion tube served both as a container for the stationary and an ion exchange membrane to separate the working electrode from the counter and reference electrodes. The disadvantage of this design is that column body could not stand pressures higher than 10 psi. In order to overcome this issue, a new column design was developed [25, 26], leading to the first generation of high performance EMLC. In this design, the column consists of a Nafion cation-exchange membrane in tubular form that is placed inside a porous stainless steel cylinder. The Nafion tubing serves as a container for the stationary phase. The stainless steel cylinder both prevents the deformation of the Nafion tubing under the high pressure of

chromatographic flow and acts as the auxiliary electrode in a three-electrode electrochemical cell.

This revolution in the column design resulted in a major improvement in chromatographic performance, and therefore opened the door for the application of this technique to a wide area. Efforts in our group [25-28] have demonstrated that this approach can be applied to manipulate the separations of a variety of different analytes, including aromatic sulfonates, corticosteroids, and benzodiazepines. It was also applied for enantiomeric separations [29]. However, to demonstrate the potential of EMLC as a mainstream technique in analytical chemistry, more developments and demonstration are needed.

However, in spite of this progress in our development and application of EMLC technique, one of the major problems is that porous graphitic carbon (PGC) and GC have to be used as stationary phases because of their high electrical conductivity, excellent chemical stability, and ability to withstand the high pressures needed for chromatographic flow. Many types of aromatic species, such as polycyclic aromatic hydrocarbons (PAHs), adsorb strongly to carbon surface through donor-acceptor interactions [30-32]. Retention is often so strong that mixtures of PAHs composed of more than three rings are poorly resolved [33-35]. Besides, carbon bears a variety of polar functional groups that originate from the reaction between active carbon surface atoms and oxygen. This surface heterogeneity is one of the causes responsible for poorly shaped elution bands [36]. All of the above-mentioned problems motivated us to pursue the development of alternative stationary phases. One of the possible route is the chemical modification of the surface of carbon stationary phase.

The fabrication and manipulation of interfacial architectures are of importance to many research areas in analytical chemistry [37-39]. The importance stems from the critical role of liquid- and gas-solid interfaces in a host of transduction mechanisms that rely on the specificity and extent of the interactions between an analyte and a modified surface. For example, the modification of an electrode surface can transform a comparatively nonselective electron-transfer process to one with an enhanced specificity based on the identity [40], size [41-46], and hydrophobicity/hydrophilicity [47] of an electroactive species. Similar advances have been realized through the manipulation of the molecular architecture at the surface of piezoelectric [48, 49] and fiber optic-based sensors [50] and of chromatographic stationary phases [51].

Over the past several years, there has been a continued interest in the use of carbon-based materials for electrochemical purposes [52-54]. The impetus for this interest is the potential of such materials as replacements for precious metal electrodes. The modification of carbon surfaces is therefore an important objective in electrochemistry and materials science. For example, the surface modification of carbon fibers aims to improve the mechanical properties of carbon composite materials, particularly carbon-epoxy composites [55]. More broadly, the interest in this area spans the use of covalently modified electrodes in catalytic, analytical, and biotechnological applications [56-62].

Studies in the design and fabrication of carbon-based material surfaces are the focus of Chapters 1 and 2. The principal modes of the modification process generally consist of two main steps: (1) the generation of surface groups such as carboxyl, quinone, ketone or hydroxyl groups, and (2) the further transformation via coupling reactions through these surface functional groups. The procedures employed in the first step often involve vigorous

oxidation processes, such as: (i) boiling in oxidizing acid solutions like H_2SO_4 or HNO_3 [63], (ii) oxidation by air or oxygen at 500-800 °C [63] or by radio frequency O_2 plasmas [64, 65], and (iii) electrochemical oxidation where the carbon electrode serves as the anode in aqueous HNO_3 or $K_2Cr_2O_7$ solutions [66, 67]. In addition to increasing surface roughness, all of these procedures lead to the formation of oxygenated functional groups on the carbon surface whose identity and number are not only difficult to identify but to control as well. It is therefore desirable to design a more facile, less complex, and less vigorous pathway for modifying carbon surfaces.

Recently, four novel routes for the modification of carbon surfaces have appeared in the literature. The first route is based on the electrooxidation of amine-containing compounds [55, 68]. This process proceeds initially via the one-electron oxidation of an amine functionality to its corresponding cation radical, which subsequently forms a carbon-nitrogen linkage with the carbon surface. The second route is based on the electrochemical reduction of diazonium salts [69], which leads to the covalent attachment of aryl radicals to the carbon surface. The third route is based on the electrochemical oxidation of carboxylates [70], which attaches arylmethyl groups to the carbon surface. The fourth route is based on the attachment of enzymes through a light-directed derivatization using photobiotin [71], which photolytically generates a nitrene for insertion into C-H bonds. All of the above approaches provide excellent pathways for the construction of interfaces with specific structures and chemical and physical properties.

The work in Chapter 1 targets the creation of an interfacial structure that exhibits redox transformable properties. A large number of chelating ligands, polymers, resins, and adsorbents have been developed for the extraction, separation, and preconcentration of trace

metals [72-77]. Regeneration of the solid-phase chelating material generally requires treatment with a stronger chelating agent, such as EDTA, or with mineral acid to strip the metal ions from the system [73-75, 77]. On a large process scale, however, this approach can potentially generate a large amount of waste that contains acidic or organic components.

An alternative approach to the above process would be the redox transformation of metal-selective chelating agent immobilized onto a carbonaceous chromatographic packing that is configured as an electrochemical cell. This concept is based on the ability of such ligands to bind more strongly to metal ions when the ligand is in its reduced form than when in its oxidized form. For example, the chelating ligand Tiron (4, 5-dihydroxybenzene-1, 3-disulfonate) coordinates through its oxy groups to the uranyl dication (UO_2^{2+}) with a formation constant (K_f) of $\sim 10^{16.7}$. Oxidation of Tiron to its 4, 5-diketone analog results in a decrease in the electron density at the chelating oxygens, a change that translates to a large decrease in the ability of the chelate to bind UO_2^{2+}. As a consequence, the K_f for the 4, 5-diketone complex is only $\sim 10^{6.8}$. The same destabilization of the Tiron-bound UO_2^{2+} complex by acidification would require an increase in hydrogen ion concentration of five orders of magnitude [78, 79]. Thus, it should be possible to facilitate markedly the release of metal ions by oxidizing electrochemically the chelating species, reducing its effectiveness as a ligand [74]. This type of process would generate a concentrated solution of the target metal ion that could potentially be devoid of acidic or organic additives. The ability to concentrate dilute solutions of trace metal ions using a surface-immobilized chelating agent that is electrochemically transformable is therefore of fundamental and technological interest.

Our long range strategy is to develop redox transformable coatings that can be immobilized on the carbon packing material utilized as the stationary phase in EMLC. A

dilute solution of target ion would be passed through the column with the surface-bound chelating agent in the reduced form. After loading the column, the potential applied to the stationary phase would be adjusted to oxidize the ligand and ideally release the metal ions in a small volume of concentrated solution. As a starting point, catechol-based ligands were chosen as the immobilized species. Chapter 1 demonstrates a method to immobilize dopamine on a glassy carbon electrode (GCE) by combining control over the acidity of electrolysis solution with the formation of a metal catechol complex. The former ensures that the amine group is present in its deprotonated form, whereas the latter protects the catechol functionality during electrolysis.

The work in Chapter 2 focuses on the creation of a highly hydrophobic layer of alkyl chains on the GCE surface. This approach can potentially be used for the fabrication of a reversed phase material. Of the reversed phases, the octadecyl silica (ODS) stationary phase is by far the most widely used in liquid chromatography. However, the limited stability of such stationary phases in strongly acidic and strongly alkaline solutions precludes their use at extremes in pH, conditions especially important for the separation of materials of biological origin [80]. The work described in Chapter 2 demonstrates the feasibility to create a highly hydrophobic GCE surface by the electrooxidation of alkylamines. The chemical modification of carbon surface by linear alkyl chains my improve the separation strongly retained species such as PAHs since the solutes will be spaced away from the carbon surface and the surface oxides will be shielded by a layer of alkyl chains. We also found that the alkylamine modified GC surface has excellent chemical stability in strong acid and in strong base, suggesting the viability of such modified carbons as replacements for silica-based reversed phases.

In Chapters 3 and 4, we demonstrate the capability of EMLC to facilitate the separation of catecholamines and indoleamines, respectively. The aim of our study is to explore the feasibility of using EMLC for the separation of these classes of compounds and lay the foundation for the future application of EMLC for the analysis of biological matrices. Cellular and extracellular fluid of all living organisms usually contain complex mixtures of biogenic amines, and there currently is great interest in the development of rapid separations and sensitive detection methods to monitor these compounds. Biogenic amines, such as catecholamines and indoleamines, play an important role in the regulation of the nervous system, in both vertebrates and invertebrates. These amines act as neurotransmitters through chemical synapses. Study of these amines in physiological fluids is of importance in pathobiochemistry and clinical chemistry as they often serve as diagnostic marker molecules for a variety of metabolic and neurological disorders [81, 82].

Of the many analytical techniques, HPLC are now widely used for the determination of biogenic amines and their metabolites in various brain tissues and body fluids. The separation of these compounds by LC is often performed using reversed-phase systems composed of silica support materials and chemically-bonded coatings of alkyl chains. Normal-phase chromatography with medium polarity stationary phases has in several cases also been employed [83-88]. In our study, we show that the procedures required for the optimization of such separation by EMLC are very facile and effective. Results indicate that the retention of these analytes can be markedly and effectively manipulated through alterations only in the value of E_{appl}. These changes are realized through the dependence of the donor-acceptor interactions between the analytes and PGC on E_{appl}.

Dissertation Organization

Centered on the main theme, four specific topics are presented as four chapters in this dissertation following the general introduction. The introduction section provides a brief literature review of the EMLC technique and modification of carbon-based materials as well as a brief discussion of the research in each of the next chapters. The first two chapters explore the role and manipulation of the interfacial structure at the surface of glassy carbon by the electrochemical oxidation of amine-containing compounds. Of them, chapter 1 investigates the application of the electrooxidation process for the creation of a redox transformable coating on the glassy carbon surface. Chapter 2 examines the feasibility of creating a reversed-phase architecture on carbon materials by the electrochemical oxidation of various alkylamines. Chapter 3 and 4 investigate the EMLC-based applications to two important classes of biogenic amines — catecholamines and indoleamines, respectively. The final section presents general conclusions and a prospectus for future studies. Literature citations are compiled in the reference section of each chapter.

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CHAPTER 1. ELECTROCHEMICAL OXIDATION OF AMINE-CONTAINING COMPOUNDS: A ROUTE TO THE DEVELOPMENT OF REDOX TRANSFORMABLE COATINGS

A paper to be submitted to *Langmuir*

Bin Lin, Jennifer A. Harnisch, James W. Anderegg, Hajime Takano, Marc D. Porter, and Robert J. Angelici

Abstract

A method for the fabrication of redox electroactive surfaces on glassy carbon electrodes (GCEs) with amine-containing compounds (i.e., dopamine) is investigated. The method utilizes the electrooxidation of amines to form a chemically stable covalent linkage between the nitrogen atom of the amine and the carbon surface. The work in this paper focuses specifically on issues related to the preparation of coatings with a high coverage. Results show that the use of a high pH for enforcing the amine in its deprotonated form and the protection of the catechol group of dopamine enhance the coverage and stability of the resulting coating.

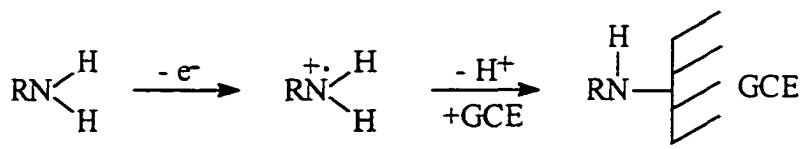
Introduction

Surface chemistry is of prime importance to the understanding of electrode Reactions [1, 2]. In the last decade, modified electrodes have been the focus of attention in many electrochemical laboratories. For the design and fabrication of electrodes at the molecular level, quinone/hydroquinone couples have occupied an important position in such efforts because of their well behaved electrochemical characteristics. In addition, they can serve as models for biological redox processes, many of which involve quinone couples of

varying degrees of complexity [3-5].

To date, approaches for preparing electrodes with reagents covalently bound at the surface have largely involved attempts to couple through surface oxides. Such approaches are, however, complicated by the inhomogeneity of the oxide functionalities [6] and often involve a lengthy sequence of steps. Examples of the latter include oxidative [7-9] and coupling agent [10-12] pretreatments that are followed by the immobilization of a target moiety via the coupling agent. Several reports have shown that glassy carbon electrodes (GCEs) modified with dopamine (DA) can catalyze the oxidation of the cofactor β-NADH via a surface electrocatalytic mechanism [13, 14]. Although different approaches have been devised for the pretreatment of carbon materials to enhance surface oxides [9, 13, 15-17], the development of a means to create a surface of only one type of oxide remains a challenge. Therefore, it is of fundamental importance to develop new, less complex routes for the modification of carbon surfaces.

Recently, a novel route has been devised for modifying carbon-based materials [18]. This route is based on the electrooxidation of amine-containing compounds and is generalized in Scheme 1. As proposed [18, 19], the process proceeds initially via the one-electron oxidation of an amine to its corresponding cation radical, which subsequently forms a carbon-nitrogen linkage at the carbon surface.



Scheme 1

Our long range interest is to develop redox transformable coatings that can be immobilized on the carbon packing material utilized as the stationary phase in electrochemically-modulated liquid chromatography (EMLC). In EMLC, the stationary phase also functions as a working electrode whereby changes in applied voltage alter the retention characteristics of the column [20-22]. Based on this interest, we have explored the utility of Scheme 1 as a facile means for the immobilization of redox electroactive groups on carbon surfaces [19]. The following sections build on our earlier investigation [19] by continuing to assess the conditions that enhance the surface coverage of redox modifiers on glassy carbon electrodes (GCEs). We have investigated: (1) the coordination ability of catechol with Co(II), (2) the electrochemical behavior of catechol and its cobalt complex in solution, (3) the adsorption behavior of catechol and dopamine on GCEs as a function of pH, and (4) the feasibility of creating an electroactive surface via the immobilization of dopamine. Cyclic voltammetry was used to ascertain whether or not the pendant catechol groups maintain their electroactivity upon immobilization. The coverage of the dopamine-modified GCE was determined electrochemically. X-ray photoelectron spectroscopy (XPS) was also used as a tool for coverage assessment. The focus will be put on the hard nuts we met for the immobilization of dopamine by electrooxidation of the amine and our efforts for overcoming them.

Experimental Section

A. Reagents and Chemicals. Butylamine, *N*-acetylethylenediamine, phenethylamine, 3-hydroxytyramine hydrochloride (dopamine hydrochloride), pyrocatechol, and lithium perchlorate were from Aldrich. Cobalt chloride, potassium chloride, perchloric

acid (70%), and lithium hydroxide were from Fisher. α-Alumina (0.3 μm) was from Buehler. Absolute ethanol (punctilious grade) was from Quantum Chemical Co. Lithium perchlorate was dried under vacuum, and pyrocatechol was purified by sublimation. All other chemicals were used as received. House-distilled water was further processed using a Millipore Milli-Q water purification system and was used in all solution and electrode preparations.

Buffer solutions were prepared using acetic acid and 4-morpholine propanesulfonic acid (MOPS) by adding perchloric acid or lithium hydroxide until the desired pH was obtained. These buffering systems were used to minimize the effect of any complexing action by the buffer with catechol [23].

B. Carbon Substrate Preparation. The GCEs (Tokai Carbon, grade 20) were prepared by polishing first with silicon carbide powder (600 grit) followed by 1.0 μm and 0.3 μm alumina on a polishing cloth (Buehler), respectively. The GCEs were sonicated in water for 20 min after each polishing step. Between experiments, the GCEs were polished using only 0.3 μm alumina. All GCEs were sonicated for 20 min in water, rinsed with water and ethanol, and dried with a stream of high-purity nitrogen immediately before use. After modification in the amine-containing electrolytic solutions, the GCEs were rinsed with ethanol and water and sonicated for 20 min in ethanol and for 20 min in water. This process was used to remove any physisorbed or unreacted materials from the electrode surface. Samples were then characterized using XPS or electrochemical techniques.

C. Electrochemistry. Electrochemical experiments were performed using a CV-27 potentiostat (Bioanalytical System) and a Houston Instruments Omnigraphic 2000 XY recorder. A conventional three-electrode cell was used with the geometric area of the

electrode defined by the circular opening in an inert elastomer gasket (1.13 cm^2). A Pt coil auxiliary electrode and Ag/AgCl/saturated KCl reference electrode (-51 mV vs SCE) were used; all voltages are given herein with respect to this reference. All electrolysis solutions were 0.1 M $LiClO_4$ in absolute ethanol or 0.5 M $LiClO_4$ in water.

D. X-ray Photoelectron Spectroscopy. The XPS data were acquired with a Physical Electronics Industries Model 5500 multitechnique surface analysis system equipped with a hemispherical analyzer, a monochromator, and a multichannel detector. Monochromatic Al Kα radiation (1486.6 eV) at 300 W was used for excitation. The photoelectrons were collected at 45° from the surface parallel. Binding energies were referenced to the C(1s) emission band at 284.3 eV. Acquisition times for the spectra were between 2 and 7 min for the N(1s) region. The pressure of the ion-pumped ultrahigh vacuum (UHV) chamber was less than $3x10^{-9}$ Torr during analysis. The elemental nitrogen-to-carbon ratio (N/C) was used as the major parameter for assessing the extent of modifier coverage. Values for N/C were calculated, after accounting for differences in sensitivity factors [24], by dividing the total number of counts under the N(1s) band by that under the C(1s) band and multiplying the result by 100. The N/C values are reported as averages of two to six samples and varied by ~10-15% between similarly prepared samples.

E. UV Spectroscopy. The pH measurements of the solutions were carried out with an Orion Research Digital Ionalyzer (Model 701A) and an Orion combination glass pH electrode (Model 91-02). The pH electrode was calibrated with a set of standard aqueous buffer solutions (Fisher). Solution pH was controlled by adding aqueous LiOH solution containing added $LiClO_4$ and of the same overall ionic strength. Since catechol compounds are sensitive to light and air [25], the pH adjustment of the solutions were carried out in a

glass jar wrapped in black tape to avoid the photodecomposition of catechol at high pH. All the solutions were deaerated 1 hr in the glass jar with UHP argon prior to pH adjustment and spectroscopic studies, and a small positive argon pressure was maintained to minimize oxidation. Solutions were transported from the glass jar to a quartz, 1-cm flow cell by using a Cole Parmer 7518-00 peristaltic pump for absorbance measurements. Absorbance measurements were taken with a computer-controlled Hewlett Packard diode array UV-Vis spectrophotometer (HP8452A). Connections between the glass jar, peristaltic pump, and flow cell were made with Teflon tubing. Catechol solutions were prepared in dilute perchloric acid with the ionic strength adjusted with $LiClO_4$.

Results and Discussion

Optical Properties of Pyrocatechol and its Metal Complex as a Function of Solution pH. Before investigating the electrochemical behavior of pyrocatechol (CAT) and its metal complex, the ability of CAT to bind Co(II) was studied. Co(II) was selected because of its relatively high stability constant for the formation of complexes with CAT, its negligible absorbance in the UV region, and its simple complexation chemistry with CAT in comparison to the other metal ions [25, 26].

CAT can bind to metal ions by using the ortho-diphenol group as a binding site. Figure 1 details the absorption spectra between 250 and 350 nm of CAT (A) and its complex of Co(II) (B) as a function of pH. The spectra in Figure 1A indicate that CAT undergoes a succession of deprotonation steps as the pH increases. These transformations result in marked differences in the solution spectra, with the absorbance shifting to longer wavelengths as level of the ionization increases. These spectra also show that there are two

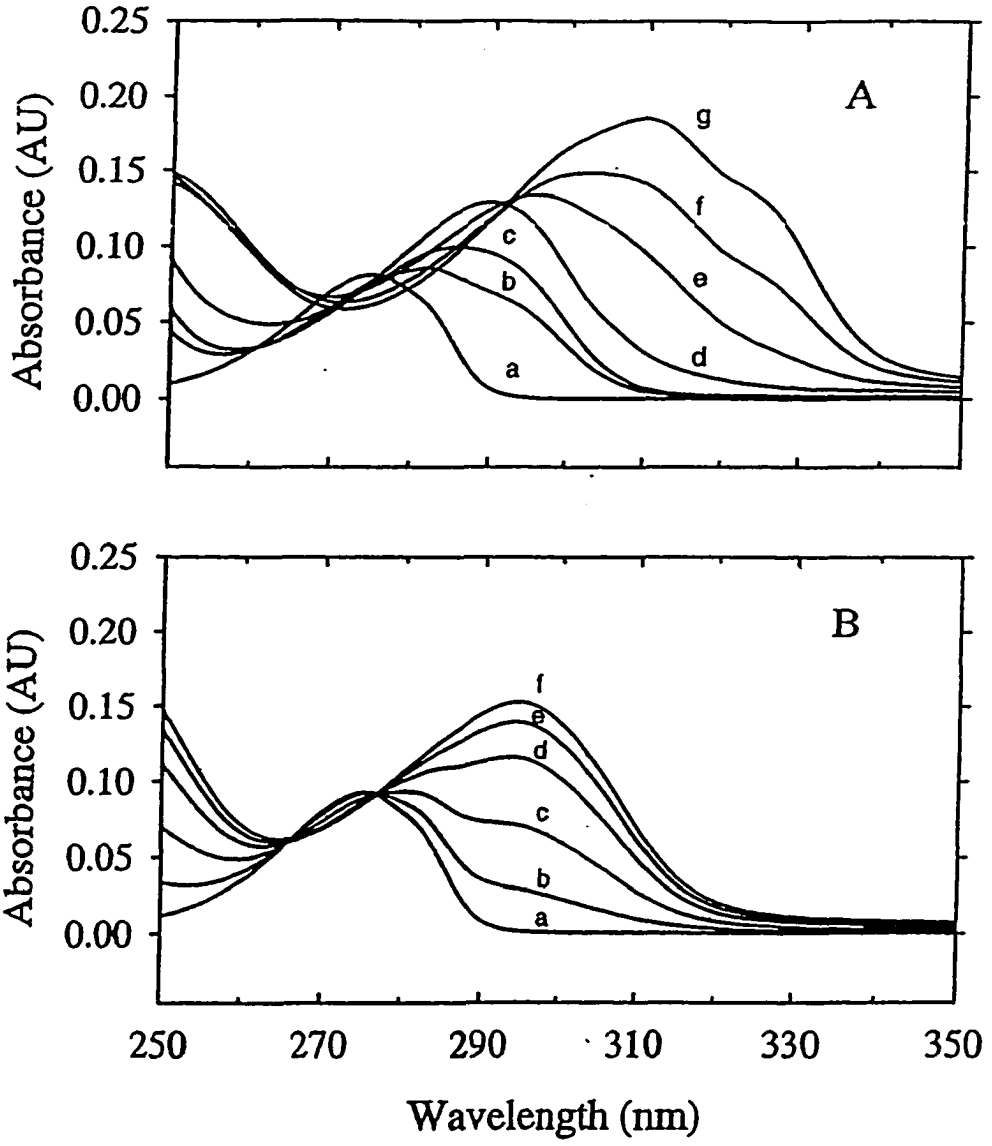


Figure 1. Absorption spectra for 0.1 M $LiClO_4$ aqueous buffer solutions containing (A) 4.0×10^{-5} M CAT, (B) 4.0×10^{-5} M CAT and 4.0×10^{-5} M $CoCl_2$ at several pH values: A. (a) 2.9, (b) 9.6, (c) 10.1, (d) 12.6, (e) 13.1, (f) 13.4, and (g) 13.7; B. (a) 3.0, (b) 8.4, (c) 8.8, (d) 9.1, (e) 9.4, and (f) 9.6.

acid-base equilibria in this pH range. At pH 7 and below (Figure 1A), CAT has a well defined band with an absorption maximum at 276 nm. We attribute the spectrum below pH 7 to diprotonated CAT with an extinction coefficient of 2085 $M^{-1}cm^{-1}$ at 276 nm. Increases in pH result in the appearance of two new features at longer wavelengths that undergo a continuous evolution as the solution alkalinity increases. One feature has an absorption maximum at 289 nm with an extinction coefficient of 3275 $M^{-1}cm^{-1}$, which we attribute to the mono-deprotonated CAT. Another feature has an absorption maximum above 320 nm, but it was not characterized further since the oxidation of CAT occurs in aqueous solutions above pH 9.5. We nevertheless attribute this feature to the fully deprotonated CAT. We note that, using the differences in absorption spectra in the ultraviolet between the neutral and ionized species and the method described by Albert and Serjeant [27], the value of pK_{a1} was found to be 9.4 and that of pK_{a2} to be 12.8. Both are in good agreement with literature data determined potentiometrically [25].

Figure 1B shows the spectra for the 1:1 Co(II)-CAT system at progressive stages of formation. As Co(II) was added to the solution, the uncomplexed CAT still exhibits a maximum absorbance at 276 nm at low pH. As the solution pH increases, the formation of the CAT complex (CoL) causes a shift in the absorbance maximum to longer wavelengths and an increase in the magnitude of the absorbance. These changes are diagnostic of an increase in the amount of CoL. Upon complete complexation, the absorbance maximum has shifted to 294 nm. The extinction coefficient for this feature at 294 nm is 3825 $M^{-1}cm^{-1}$. We assign this band as a $\pi \rightarrow \pi^*$ absorption in the catechol ligand [25, 28]. Two well-defined isosbestic points indicate that only one form of the complex is present under the experimental conditions employed.

For the Co(II)-CAT complex system, only the formation of the simple complexes CoL and CoL_2 has been suggested [25, 29-32]. In addition, the binding of the second ligand is less favored because of the electrostatic repulsion that occurs between the negatively charged oxygen atoms of the two ligands. Thus, we assume that Co(II) complexes with CAT in a 1:1 binding stoichiometry under these experimental conditions, although other possibilities cannot be fully ruled out.

Electrochemical Behavior of CAT and its Cobalt Complex in Aqueous Solution. The electrochemistry of CAT and its cobalt complexes at a stationary GCE was examined in 0.5 M $LiClO_4$/buffer pH 9.1 aqueous solution prior to exploring the immobilization process. In Figure 2, the cyclic voltammetric (CV) current-potential (i-E) curves between -0.20 and +0.80 V are presented for CAT prior to (trace a) and after (trace b) addition of Co(II) into the solution. The scan was initiated at -0.20 V. A set of well defined redox waves for the two-electron conversion between the dihydro and the quinone form of CAT is observed in solution in which the cathodic current is slightly smaller than the anodic current by ~25%. Since we used CAT as a starting material in our experiment and the scan rate is relatively slow, the larger anodic current is understandable. The peak current separation is 30 mV and is in good agreement with a two-electron, one proton reversible process that is electrochemically reversible.

We repeated the above experiment with Co(II) added to the CAT solution at pH 9.1. From the cyclic voltammograms, it was found that the electrolysis of CAT is greatly diminished. The absence of the redox wave is indicative of the formation of a complex between Co(II) and CAT. When we extended the potential scan range to +1.8 V, we were still unable to observe any redox waves that can be assigned to the complex. This result

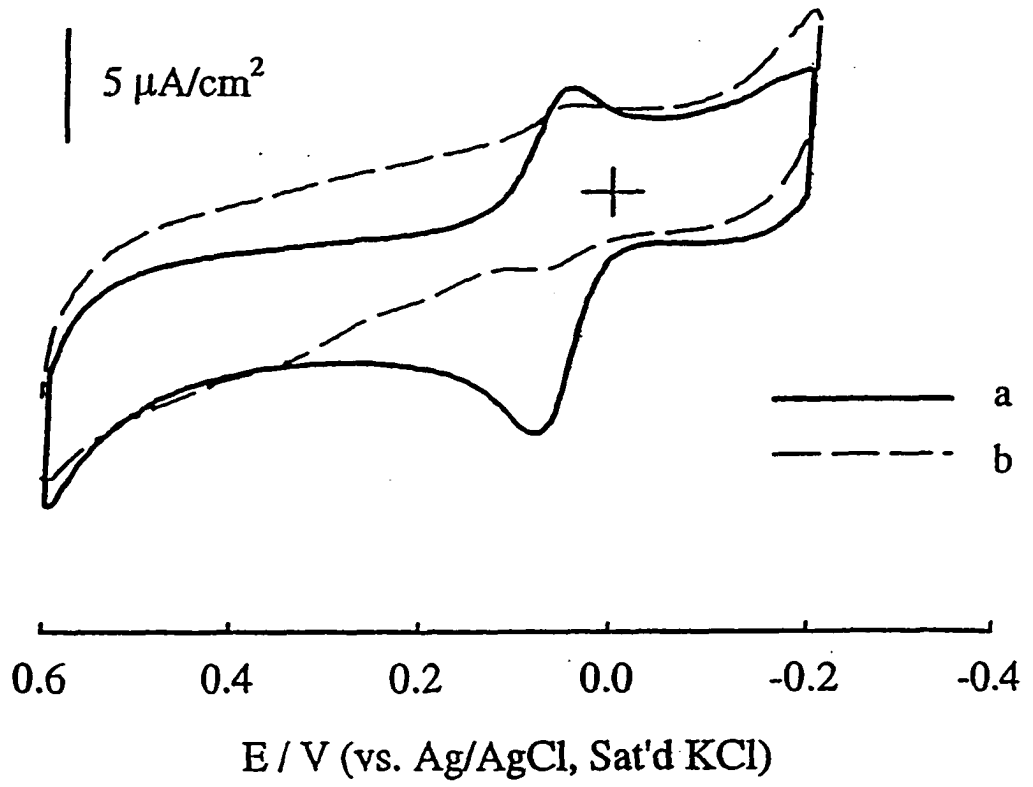


Figure 2. Cyclic voltammograms obtained at freshly polished GCEs in aqueous 0.5 M $LiClO_4$ buffer solution (pH ~ 9.1) for (a) 4.0×10^{-5} M CAT, and (b) solution a plus 4.0×10^{-5} M $CoCl_2$. The scan rate was 20 mV/s.

indicates the cobalt complex of CAT is very stable and cannot be readily oxidized to its quinone form. We attribute this stability to the strong interaction between the π-electron system of CAT and the d-orbitals of cobalt (II) [33, 34].

Adsorption Behavior of Dopamine (DA) on GCE. One can imagine the levels of interaction with the electrode surface increasing as follows: physical adsorption < chemical adsorption < covalent bonding. In view of the possibility that the types of immobilization do not significantly affect the barrier for electron transfer, their electrochemical responses may be quite similar. In order to better understand the behavior of covalently bound DA and differentiate it from absorbed DA, we investigated the electrochemical characterization, surface coverage, and possible orientation of the adsorbed electroactive material as a function of pH.

Freshly polished GCEs were immersed for 30 min in 10 mM DA or CAT aqueous buffer solutions at different values of pH. The GCEs were then rinsed three times and sonicated for 20 min in distilled, deionized water to remove residual DA or CAT. Electroactive material adhering to the electrode was then detected by cyclic voltammetry in 0.1 M $HClO_4$ aqueous solution and the charge (Q) passed in the two-electron reaction was determined by graphical integration of the area under the voltammetric wave after subtraction of an extrapolated background current. In some cases, surface coverages close to saturation were observed, revealing that a significant amount of adsorbed material adhered strongly to electrode surface. The curves in Figure 3 are cyclic voltammograms recorded for DA irreversibly adsorbed on GCEs at three different values of pH.

Figure 4 summarizes the results of the above study, with adsorbed amounts expressed as interfacial concentrations, (Γ, mol cm^{-2}). The geometric area of the GCE was used for

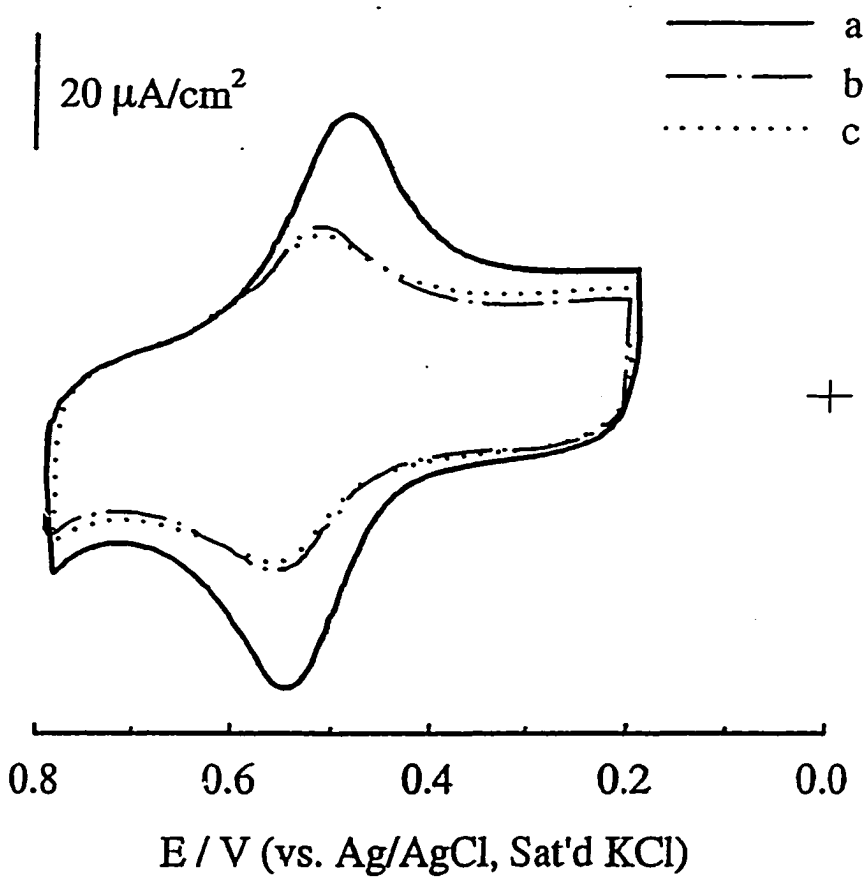


Figure 3. Cyclic voltammograms obtained in aqueous 0.1 M $HClO_4$ solution for dopamine adsorbed on GCE in different pH solution: (a) 9.5, (b) 7.0, and (c) 2.0. The scan rate was 100 mV/s.

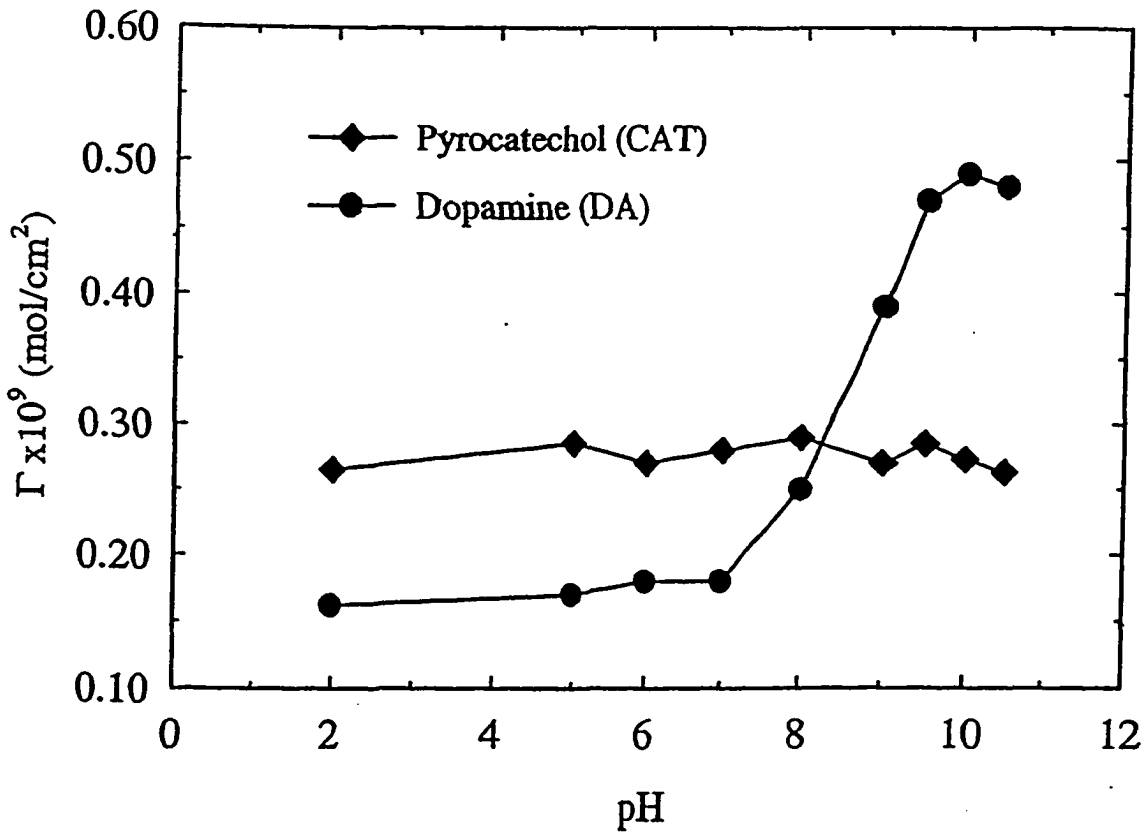


Figure 4. Adsorption data for CAT and DA on GCEs, expressed as Γ (mol cm^{-2}), as a function of solution pH. The solid lines serve only to connect the experimental data.

these calculations. The DA displays two plateaus separated by an abrupt transition to higher Γ beginning at pH of ~8. For CAT, surface coverages are essentially unchanged by changes in pH. In comparison to CAT, DA contains a primary amine side chain which is probably responsible for the difference in adsorption behavior. This assertion is supported by the presence of the transition in Γ at the pH values that are considerably close to the deprotonation of amine group of DA (pK_{a2}= 10.3) [26]. We believe the difference between the observed pH range for transition in Γ and the pH range for deprotonation of amine group of DA comes from the decomposition of DA at high pH which causes deactivation of adsorbed DA.

The transition in Γ for DA could be due to: (i) multilayer condensation, or (ii) changes in orientation from a low packing architecture to one with more dense packing. After adsorption from a DA solution at high pH, the GCEs were rinsed and sonicated in acidic, neutral, or basic solutions. The Γ data show no significant effect of the rinsing solution. This result indicates that the transition from the low-coverage to the high-coverage form of DA at GCE is irreversible unlike those found for multilayer formation whereby the adsorption of organics from solution onto nonporous adsorbents is reversible [35]. Therefore, molecular reorientation within the adsorbed monolayer emerges as the most possible explanation for the pH-induced changes in Γ.

In order to translate surface coverage data to insights about potential details on molecular orientation, it is necessary to calculate the limiting surface coverage for each possible orientation. Comparison of calculated coverages with those found provides the needed quantitative insight. Examination of molecular models, as described by Soriaga and

Hubbard [35, 36], provide a basis for a possible explanation. A detailed tabulation of experimental ($\Gamma_{experimental}$) and theoretical ($\Gamma_{calculated}$) values for DA and CAT adsorption surface coverages with various orientations appears in Table 1. As evident, adsorption of DA from acidic solution yields coverages (~1.8×10^{-10} mol/cm^2) comparable to adsorption exclusively through the phenyl ring in a η^6 (flat) orientation (2.4×10^{-10} mol/cm^2). Adsorption of DA from basic solutions, however, gives a substantial increase in coverage (~4.9×10^{-10} mol/cm^2), which is close to that expected for adsorption in a η^1 (endwise) orientation by attachment through the amine side chain (5.5×10^{-10} mol/cm^2).

Experiments performed with CAT, which does not have an amine side chain, confirms that the observed coverage is not pH dependent. The surface coverage observed (2.8×10^{-10} mol/cm^2) corresponds rather closely to the calculated value for η^6 (flat) orientation (3.1×10^{-10} mol/cm^2).

If the surface roughness factor is considered in the calculation[37], the observed values would be much smaller than the calculated values. There are two possibilities responsible for this deviation: (i) the existence of specific sites for the adsorption, and (ii) the mixture of the physisorbed and chemisorbed species on the surface in the adsorption step. Weakly physisorbed species, which compete with the chemisorbed species for the surface sites, are removed largely by rinsing and sonication (especially latter one), as evidenced by the appearance of a much larger surface redox peak when we use a rinse as the only cleaning procedure. This treatment leads to an unsaturated coverage on surface, but adsorbed species left on surface still maintain their original orientation. We also note that adsorption can be a potential-dependent phenomenon. Our preliminary experiments in which the potential was controlled during adsorption indicated, however, that the surface coverage did not vary

Table 1. Adsorption Data: Pyrocatechol and Dopamine Adsorbed on Glassy Carbon Surface from Solution at Various pH Values

	$\Gamma_{experimental}$ (nmol/cm^2)		$\Gamma_{calculated}$ (nmol/cm^2)[c]		
compound	apparent[a]	actual[b]	flat	edgewise	endwise
pyrocatechol	0.28	0.15	0.31	0.64	0.71
dopamine	0.18 (pH<7.0)	0.09	0.24	0.46	0.55
	0.49 (pH>9.5)	0.26			

[a] Average apparent interfacial concentration calculated by using geometric area of GCE.

[b] Average actual interfacial concentration calculated by considering the estimated roughness factor of polished GCE as 1.9[37].

[c] See references 35 and 36.

substantially over the range of potential from -0.2 to +0.6 V.

Immobilization of Dopamine at the GCE Surface. The feasibility of immobilizing amine-containing molecules at GCEs via the electrooxidation of amines has been assessed in our group [19], building on the earlier literature [18]. The presence of the primary amine functionality that is linked through a short alkyl chain to its catechol moiety makes DA an attractive candidate for our overall goal. Figure 5 shows CV curves obtained at a GCE in ethanolic 0.1 M $LiClO_4$ solutions containing butylamine (a), *N*-acetylethylenediamine (b), phenethylamine (PA) (c), or dopamine hydrochloride (DA) (d); all at 2 mM. The voltage scan was initiated at 0.00 V and reversed at +1.80 V. The scan rate was 10 mV/s. A broad, chemically irreversible oxidation wave is apparent in all cases except for DA, with a peak-current voltage (E_p) of ~+1.4 V. We attribute this wave to the one-electron oxidation of the amine group to its corresponding cation radical. The waves that appear in Figure 5d for DA are assigned to the redox transformation of the catechol functionality in DA; there is, however, no detectable presence for the oxidation of the amine group of DA.

The relative nitrogen content at the GCE surface after potential cycling in different amine-containing compound solutions are represented in Figure 5 as the values of nitrogen-to-carbon ratios (N/C) from XPS spectra. The N(1s) signal observed in XPS spectra is assigned to molecules bound through a C-N linkage. The results show that only trace levels of nitrogen are detected at the GCE modified in DA solution in comparison to that for butylamine (N/C = 6.2%), *N*-acetylethylenediamine (N/C = 10.2%), and phenethylamine (PA) (N/C = 6.1%). These results suggest that, of the four compounds, the DA is much less effectively attached to GCE. These data further support that the oxidation of the amine functionality is requisite for immobilization at the GCE surface, as suggested in Scheme 1.

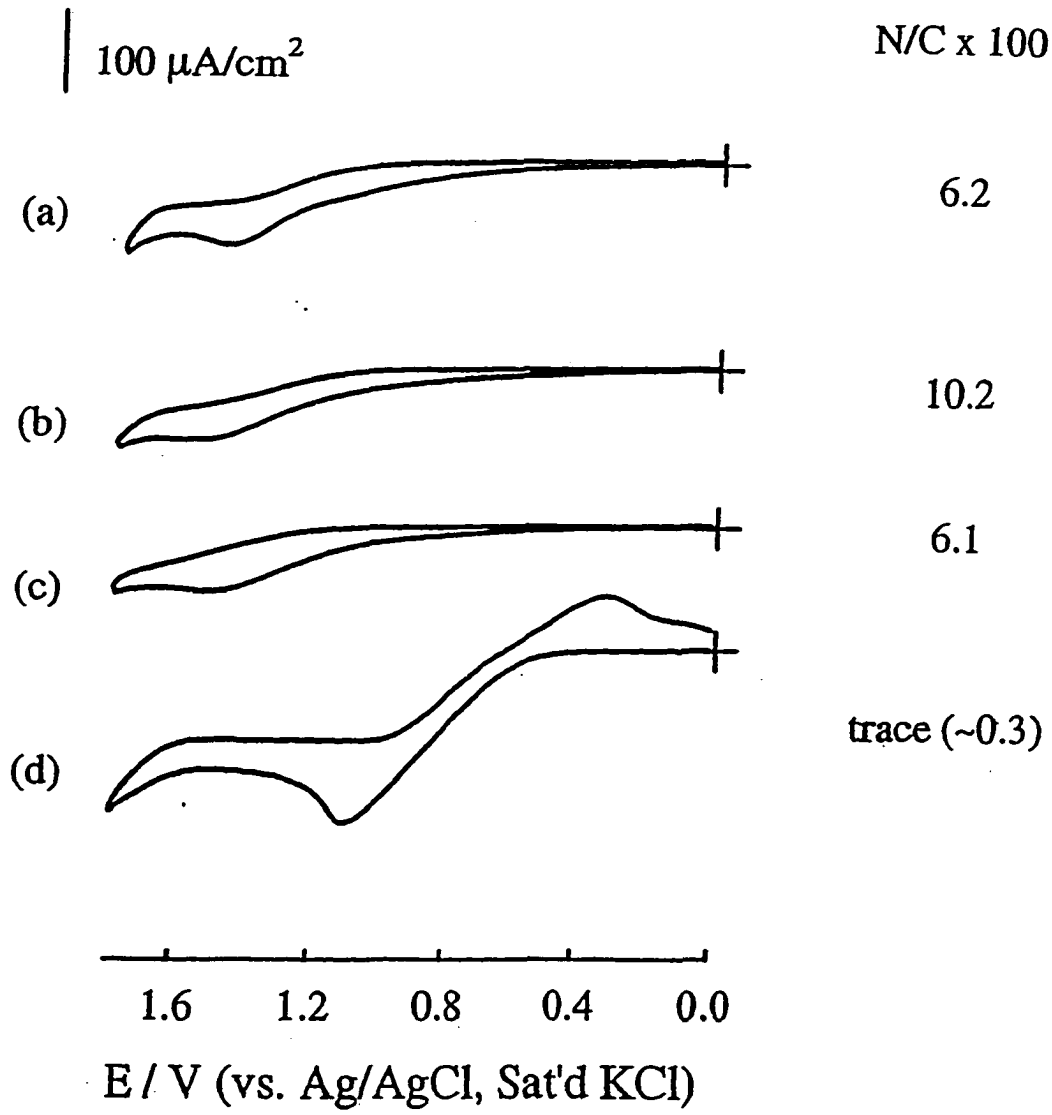


Figure 5. Cyclic voltammograms and N/C values obtained at freshly polished GCEs in ethanolic 0.1 M $LiClO_4$ solutions containing 2 mM of (a) butylamine, (b) *N*-acetylethylenediamine, (c) phenethylamine, and (d) dopamine hydrochloride. The scan rate was 10 mV/s in each case.

By comparing two structurally-related compounds, an electrochemically irreversible oxidation wave is apparent for PA, but not for DA, which is reflected on the surface coverage as relatively high N/C ratio for PA and very low N/C ratio for DA. Thus, the following explanation was developed.

In comparison to PA, DA was used as its hydrochloride salt. The amine group of DA is therefore protonated. Possible other sources of protons on the GCE surface that may result in protonation of the amine group include: (i) the acidic nature of the hydroxyl groups of catechol (i.e., the first phenolic group is more acidic than the protonated amino group [38]), and (ii) proton release from the two-electron oxidation of the catechol functionality of DA (this process occurs at a less positive potential than that for the oxidation of the amine group). All of the above may induce a high surface H^+ concentration which favors the protonation of the amine group. It is therefore likely that the protonated amine group leads to electrostatic repulsion to the positively charged GCE surface at extremely anodic potentials used in the electrooxidation process. This causes the protonated amine to be directed away from the surface when it closes to surface and makes it unavailable for electrooxidation. Another possibility is that the H^+ has a strong electron withdrawing ability which cause electrons of the nitrogen to be less available for reaction by protonation.

From the above proposition, we speculate that amine-containing compounds should be more easily oxidized in basic solutions. We designed the following experiment to confirm this speculation. We repeated the above experiment for PA except the amount of perchloric acid that was purposely added into the solution was varied, as shown in Figure 6. The influence of solution acidity on the electrooxidaton of the amine is clearly evident as the peak current decreases with the increased acidity of the electrolyte solution until disappearing at

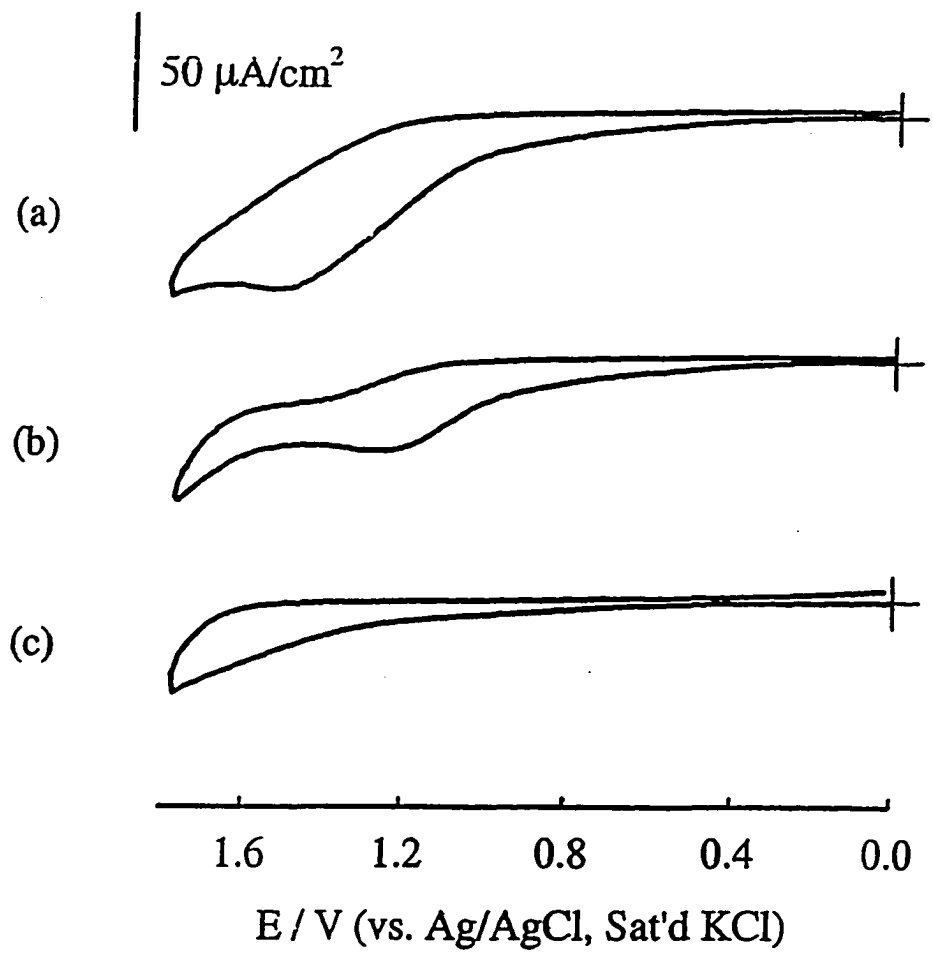


Figure 6. Cyclic voltammograms obtained at freshly polished GCEs in ethanolic 0.1 M $LiClO_4$ solutions (ethanol:water; 100:1 (v/v)) containing (a) 2 mM phenethylamine (PA) only, (b) solution a plus 1.0 mM $HClO_4$, and (c) solution a plus 3.0 mM $HClO_4$. The scan rate was 10 mV/s.

high H^+ concentration completely. As expected, examination by XPS indicated a decrease in the N/C ratio from 6.1% to a trace level as shown in Figure 7. The above results also explain our previous experimental finding [19], in which the modification of GCE surface by *N*-acetylethylenediamine in the presence of catechol is much less effective than that in electrolytic solution without catechol, which is known to be weak acid. At that time, however, we attributed this difference to a blocking of surface binding sites by the adsorption of the catechol moiety. These new results correct our earlier conclusion.

In light of the above findings, we altered the previously mentioned procedure for immobilizing DA by adding an appropriate amount of aqueous LiOH solution into the electrolyte solution in an attempt to deprotonate the amine group of DA. The resulting CV curves and N/C values from the XPS data are shown in Figure 8.

We note that aqueous LiOH solution was used to adjust the H^+ concentration in order to avoid possible interferences from other amine-containing species that may be used to manipulate the solution basicity. In practical applications, similar results can be achieved by the use of a non-hydroxylic organic base such as triethylamine. Although tertiary amines can also be oxidized, its corresponding cation radicals cannot bind effectively to the GCE surface under these conditions [19].

From Figure 8b, a chemically-irreversible oxidation wave is observed at ~+1.6 V after adding appropriate amounts of LiOH. In Figure 8c, poorly-defined waves were obtained by adding more LiOH into the solution that resulted from the overlap of the waves for the amine oxidation, the catechol functionality oxidation, and OH^- oxidation. A layer of surface-bound DA on GCE should, however, be formed in this case. This expectation is confirmed by the increase of N/C ratio from trace amounts to 6.6% in the XPS data. After

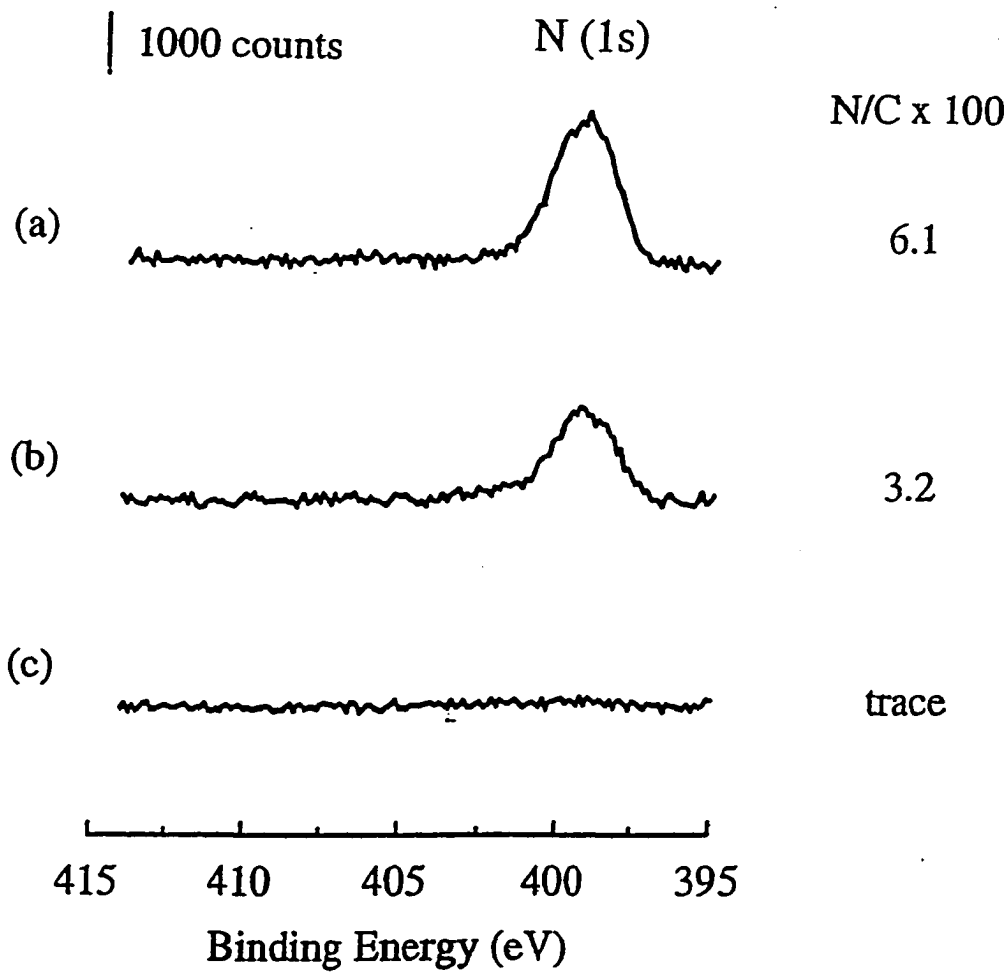


Figure 7. X-ray photoelectron spectra in the N(1s) region for freshly polished GCEs immersed in ethanolic 0.1 M $LiClO_4$ solutions (ethanol:water; 100:1 (v/v) containing (a) 2 mM phenethylamine (PA) only, (b) solution a plus 1.0mM $HClO_4$, and (c) solution a plus 3.0 mM $HClO_4$. The applied voltage was cycled once between 0.00 V and +1.80 V at 10 mV/s in each case. The exposure time of GCEs to DA solutions is ~15min.

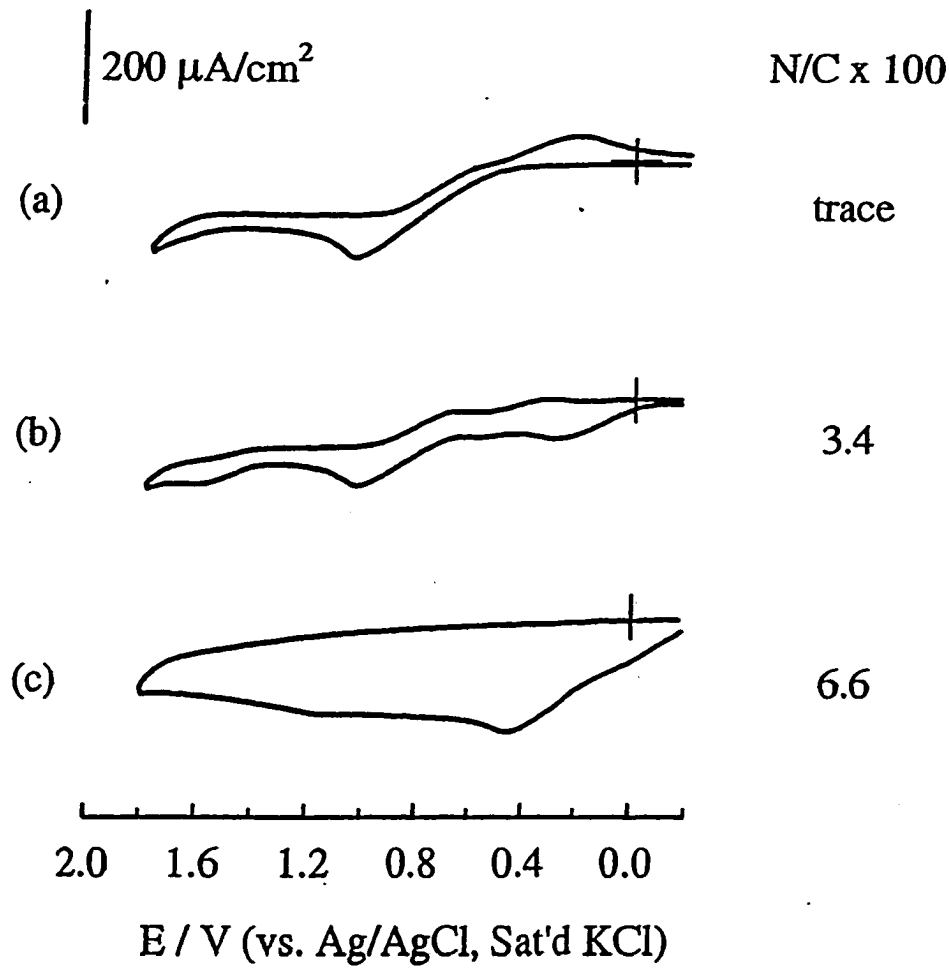


Figure 8. Cyclic voltammograms obtained at freshly polished GCEs immersed in ethanolic 0.1 M $LiClO_4$ solutions (ethanol:water; 100:1 (v/v) containing (a) 2 mM dopamine hydrochloride (DA) only, (b) solution a plus 2.0 mM LiOH, and (c) solution a plus 6.0 mM LiOH. The applied voltage was cycled once between -0.10 V and +1.80 V at 10 mV/s in each case.

immobilization, we expected that the electrochemically reversible oxidation-reduction behavior observed for the "dissolved" species might persist after immobilization. However, the expected reactivity is not observed. The response for a surface-bound redox DA is barely distinguishable from background current, as shown by the CV curve in Figure 9 for a GCE modified with DA. The curve was recorded in aqueous 0.1 M $HClO_4$ solution at a scan rate of 100 mV/s.

Possible mechanisms to account for the loss of electroactivity include: (i) desorptive loss involving nonbonded dopamines; (ii) breakage of covalent bond(s) linking the catechol to the carbon electrode; and (iii) reaction of the oxidized form of the catechol functionality of DA through nucleophilic attack to generate compounds that are electroinactive at or near the potentials of the quinone/hydroquinone couple.

The desorptive loss is believed to be minimal since the high N/C ratio (6.6) from XPS indicates the existence of surface-bound DA. Loss due to rupture of the attachment bond is also considered minimal, as described by Barbier *et al* [18]. Barbier and co-workers immobilized 2-amino-4-methyl thiazole (i.e., a compound with one sulfur and two nitrogens) on the carbon surface and found the expected nitrogen to sulfur ratio of two. Besides, our XPS data show that the O/C ratio after immobilization of DA greatly increases to 33% in comparison to that for control experiment without adding DA (O/C = 17%) as well as that for the immobilization of PA (O/C = 15%). We believe this higher oxygen content comes from hydroxyl groups on DA, which further supports the opinion that the deactivation of the DA-immobilized carbon surface is not due to the cleavage of the covalent bond linking the catechol to the carbon electrode.

Hence, the most likely route for deactivation is from the instability of the oxidized

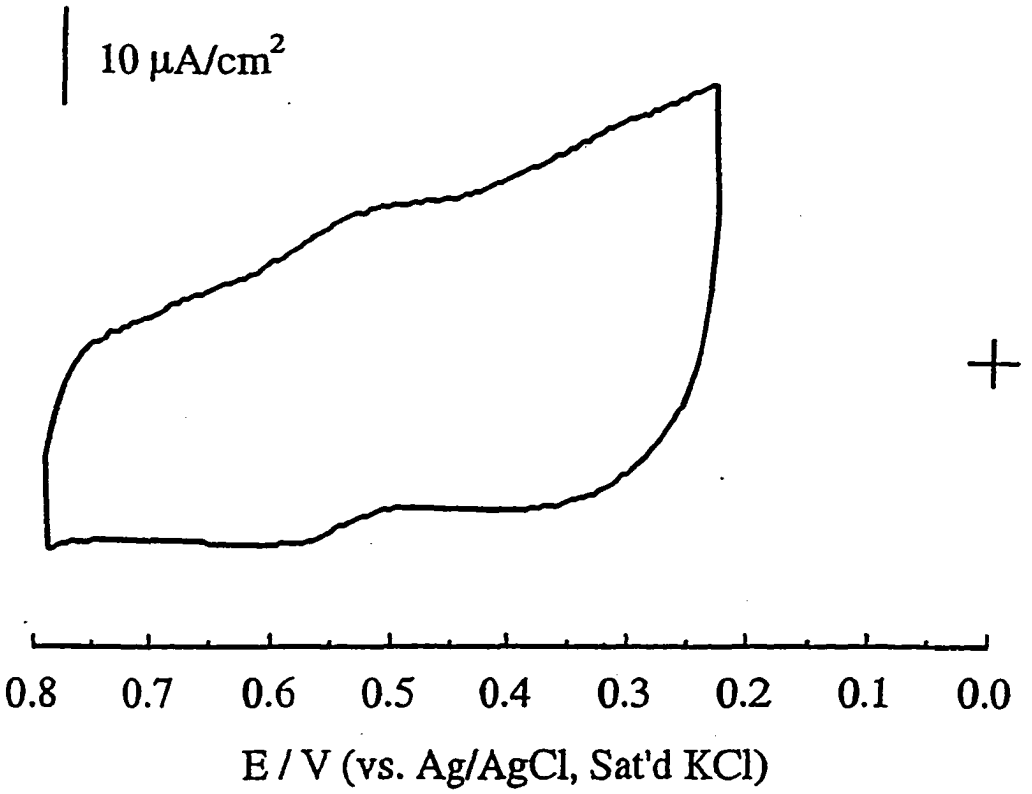
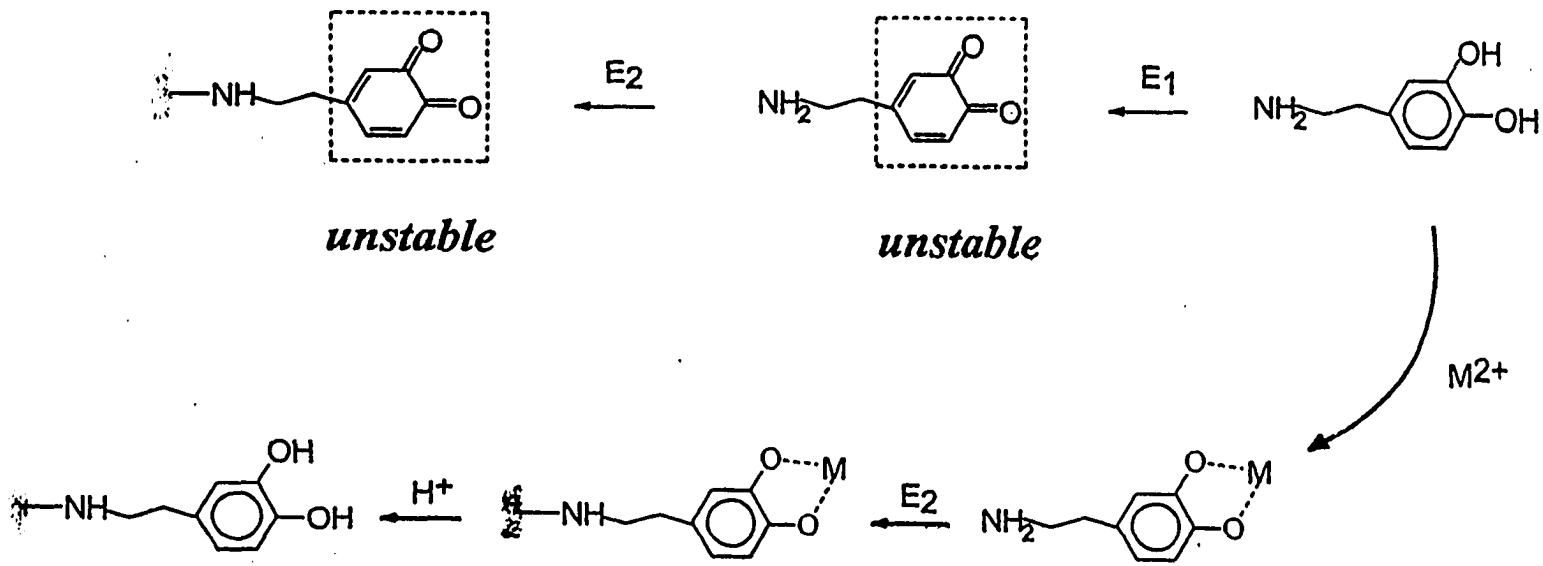


Figure 9. Cyclic voltammogram obtained in aqueous 0.1 M $HClO_4$ at a DA-modified GCE from Figure 8c. The scan rate was 100 mV/s.

form of the catechol functionality of DA in strong alkaline medium which is oxidized before the amine group. It is known that catechol and molecules containing the catechol moiety can be unstable in the presence of base, oxygen, and nucleophilic solvents such as water [39-44]. Reactions of *o*-quinones to form hydroxylated products, which then react with parent hydroquinone (QH_2) or other nucleophiles, have been documented [45-48]. Another possibility is a follow-up cyclization [49]. The unstability of the oxidized form of catechol in alkaline solution was also confirmed by the other members in our group [50] in experiments in which a gold electrode modified with 3,4-dihydroxyphenylethyl mercaptan (DHPM) was potentiostated at different fixed potentials and CV i-E curves were obtained at various time intervals. The peak heights of the i-E waves were found to decrease notably when the potential was maintained at values where the catechols were in their quinone form. In the next section, we will attempt to solve this problem.

Creation of Electroactive GCE Surface Immobilized by DA. The results presented in this section focus on demonstrating the utility of the formation of a stable Co(II)-DA complex for the creation of an electroactive layer of immobilized DA. We showed earlier that the formation of a Co(II)-CAT complex in basic solution can prevent the transformation of CAT to its oxidized quinone form over a wide potential range. We were intrigued by this result and have exploited this binding to stabilize the catechol functionality in DA during the electrooxidation step for immobilization. After immobilization, Co(II) can be released from the GCE surface in acidic or neutral solution. A simplified scheme is presented in Scheme 2. Pertaining to the metal complexes with DA, it was found that only the ortho phenolic hydroxy groups take part in the coordination, displaying similar coordination characteristics as CAT [51].


NH
O
O
E2
NH2
O
O
E1
NH2
OH
OH
unstable
unstable
M2+
NH
OH
OH
H+
NH
O
M
O
E2
NH2
O
M
O

Scheme 2

Figure 10 shows CV curves obtained at a GCE in 0.5 M $LiClO_4$ aqueous solutions adjusted to pH ~9.6 containing 0.2 mM DA with (b) and without (a) the presence of Co(II). The choice of pH 9.6 is a compromise between the required pH for deprotonation of the amine group, the formation of Co(II)-DA complex, and the decomposition of DA. We found that the oxidation peak for the catechol functionality, which is evident in curve a, vanished in curve b when Co(II) is present in solution. We attribute this difference to the stabilization from cobalt complex formation which prevents the oxidation of the catechol functionality. Thus, a much stronger response for the surface-bound DA is observed in comparison to that for surface-bound DA immobilized without the presence of Co(II); this situation is evident in curve a and curve b, respectively, in Figure 11. The anodic and cathodic waves located at ~+0.54 V in curve b correspond to the two-electron oxidation and reduction of the surface-bound DA.

The dependence of the catechol redox peaks on both scan rate and electrolyte pH was also investigated. The peak currents for the observed waves were found to be linearly dependent on scan rate (tested range: 20 to 200 mV/s) which is characteristic of a surface immobilized redox reactant. The immobilized species was also cycled in several different buffers containing 0.5 M $LiClO_4$ supporting electrolyte. The buffer pH was varied between 2.0 and 6.0 by the addition of LiOH or $HClO_4$. The slope for a plot of $E^{0'}$ vs electrolyte pH was -0.055 V/pH unit which is very close to the theoretical value of -0.059 V/pH unit for a $2e^-$, $2H^+$ process. The surface coverage of DA, as estimated by integration of the area under either the anodic or cathodic waves, is $3.3 \pm 0.2 \times 10^{-10}$ mol/cm^2. This coverage compares favorably with that obtained previously (~1.3×10^{-10} mol/cm^2) for GCEs modified with DA through amidization reaction [13]. Geometric areas of GCEs were used for the above

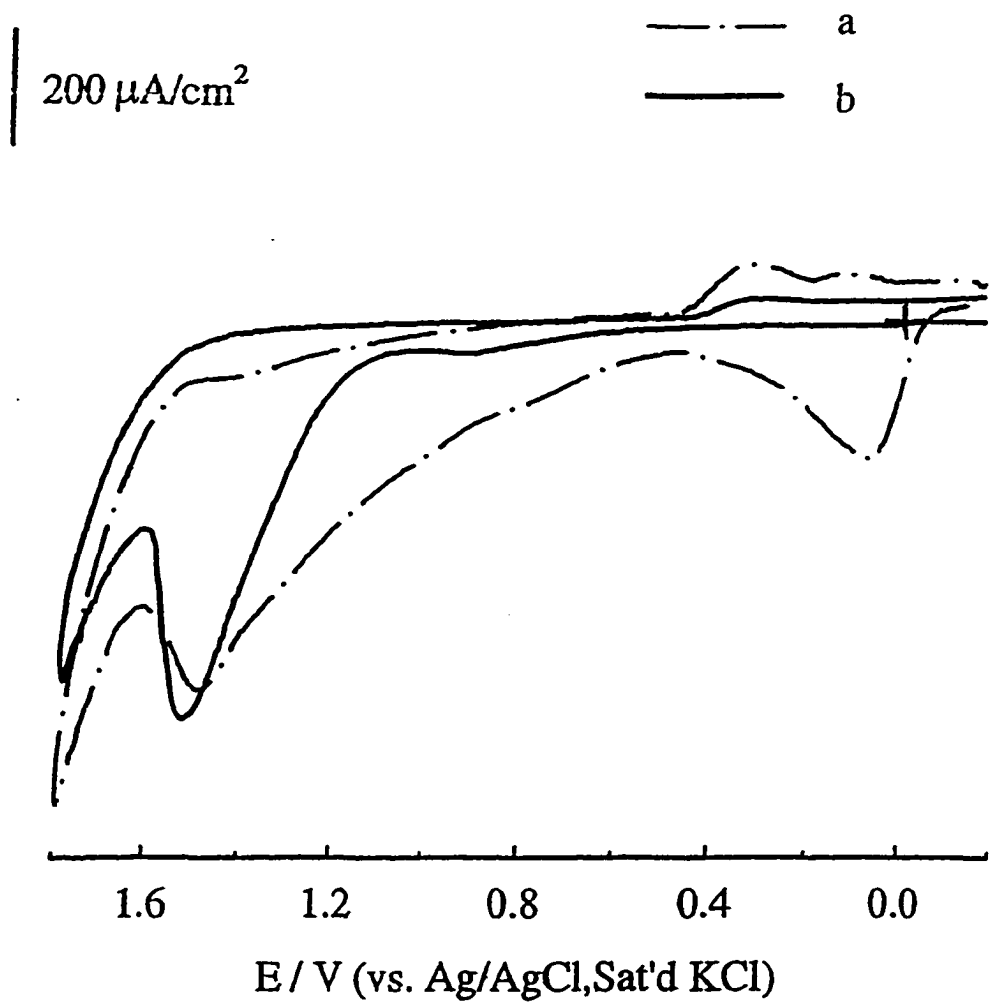


Figure 10. Cyclic voltammograms obtained at freshly polished GCEs in aqueous 0.5 M $LiClO_4$ solutions containing (a) 0.2 mM dopamine, (b) solution a plus 0.4 mM $CoCl_2$. The pH values of solutions were adjusted to 9.6 before potential scannings. The applied voltage was cycled once between 0.00 V and +1.80 V at 10 mV/s in each case.

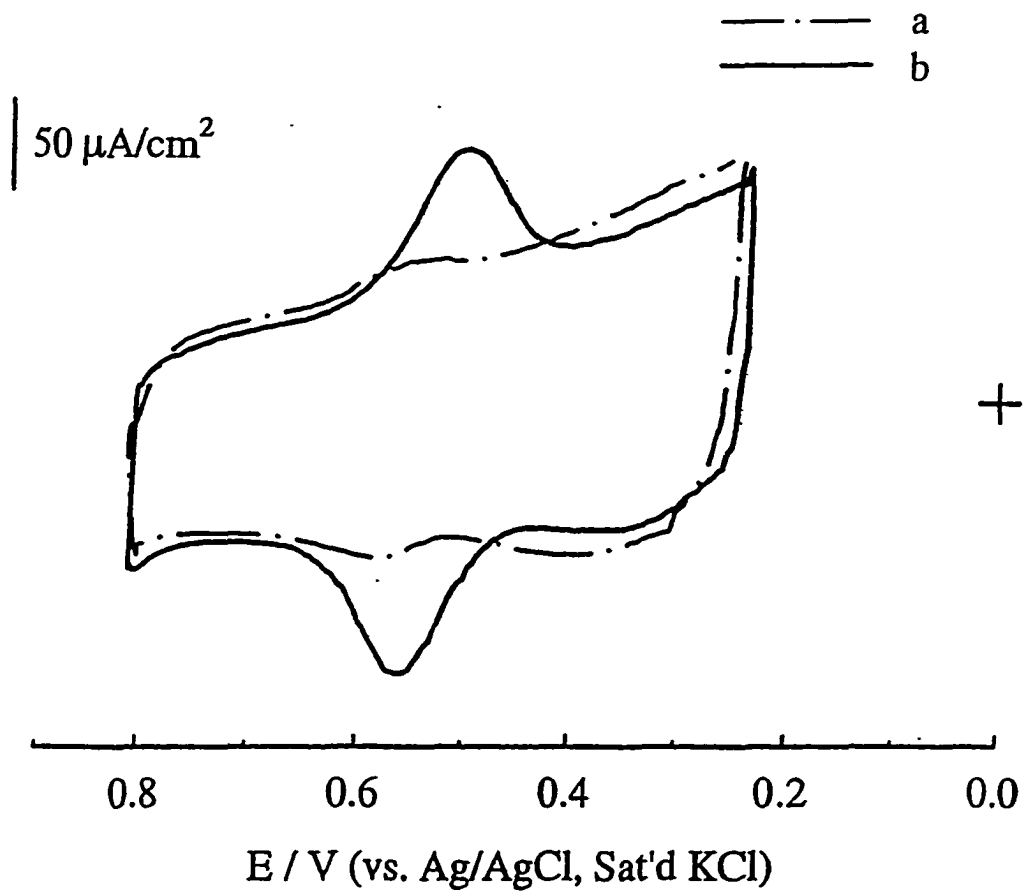


Figure 11. Cyclic voltammograms obtained in aqueous 0.1 M $HClO_4$ at DA-modified GCEs from (a) Figure 10a and (b) Figure 10b. The scan rate was 100 mV/s.

calculations. However, one cannot rule out the possibility that the relatively high coverage herein may be due to the increase in the effective surface area of the electrode. This area increase is manifested by an increase in the background charging current of the modified surface over that of the freshly polished one which results from the introduction of oxygen functionalities on GCE surface by applying a high anodic potential to GCE.

Examination by XPS showed that the N/C value of 2.5% for the GCE modified in aqueous solution is much less than that obtained for the GCE modified in ethanolic solution (N/C= 6.6%), although the latter coating is electroinactive. We believe the decrease in surface coverage is because of the electrochemical oxidation of water which introduces the surface oxygen functionalities that occupy surface binding sites. This situation is supported by the much higher O/C ratio (33.6%) obtained in aqueous solution compared to that obtained in ethanolic solution (17.4%) for control experiments without adding DA.

Tests of the stability of immobilization support the formation of a covalent linkage. The CV curves show no observable change when cycled twice within the time interval of 20 min. Repetitive potential scans reproduced the original curves almost exactly. The curve changed slightly, however, when repetitively cycling 10 or more times within the same time interval, presumably due to the instability of the quinone form produced during the oxidation excursion in the cycles. For adsorbed DA, a second cycle taken 20 min after the first cycle in 0.1M $LiClO_4$ showed a decreased response (~10%). Cycling 10 or more times leads to a larger decrease in the response (15%~20%). Evidently, there is a slow equilibrium between the surface adsorbed and solution species.

Further support for covalent-bound immobilization is derived from the XPS data. The position of the peak maximum (399.3 eV) is consistent with the formation of a carbon-

nitrogen bond between the amine cation radical and an aromatic moiety of the GC surface. Comparatively, the N(1s) binding energies for aliphatic primary amines are ~398.0 eV [52]. Together, we conclude that the surface modification procedure employed results in the covalent binding of catechol functionalities on GCE which retain their ability to undergo reversible redox reactions.

Conclusions

This paper has demonstrated the ability to create electroactive GCEs via the electrooxidation of amine-containing compounds. Importantly, this procedure dramatically simplifies the fabrication of modified electrodes, a process which often involves an extended series of pretreatment, activation, and functionalization steps. This procedure also provides a much higher coverage of the immobilized species.

We also found the adsorption and surface orientation of DA on GCEs depends strongly on pH. The electrochemical oxidation of the amine group can be controlled by changing the $[H^+]$ of the electrolyte solution. It is, however, possible to protect the catechol functionality from oxidation in basic solutions by forming a metal complex.

Studies are underway to investigate the complexing ability of catechol in ethanolic solution in order to improve the coverage. To increase the stability of these tethered catechol systems, the synthesis of similar molecules is currently being explored. Inclusion of substituents at various ring positions to "cap" reactive sites of the catechol is being investigated in attempts to increase the lifetime of the immobilized catechol/quinone couple.

Acknowledgments

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CHAPTER 2. PRELIMINARY STUDY OF CREATING REVERSED-PHASE COATINGS ON CARBONACEOUS MATERIALS BY THE ELECTROCHEMICAL OXIDATION OF AMINE-CONTAINING COMPOUNDS

A paper to be submitted to *Langmuir*

Bin Lin, James W. Anderegg, Chuan-Jian Zhong, and Marc D. Porter

Abstract

This paper presents the results of our ongoing investigations on the modification of the surfaces of glassy carbon electrodes (GCEs) by the electrooxidation of amine-containing compounds. In addition to primary amines, a strategy to immobilize secondary and tertiary amines on GCEs is developed. To implement this strategy in achieving a high surface coverage, the correlation between the surface coverage of amines and surface oxides was studied. In particular, we focus on the feasibility of utilizing such a modification strategy to create reversed-phase architectures on the carbon materials. It was found that the wetting properties of modified GCE surfaces were dependent on the chain length of alkylamine. The resulting glassy carbon surfaces showed high hydrophobicity and excellent chemical stability in strong acid and strong base, demonstrating the viability of the alkylamine-modified carbons as a new class of reversed-phase materials for liquid chromatography.

Introduction

Bonded phases are the most used types of stationary phase in liquid chromatography (LC) and have a very broad range of applications [1, 2]. Many of them are chemically

modified forms of silica. The most popular bonded phases are, without doubt, the reversed-phases which consist solely of aliphatic hydrocarbon chains bonded to silica. Of the reversed-phases, the octadecyl silica (ODS) stationary phases are by far the most widely used. However, the limited stability of such phases in strongly acidic and strongly alkaline solutions limits their use at extremes in pH, conditions especially important for the separation of materials of biological origin [3]. This situation led us to pursue the development of new bonded phases that are chemically stable in strong acid and in strong base.

In the novel separation technique termed electrochemically modulated liquid chromatography (EMLC) developed in our group [4-7], porous graphitic carbon (PGC) and glassy carbon (GC) are used as stationary phases because of their high electrical conductivity. Because of their chemical inertness, PGC and GC also allow the use of a wider range of mobile phase pH values. However, one of the major problems in the application of PGC and GC in separations is that many types of aromatic species, such as polycyclic aromatic hydrocarbons (PAHs), adsorb strongly to their surfaces through donor-acceptor interactions [8-10]. Retention is often so strong [11-13] that mixtures of PAHs composed of more than three rings are poorly resolved. Besides, carbon bears a variety of polar functional groups that originate from the reaction between active carbon surface atoms and oxygen. This surface heterogeneity is one of the causes responsible for poorly shaped elution bands [14]. All of the above-mentioned problems motivated us to pursue the development of alternative stationary phases. We speculated that the chemical modification of carbon surfaces by linear alkyl chains may improve the separation of strongly retained species such as PAHs since the solutes will be spaced away from the carbon surface and the surface oxides will be shielded

by a layer of alkyl chains.

Modification of carbon surfaces is an important objective in electrochemistry and material science. Up to now, most of the methods used for modifying the carbon surface involve vigorous oxidation processes leading to the formation of carboxyl, quinone, ketone, or hydroxyl groups on the surface that can then be coupled with the molecule to be immobilized [15-17]. However, all of these procedures for oxidation lead to the formation of oxygenated functional groups on the carbon surface whose nature and number are difficult to identify and control [18-21]. Besides, modified carbon obtained by this way is not pH-stable. It would therefore be desirable to design a more facile and less vigorous way of modifying carbon surfaces.

Over the past several years, a novel route has been devised and tested by several groups [22], including our own [23, 24], for modifying carbon-based materials. This route is based on the electrooxidation of amine-containing compounds. The work described in this paper extends our previous study where we found secondary and tertiary amines are not effectively immobilized to the glassy carbon electrode (GCE) surface under the experimental conditions used [23]. We show herein that secondary and tertiary amines can be immobilized on carbon. The relationship between the surface coverage of amines and surface oxides has been explored, and the idea for creating a reversed-phase on carbon materials was demonstrated in preliminary experiments by fabricating a highly hydrophobic glassy carbon surface. X-ray photoelectron spectroscopy (XPS) was used as the primary tool for coverage assessment, and the wetting properties of the various alkyl chain modified GCEs were examined by contact angle measurements. Contact angle measurements have proven

remarkably useful and sensitive in characterizing surface functionality and structure [25].

Experimental Section

A. Reagents and Chemicals. Butylamine, hexylamine, octylamine, dodecylamine, hexadecylamine, octadecylamine, phenethylamine, tetrabutylammonium tetrafluoroborate (NBu_4BF_4), and lithium perchlorate were from Aldrich. Potassium chloride, acetonitrile (HPLC grade), formamide, and glycerol were from Fisher. α-Alumina (0.3 μm) was from Buehler. Absolute ethanol (punctilious grade) was from Quantum Chemical Co. The acetonitrile (ACN) was dried over 3 Å molecule sieves before use. Tetrabutylammonium tetrafluoroborate and lithium perchlorate were dried under vacuum. All other chemicals were used as received. House-distilled water was further processed using a Millipore Milli-Q water purification system and was used in all solution and electrode preparations.

B. Carbon Substrate Preparation. The GCEs (Tokai Carbon, grade GC-20) were prepared by polishing first with silicon carbide powder (600 grit) followed by 1.0 μm and 0.3 μm alumina (Buehler). The GCEs were sonicated in water for 20 min after each polishing step. After the initial polishing, the GCEs were resurfaced using 0.3 μm alumina only. All GCEs were sonicated for 20 min in water, rinsed with water and ethanol, and dried with a stream of high-purity nitrogen immediately before use. After electrochemical treatment in the amine-containing electrolytic solutions, the GCEs were rinsed with ethanol and water and sonicated for 20 min in ethanol and for 20 min in water. This process was used to remove any physisorbed or unreacted materials from the electrode surface. Samples were then characterized using XPS or contact angle measurements.

C. Pretreatment of Glassy Carbon Surface

Electrochemical pretreatment (ECP): The freshly polished GCE was immersed in aqueous 0.1 M $LiClO_4$ solution with the applied voltage held at +1.8 V for 30 min.

Vacuum heat treatment (VHT): The heat treatment was done in the XPS system by heating the GC substrate to 725 °C on a molybdenum sample holder. The temperature, which was monitored by a chromel-alumel thermocouple, was maintained at 725 °C for about 20 min at a pressure $<10^{-8}$ torr. After heat treatment, the GCE was allowed to cool to 30 °C before removed through the sample load lock.

D. Electrochemistry. Electrochemical experiments were performed using a CV-27 potentiostat (Bioanalytical System) and a Houston Instruments Omnigraphic 2000 XY recorder. A conventional three-electrode cell was used with the geometric area of the electrode defined by the circular opening in an inert elastomer gasket (1.13 cm^2). A Pt coil auxiliary electrode and Ag/AgCl/saturated KCl reference electrode (-51 mV vs SCE) were used; all voltages are given with respect to this reference.

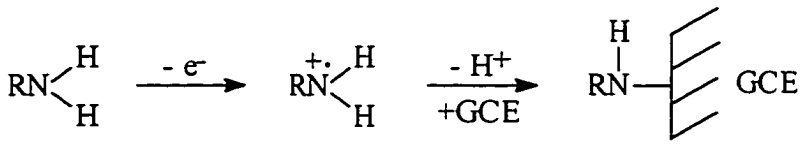
E. X-ray Photoelectron Spectroscopy. The XPS data were acquired with a Physical Electronics Model 5500 multitechnique surface analysis system equipped with a hemispherical analyzer, a monochromator, and a multichannel detector. Monochromatic Al Kα radiation (1486.6 eV) at 250 W was used for excitation. The photoelectrons were collected at 45° from the surface parallel. Binding energies were referenced to the C(1s) emission band at 284.3 eV. Acquisition times for the spectra were between 2 and 7 min for the N(1s) region. The pressure of the ion-pumped ultrahigh vacuum chamber was less than $3x10^{-9}$ Torr during analysis. The elemental nitrogen-to-carbon ratio (N/C) was used as the

major parameter for assessing the extent of modifier coverage. Values for N/C were calculated, after accounting for differences in sensitivity factors, by dividing the total number of counts under the N(1s) band by that under the C(1s) band and multiplying the result by 100. The N/C values are reported as averages of two to six samples and varied from 10 to 15% between similarly prepared samples.

F. Contact angle measurements. Contact angles were measured in the laboratory ambient using a Rame-Hart model (100-22) goniometer and were reported as advancing contact angles. Briefly, the tip of the pipette was moved close to the surface, a drop of a fixed volume (~3 μL) was formed on the end of the pipette, and lowered until the liquid contacted the surface. As the pipette tip was raised, the drop detached from the tip and advanced across the surface. The contact angles were measured within 10 seconds after detachment. The influence of the adsorption of impurities from the air to GCE was minimized by performing the contact angle measurements immediately after the electrochemical immobilization and cleaning procedures.

Results and Discussion

Immobilization of Various N-Substituted Alkylamines at the GCE Surface. The general method for the modification of glassy carbon electrodes (GCEs) via the electrochemical oxidation of amine-containing compounds has been described elsewhere [22, 23]. Briefly, the process proceeds initially via the one-electron oxidation of an amine functionality to its corresponding cation radical, which subsequently forms a carbon-nitrogen linkage at the carbon surface as generalized in Scheme 1.



Scheme 1

In our previous study [23], the utility of this route was well demonstrated by the immobilization of a simple primary amine at the GCE surface. However, an investigation of the influence of substituents on the nitrogen atom (e.g., primary, secondary, tertiary amines) revealed that this route is not effective for the immobilization of secondary and tertiary amines. Figure 1 shows cyclic voltammetric (CV) curves obtained at a GCE in ethanolic 0.1 M $LiClO_4$ solutions containing 2 mM of either butylamine (Figure 1a), *N*-ethylbutylamine (Figure 1b), or triethylamine (Figure 1c). Chemically irreversible oxidation waves are apparent for all three of the amine-containing compounds. The negative shift in peak-current voltage (E_p) (cf. Table 1) as alkyl substituents are added to the amine group indicates that the electrooxidation of the amine to its cation radical intermediate is more facile for secondary and tertiary amines than that for primary amines. However, the N/C values from XPS spectra (cf. $(N/C)^a$ in Table 1) show that the highest surface coverage of primary amines (N/C = 11.3% for butylamine) was ~9 times higher than that of secondary amines (N/C=1.3% for *N*-ethylbutylamine) and of tertiary amines (N/C = 1.4% for triethylamine). The N/C values for freshly polished GCE surfaces varied from 0.0% to 0.6%. These results show that, in spite of the more facile and more extensive oxidation of secondary and tertiary amines, their corresponding cation radicals are ineffective in binding to the GCE surface under these experimental conditions. We previously attributed this behavior to a steric effect whereby

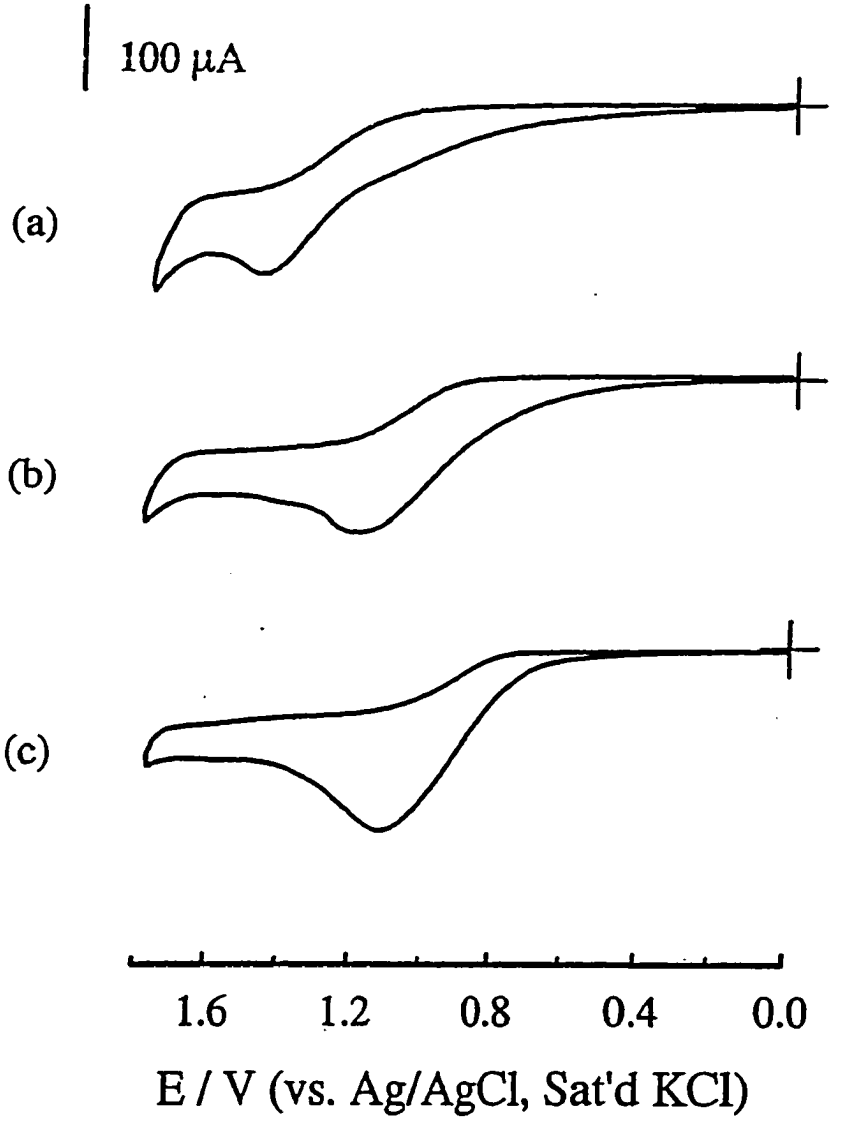
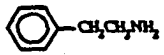


Figure 1. Cyclic voltammograms obtained at freshly polished GCEs in ethanolic 0.1 M $LiClO_4$ solutions containing 2 mM of (a) butylamine, (b) *N*-ethylbutylamine, and (c) triethylamine. The scan rate was 10 mV/s in each case.

Table 1. Chemical Structures, Anodic Peak Voltages (E_p), and Maximum N/C Values for Various Nitrogen-Containing Compounds

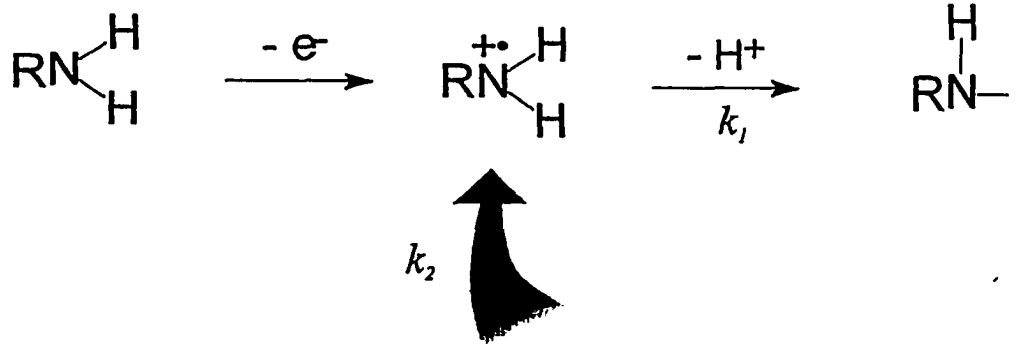
chemical name	chemical structure	E_{pa} (V)	max. (N/C) x 100[a]	max.(N/C) x100[b]
butylamine	$CH_3(CH_2)_3NH_2$	+ 1.45	11.3	17.8
phenethylamine		+ 1.48	9.6	15.5
N-methylbutylamine	$CH_3(CH_2)_3NHCH_3$	+ 1.19	1.4	12.1
N-ethylbutylamine	$CH_3(CH_2)_3NHCH_2CH_3$	+ 1.20	1.3	11.3
triethylamine	$(CH_3CH_2)_3N$	+ 1.10	1.4	13.3

[a]Maximum N/C ratio obtained by electrochemical treatment in ethanolic solution.
[b]Maximum N/C ratio obtained by electrochemical treatment in methylene chloride solution.

bulky substituents on the nitrogen atom hinder accessibility of the reactive amine cation radical to surface binding sites. The following shows, however, that while steric issues may still play a role, the use of a weaker nucleophilic solvent enhances the ability to modify GCEs by the electrooxidation of secondary and tertiary amines.

From Scheme 1, we see that the one-electron oxidation of an amine functionality generates its corresponding cation radical, which is a highly electrophilic species. On the other hand, the solvent ethanol is highly nucleophilic [26]. Thus, there is a competition between the surface and solution reactions of the cation radical, as shown in Scheme 2. The success of the derivatization of the carbon surfaces by the electrochemical oxidation of amines hinges upon the fact that the cation radical intermediates produced by Scheme 1 are not lost by a reaction with solvent. For secondary and tertiary amines, the amine cation radicals produced are stabilized by the alkyl substituents [27, 28] and do not react as rapidly with the carbon surface. The steric factor caused by the presence of the additional substituents on the amine functionality may also contribute to the slow reaction rate for immobilization. We believe that these factors, acting either in concert or independently, are the most likely causes of the lower coverage. The next series of experiments were carried out in a nucleophilic solvent with a weaker nucleophilicity (i.e., methylene chloride) to assess the role of the solvent.

To test whether surface coverage was affected by solvents with different nucleophilicities, XPS was used to follow the changes in the relative nitrogen content at the GCE surface as a function of number of voltage cycles in different solvent systems. The values of N/C are plotted vs. the number of voltage cycles in Figure 2. Figure 2A is the result



Scheme 2

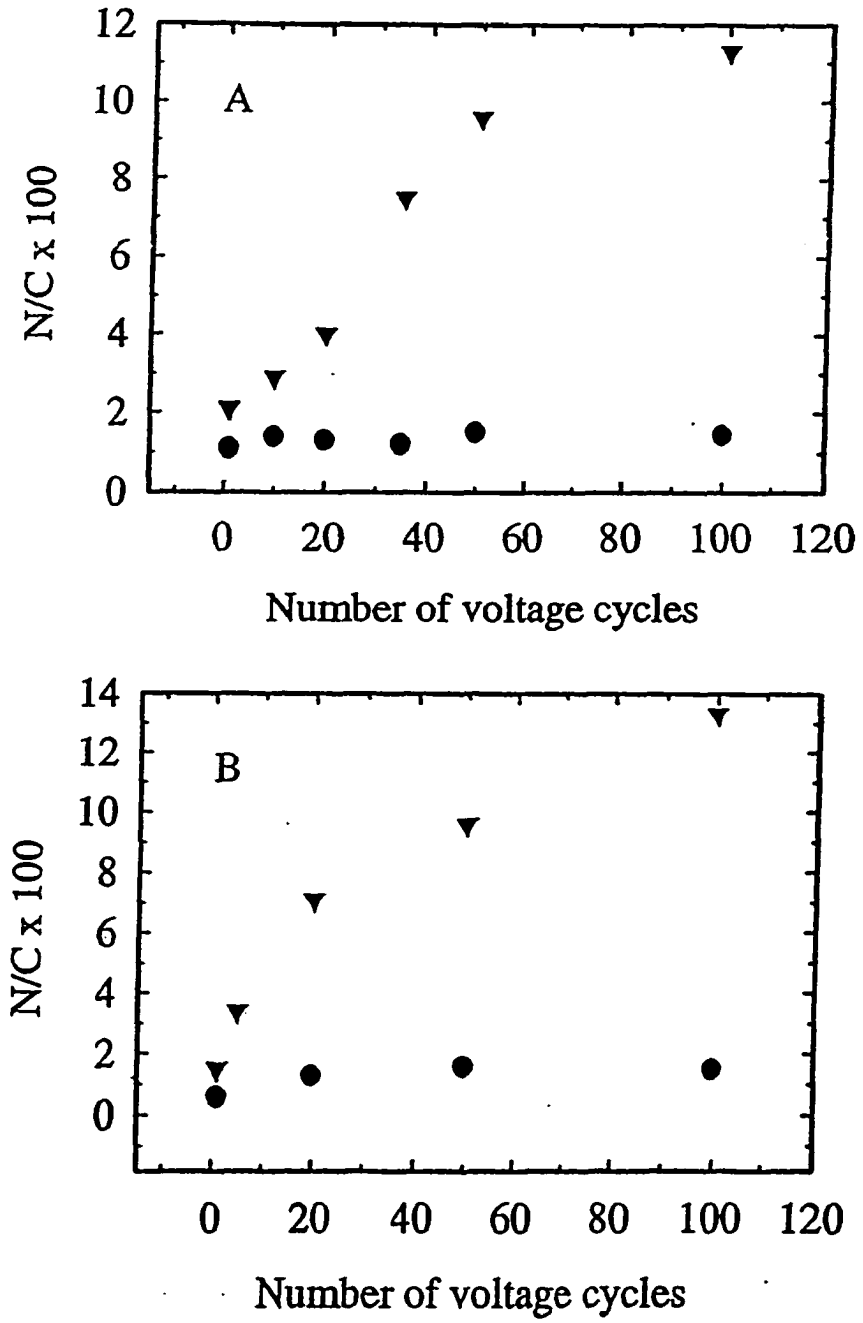


Figure 2. Variation of N/C values with the number of voltage cycles for the immobilization of amines on GCEs in 0.1 M NBu_4BF_4 ethanolic solutions (●) or methylene chloride solutions (▼) containing 2mM (A) *N*-ethylbutylamine, and (B) triethylamine. The applied voltage scanned between 0.00 V and +1.80 V at 10 mV/s.

for freshly polished GCEs immersed in an ethanolic solution (·) or a methylene chloride solution (·) containing 2 mM *N*-ethylbutylamine and 0.1 M NBu_4BF_4 with the applied voltage scanned at 10 mV/s between 0.00 V and +1.80 V for differing numbers of cycles. The data show that the surface coverage of *N*-ethylbutylamine dramatically increases with the number of cycles in methylene chloride. The highest coverage (N/C = 11.3%) was obtained after 100 voltage cycles. In contrast, the highest coverage (N/C = 1.3%) in ethanol was obtained within the first two voltage cycles. Successive scans did not detectably increase the N/C value.

There are two important conclusions from these results. First, most of cation radicals produced in ethanol by the electrooxidation of secondary amines are consumed by the stronger nucleophilic solvent. There is, however, a small but detectable level of immobilization (N/C = 1.3%). We suspect, since the possibility of adsorption can be ruled out by control experiments, that the observed level of coverage may be due to the relatively fast reaction rate between radicals and carbon surface within the first few scans because of the presence of a large amount of active sites on the surface that are not sterically hindered. These sites are consumed and/or become sterically hindered after the first few scans, which leads to slower reaction rates between the radicals and the GCE. In other words, almost all of the radicals are consumed by the nucleophilic solvent before they can react with the carbon surface. Second, the reaction rate between radicals and methylene chloride is much lower because of the lower nucleophilicity of the solvent. Since the lifetime of the radical intermediates is longer, the probability of reacting with the GCE surface is greater, and the observed coverage significantly increases.

Figure 2B presents the results for immobilizing triethylamine under the same experimental conditions as in Figure 2A. Similar results were obtained in terms of the effect of the nucleophilicity of the solvent. The highest coverage using in methylene chloride solution (N/C = 13.3%) is much higher than that obtained in ethanolic solution (N/C = 1.4%). In ethanol, the highest coverage for triethylamine was obtained after 20 voltage cycles as compared to two cycles for *N*-ethylbutylamine. This difference suggests a slower reaction rate between tertiary amine radicals and the GCE surface than that for secondary amine radicals, and is consistent with the stabilization of amine cation radicals by an additional alkyl group [27, 28].

Relationship between Coverage of Amines and Surface Oxygen Functional Groups on GCEs. In our previous experiments in exploring the immobilization of dopamine (DA) on GCE [24], we found that the surface coverage of DA on GCE modified in an aqueous solution is much lower than that for the GCE modified in an ethanolic solution. We attributed this difference to the introduction of surface oxygen functionalities by the anodic oxidation of water at the high value of applied potential used in this process. This result indicates that the choice of the solvent system for immobilization is critical for the improvement of coverage. In this section, we investigated the influence of surface oxygen functionalities on the surface coverage of amines in detail.

To this end, electrochemical pretreatments (ECP) and vacuum heat treatments (VHT) of freshly polished GCEs were used to alter the oxygen content on GCE surface [15, 16, 29]. The vacuum heat treatment is based on the procedure developed by the Kuwana group [29]. Figure 3 compares the XPS survey spectra of electrochemically pretreated (a), freshly

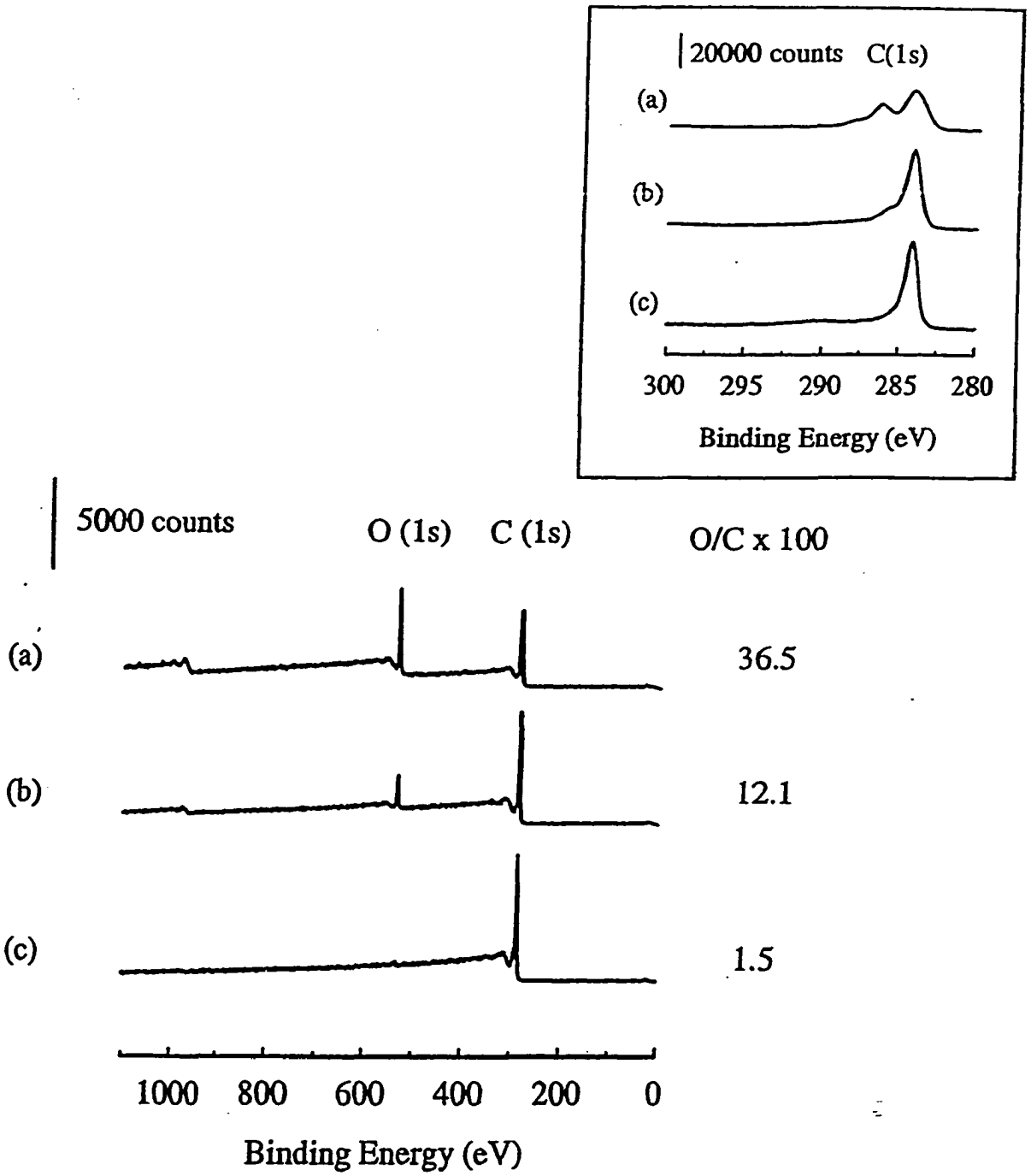


Figure 3. X-ray photoelectron survey spectra for (a) electrochemical pretreated GCE, (b) freshly polished GCE, and (c) vacuum heat treated GCE. The insert in the figure shows corresponding high resolution C(1s) spectra.

polished (b), and vacuum heated GCEs (c); the insert in Figure 3 shows the corresponding high resolution C(1s) region spectra for each of the GCEs. A comparison of the intensities of the oxygen peaks for the different pretreated GCE surfaces reveals that the oxygen content is strongly increased after ECP, and the oxygen content is greatly diminished after VHT. This result demonstrates that the VHT removes most, if not all, of the surface oxygen functionalities as evidenced by the extremely low O/C ratio.

The high-resolution C(1s) spectra in the insert of Figure 3 further details the differences between the three pretreatments of this region. ECP dramatically increases the high binding energy (BE) components which indicates that ECP has incorporated oxygen covalently onto the carbon surface (i.e., the O/C value increases from 12.1% for the polished to 36.5% for the ECP electrode). The high binding energy "tail" is taken as an indication of the presence of several different types of surface oxygen functional groups (e.g., quinone, ketone, hydroxyl groups) [30, 31]. In contrast, the band 2.5 to 3 eV higher in BE from the main C(1s) band, which is present in the "polished-only" GCE, was eliminated after VHT (i.e., the O/C value decreases from 12.1% for the polished to 1.5% for the VHT electrode). This result is expected since surface oxides are known to be removed by VHT.

Parts a-e of Figure 4 show high resolution XPS spectra in the O(1s) regions and N(1s) regions for different pretreated GCEs that were cycled five times between 0.00 V and +1.80 V in 0.1 M $LiClO_4$ solutions with different solvents containing 2 mM phenethylamine. Under these conditions, limiting surface coverages are realized. In curve a, an ECP GCE was modified in an ethanolic solution, whereas an as-polished GCE was modified in an ethanolic solution in curve b. In curve c, an as-polished GCE was modified in an acetonitrilic solution.

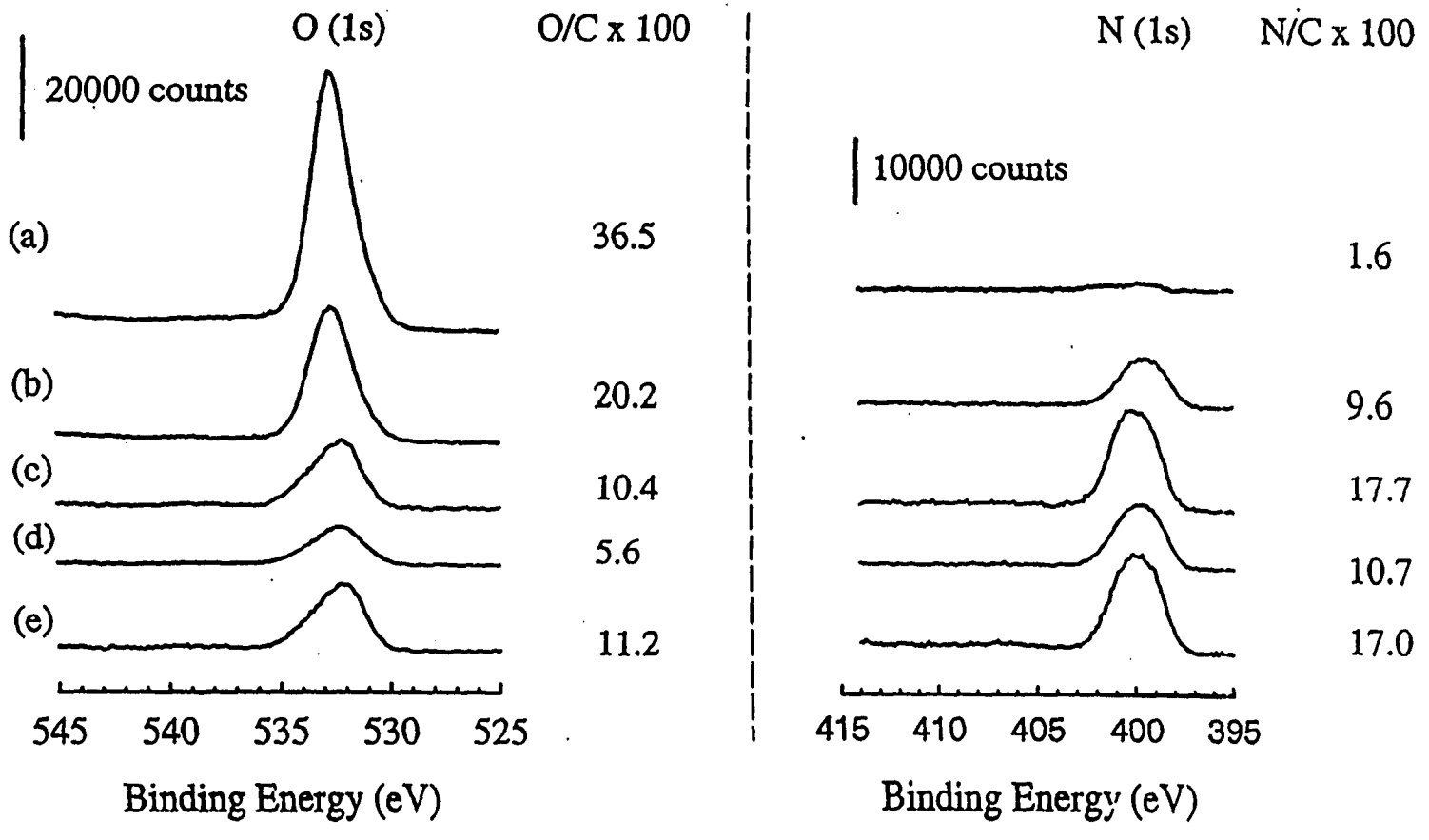


Figure 4. X-ray photoelectron spectra in the O(1s) and N(1s) region for (a) electrochemical pretreated GCE and (b) freshly polished GCE immersed in an ethanolic solution, (c) freshly polished GCE and (d) vacuum heat treated GCE in an acetonitrile solution, (e) vacuum heat treated GCE in an mixed solution of acetonitrile and water (ACN:H_2O ; 100:1 (v/v)) containing 2 mM phenethylamine. In each case, the applied voltage was cycled 5 times between 0.00 V and +1.80 V at 10 mV/s.

In curve d, a VHT GCE was modified in an acetonitrilic solution. In curve e, VHT GCE was modified in a solvent mixture of ACN:H_2O = 100:1 (v/v). From curve a to curve c, an analysis of the XPS data shows an decrease in the O/C ratio from 36.5% to 20.2% to 10.4% while the N/C ratios increases from 1.6% to 9.6% to 17.7%, respectively. It is therefore apparent that the high oxygen content on GCE formed by ECP impedes the immobilization of amines. In curve b, using ethanol as solvent causes higher oxygen content when compared to using acetonitrile as solvent in curve c. This higher oxygen content was introduced by the anodic oxidation of ethanol at the high value of applied potential used in the process. As is expected, the N/C value in curve b is lower than that in curve c. This result clearly indicates that using acetonitrile as solvent instead of ethanol is beneficial for obtaining a high coverage.

In curve d, GCE was treated by VHT prior to immobilization of amine. This pretreatment should effectively remove the surface oxygen functionalities and should expose more binding sites suitable for immobilization. A higher surface coverage was therefore expected, but the result did not support our expectation. The N/C ratio (10.7%) is much lower than that in part c (17.7%), although having a lower oxygen content (O/C=5.6%) on surface.

There are two possibilities for this unexpected result. (i) The conversion of surface oxygen functionalities to carbon-carbon bonds occurs as the temperature rises during VHT [29]. This conversion causes bond reorganization and decreases the edge plane areas which are the active sites for immobilization. This possibility is supported by the significantly smaller oxidation wave in the CV curve observed at the VHT GCE for immobilization of that

amine than that at the analogous oxidation at an as-polished GCE. (ii) Amine radical coupling reactions may be activated for ortho and para attack by electron-releasing substituents such as hydroxyl groups and ketones on the GCE surface [32-35], and VHT may remove the surface oxygen functional groups responsible for this activation and cause the decrease in coverage.

We should also mention that the GCE was briefly exposed to air during the transfer from the VHT chamber to the electrochemical apparatus and trace water in solution was oxidized during immobilization, which may account for the presence of the higher O/C (5.6%) in curve d than that in Figure 3c (1.5%). Otherwise, the coverage of amine would be even lower. In curve e, we purposely added a known amount of water to the acetonitrile solution. The oxidation of water at these high applied potentials greatly increases the oxygen content on VHT GCE; the O/C ratio (11.2%) is very close to that for as-polished GCE in curve c. Correspondingly, it was observed that the N/C ratio (17.0%) is much higher than that in curve d (10.7%), and very close to that in curve c (17.7%). This result can be explained as follows: (i) oxidation-induced fracturing of the carbon lattice creates new active edge plane sites [36, 37], and (ii) the increased number of surface oxygen functional groups actives amine coupling reactions [32-35].

From the results described above, it appears that an optimal O/C ratio exists for obtaining the highest coverage. Below this ratio, the surface coverage is lower because of the decreased number of active edge sites for binding or a deactivation of the GCE surface for amine coupling reactions. Above this ratio, the coverage is also lower because of the competition for binding sites between surface oxides and amine compounds.

Wettability for GCEs Immobilized by Primary Alkylamine with Various Chain Lengths. In this section, we present and discuss the results of a characterization of GCE surfaces immobilized by alkylamines having various chain lengths by contact angle measurements. The immobilization procedure is similar to that described in the previous section. Briefly, GCEs were cycled five times between 0.00 V and +1.80 V in ethanolic 0.1 M $LiClO_4$ solutions containing 4 mM of either butylamine, hexylamine, octylamine, dodecylamine, hexadecylamine, or octadecylamine, respectively. We used ethanol because of the higher solubility of the long chain alkylamines in this solvent.

The wetting properties of GCEs immobilized by various alkylamines were examined with contact angle measurements [25]. The results are given as a function of chain length in Figure 5 using the polar liquids formamide, glycerol, and water. Wetting properties for freshly polished GCE are included for comparison. The uncertainty in these data (sample to sample variation) is ±3°. Two observations from the data in Figure 5 are immediately apparent. First, the advancing contact angles (θ_a) for each liquid increase as the chain length increases, indicative of a decrease in surface free energy. For both glycerol and water, θ_a's reach limiting values at n~12. The limiting θ_a's are ~98° for water and ~87° for glycerol, and are indicative of a very low surface free energy. For formamide, θ_a reaches limiting value 80.5 at n~16. Second, these limiting θ_a's are less than those observed for well-ordered closely packed alkyl chain monolayers on Au or Ag [25, 38-41]. There are two possible causes for this result: (i) the relatively rough surface of GCE compared to those atomically smooth metal surface causes poor packing in the alkyl chain arrangement on the GCE, and (ii) alkyl chains are not closest packed because surface coverage is limited by the edge plane

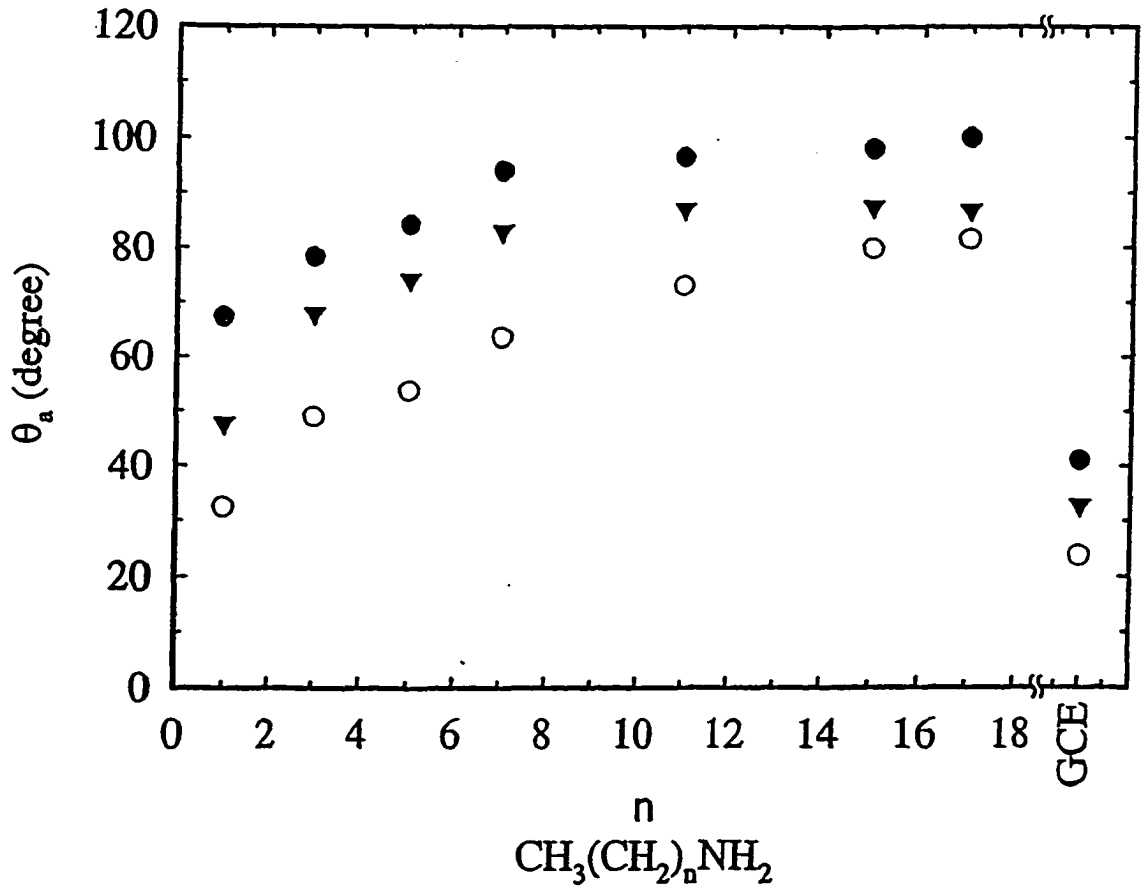


Figure 5. Advancing contact angles of formamide (○), glycerol (▼)and water (●) for alkylamine ($CH_3(CH_2)_nNH_2$) modified GCE surface.
GCE = freshly polished GCE surface.

density and surface oxides of the GCE surface.

For n <12, the θ_a's for those three probe liquids decrease with chain length. We believe this trend arises from a combination of two sources: an increasing disorder in the structure of the short-chain monolayers and the sensitivity of the probe liquid to the underlying substrate [38].

Variation of the Surface Composition with the Potential Cycles and Stability of the amine-GCE linkage. As already mentioned, there is a competition between amine and surface oxides for occupying the active sites for the coupling reactions. As depicted in Figure 6, the surface composition of amine and oxygen functional groups on GCEs can be varied by the number of potential cycles. In this case, GCEs were scanned between 0.00 V and +1.80 V in ethanolic 0.1 M $LiClO_4$ solutions containing 4 mM of octadecylamine. The values of N/C and O/C are plotted vs. number of voltage cycles in Figure 6. It is observed that N/C ratio reached the limiting value (~4.1%) after four cycles. However, the O/C ratio keeps increasing and does not reach a limiting value within 48 cycles. The difference in the trends may be explained by steric effects. After four cycles, the formation of the octadecyl monolayer may prevent additional octadecylamine to approach the remaining GCE active sites, and a determined limiting surface coverage is obtained. However, because of its relatively small size, ethanol can still move through surface layer and react with the GCE active sites. From these results, we conclude that four to five voltage cycles are needed for obtaining a limiting coverage of an octadecyl chain without further increases in surface oxides.

Based on our interests in the application of carbon-based materials coated with alkyl

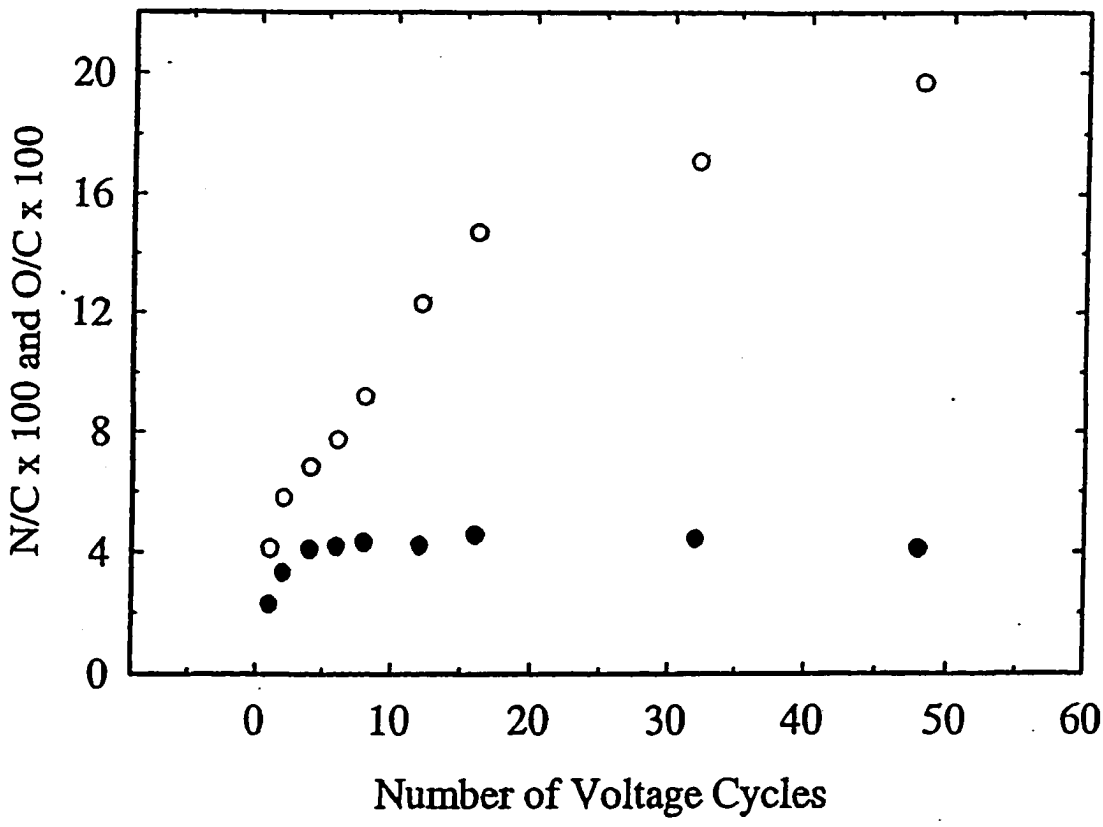


Figure 6. Variation of N/C (●) and O/C (○) values with the number of voltage cycles for immobilization of GC electrodes in 0.1 M $LiClO_4$ ethanolic solutions containing 4 mM octadecylamine with the applied voltage scanned between 0.00V and +1.80 V for different cycles. The scan rate was 10 mV/s.

chains as stationary reversed-phases for new forms of chemical separations developed in our group [4, 5], we also tested the ability of the reversed-phase obtained by the electrooxidation of amine to withstand exposure to strong acid and to strong base. The chemical stability of the immobilization was tested as follows: GCEs were cycled five times between 0.00 V and +1.80 V in ethanolic 0.1 M $LiClO_4$ solutions containing 4 mM of octadecylamine. After electrochemical treatment, the cleaned GCEs were immersed in different concentrations of H_2SO_4 and NaOH solutions for 24 h. After cleaning thoroughly by ultrasonication in deionized water and ethanol, the octadecylamine-modified GCEs were examined by XPS and contact angle measurements. The results are summarized in Table 2.

Table 2. Chemical Stability of Octadecylamine Modified GCEs in Strong Acid and Base

	in 1.0 M NaOH		in 0.5 M H_2SO_4		in 2.0 M H_2SO_4	
	before	after	before	after	before	after
100(N/C)	4.5	4.2	4.2	4.4	4.6	4.2
θ_a (H_2O)	102°	98°	97°	95°	100°	91°

Examination of N/C ratio by XPS shows no significant change in the surface coverage of octadecylamine before and after soaking in strongly acidic and basic solutions. This result demonstrates the excellent stability of the amine-GCE linkage under harsh conditions and also indicates the nitrogen-carbon bond formed is hydrolytically stable. Other types of immobilized species such as amides or salts formed with acidic surface oxides would be highly unstable under such acidic conditions. After soaking in 1.0 M NaOH and 0.5 M H_2SO_4 solutions, we observed a slight decrease in the contact angle for the modified surface. We suspect that this decrease is due to the adsorption of contaminants from air or solution

after extended immersions in acidic or basic solutions. The modified GCE exposed to air for several hours also showed the similar decrease of contact angle. After soaking in 2 M H_2SO_4 solution, however, there is a significant decrease of contact angle. The possibility of N-C bond cleavage is excluded because of no significant decrease in N/C value. We doubt this decrease is due to an increase in the roughness on the GCE surface caused by the relatively high concentration H_2SO_4, which is a strong oxidant. Further investigations along these lines are underway.

To ensure that the chemical stability of octadecylamine modified GCE was not because of protection by closely-packed long alkyl chains, we also tested a butylamine modified surface and observed a similar stability. From the results presented above, we predict that the carbon-based materials immobilized with alkyl chain by electrochemical oxidation of corresponding alkylamine will emerge as promising stationary reversed-phases for separations in extremes of pH.

Conclusions

This paper has demonstrated that secondary and tertiary amines can bind effectively to the GCE surface in solvents that are poor nucleophiles. This finding greatly extends the range and scope of utilizing the electrochemical oxidation of amine as a route for the creation of chemically modified carbon materials with various surface structures on the molecular level. The surface coverage of immobilized amine compound is closely related to the surface oxygen functionalities. There is a competition between amine and surface oxides for binding sites. Excess surface oxides hinder accessibility of amine compound to active sites at the

GCE surface and cause a lower coverage. If the amount of surface oxygen contents is, however, too small, the surface coverage is detectably lower. It was also found that the alkylamine modified GC surface is very hydrophobic and very stable even under strongly acidic and strongly basic environments, suggesting this method is a promising route for the fabrication of reversed stationary phases in HPLC as a demand for bonded phases with good pH stability for the separation of materials of biological origin becomes more urgent. Applications of this method for the modification of small carbon particles as packing materials in HPLC are underway in our laboratory.

Acknowledgment

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CHAPTER 3. SEPARATIONS OF BIOGENIC AMINE NEUROTRANSMITTER BY ELECTROCHEMICALLY MODULATED LIQUID CHROMATOGRAPHY (EMLC): I. CATECHOLAMINES AND STRUCTURALLY RELATED COMPOUNDS

A paper to be submitted to *Journal of Chromatography*

Bin Lin, Hajime Takano, and Marc D. Porter

Abstract

Electrochemically-modulated liquid chromatography (EMLC) has been applied to the separation of a mixture of structurally similar catecholamines (i.e. norepinephrine, epinephrine, isoproterenol, dopamine, dihydroxyphenylalanine) by using porous graphitic carbon as the stationary phase. Changes in the voltage applied to the column markedly affect the resolution of the separation as well as the elution order of the components in the mixture. The mixture is well resolved at a large negative value of applied potential while only marginally increasing the overall elution time. We also show that the procedure required for the optimization of this EMLC separation is more effective than those for conventional solvent elution strategies. Mechanistic aspects in terms of the influence of changes in the applied potential on the extent of the interactions between these analytes and the stationary phase are qualitatively explored by examining the retention of several compounds that are structurally similar to catecholamine (i.e., octopamine, normetanephrine, tyramine, and 3-methoxytyramine). The results show that the retention of catecholamines is affected to different degrees by the eluent pH, depending on the functional groups present on the molecule.

Introduction

Catecholamines play an important role in regulating the nervous system of both vertebrates and invertebrates. These amines act as neurotransmitters through chemical synapses. Some catecholamines also modulate synaptic transmission and muscle contraction, acting therefore as neuromodulators [1-3]. In addition, these compounds may perform a hormonal function in regulating physiological processes [4, 5].

To delineate the mechanism of such control and regulation, a wide number of analytical methods have been developed for monitoring the fate of the catecholamines and related compounds, including their precursors and metabolites [6-11]. Of these methods, high performance liquid chromatography (HPLC) is often used for the determination and separation of catecholamines and their related compounds in samples collected, for example, from brain tissue and body fluids [12-14]. However, optimization of conventional HPLC separations by manipulation of ion-pairing reagent, temperature, organic modifier, or eluent pH can be a labor intensive process. Furthermore, alterations in the separation conditions also affect the sensitivity and reliability of detection, especially when using electrochemical detection which is the most commonly used approach for the low level determination of catecholamines [15]. Therefore, efforts to improve and simplify the analytical methodology for these types of samples are of fundamental importance [13, 16].

In our laboratory, we have been examing a novel liquid chromatographic technique that can be applied to the facile separation of catecholamines and related compounds. This new technique, which we have termed electrochemically modulated liquid chromatography (EMLC), relies on alterations of the retention characteristics of conductive stationary phases

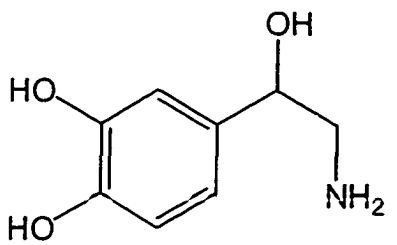
(e.g., porous graphite carbon (PGC) particles) through changes in the potential applied (E_{appl}) to a LC column that also functions as an electrochemical cell. Efforts in our [17-20] and other [21, 22] laboratories have demonstrated that this strategy can be exploited to manipulate separations for a variety of different analytes, including aromatic sulfonates, metal ions, corticosteroids, and benzodiazepines. EMLC has also been applied to enantiomeric separations by exploiting electrosorption phenomena [23]. In many of the above instances, separations with an effective resolution were readily achieved solely by changes in E_{appl}. These results argue that EMLC has the potential to become a valuable tool in the vast arsenal of separation techniques.

In this paper, we apply EMLC to the separation of a mixture of structurally similar catecholamines (i.e., norepinephrine (NE), epinephrine (E), isoproterenol (IP), dopamine (DA), and dihydroxyphenylalanine (DOPA), (see Chart 1)) at a PGC stationary phase. The goal is to assess the extent by which EMLC can be used to manipulate the separation of this class of compounds, laying a foundation for future applications of EMLC on this type of biological sample. We show that this mixture can be effectively separated by simply changing the E_{appl} applied on PGC stationary phase. We also examined the retention behavior of several other compounds that are structurally similar to catecholamine (i.e., octopamine (OCT), normetanephrine (NMN), tyramine (TYM), and 3-methoxytyramine (3-MT) (see Chart 2)). These results allow us to begin to speculate about the effect of various substituents on the retention of this class of compounds. Based on these results, the strategies that would enhance the sensitivity and selectivity toward substituent differences can be developed for the optimization of separations using EMLC.

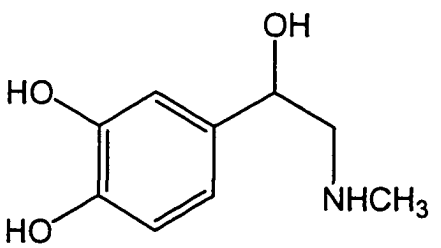
We also present the results on the dependencies of the retention of these catecholamines on the mobile phase pH. Since the degree of dissociation of these compounds depends on the pK_a values of the ionizable groups and pH of the eluent, a wide range of mobile phase pH values (1.7-9.2) is used to investigate the retentive behavior of these ionizable analytes. The results show that, depending on the various functionalities in the molecule, the retentions of catecholamines and related compounds are affected to different degrees by the eluent pH.

Experimental Section

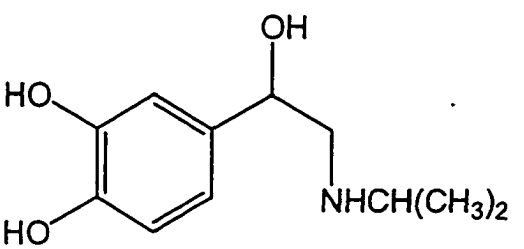
Reagents and Chemicals. Charts 1 and 2 shows the chemical structures, numerical designations, and acid dissociation constants [24] for each of the catecholamines and several related compounds. Norepinephrine, epinephrine, isoproterenol, 3,4-dihydroxyphenylalanine (i.e., DOPA), and 5-hydroxytryptamine (i.e., serotonin) were purchased from Sigma (St. Louis, MO, USA). 3-hydroxytyramine hydrochloride (i.e., dopamine hydrochloride), octopamine hydrochloride, normetanephrine hydrochloride, tyramine, and 3-methoxytyramine were purchased from Aldrich (Milwaukee, WI, USA). Sodium dihydrogen phosphate, orthophosphoric acid, sodium hydroxide, and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Dibromomethane was from Eastman Kodak (Rochester, NY, USA). All chemicals were used as received. All aqueous solutions were prepared with water that was obtained from a Millipore Milli-Q purification system (Bedford, MA, USA).



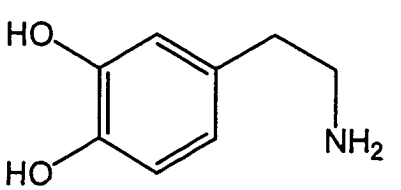
1. Norepinephrine (NE)
   pK_{a1} = 8.61
   pK_{a2} = 9.62
   pK_{a3} = 12.90



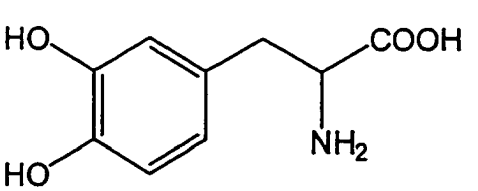
2. Epinephrine (E)
   pK_{a1} = 8.65
   pK_{a2} = 9.90
   pK_{a3} = 13.00



3. Isoproterenol (IP)
   pK_{a1} = 8.68
   pK_{a2} = 10.13
   pK_{a3} = 13.08

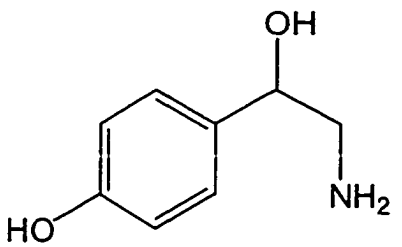


4. Dopamine (DA)
   pK_{a1} = 8.88
   pK_{a2} = 10.36
   pK_{a3} = 13.11

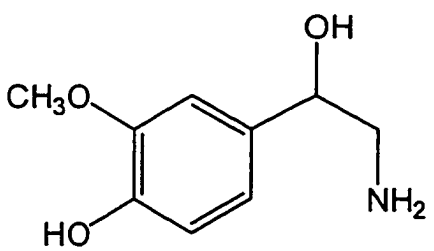


5. 3, 4-Dihydroxyphenylalanine (DOPA)
   pK_{a1} = 2.20
   pK_{a2} = 8.72
   pK_{a3} = 9.78
   pK_{a4} = 13.43

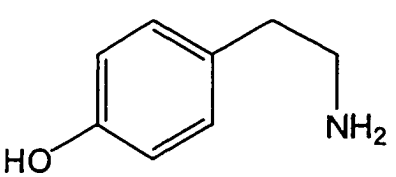
Chart 1. Structures and numerical labels for the catecholamines.



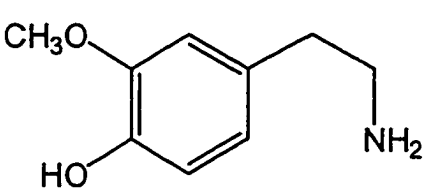
6. Octopamine (OA)
   pK_{a1} = 8.89
   pK_{a2} = 9.91



7. Normetanephrine (NMN)
   pK_{a1} = 9.54
   pK_{a2} = 9.56



8. Tyramine (TA)
   pK_{a1} = 9.45
   pK_{a2} = 10.56



9. 3-Methoxytyramine (MT)
   pK_{a1} = 9.34
   pK_{a2} = 10.47

Chart 2. Structures and numerical labels for the catecholamine-like compounds.

Chromatographic Column Construction. The details for the design of the configuration of the EMLC column has been described elsewhere [25]. Briefly, the column consists of a Nafion cation-exchange membrane in tubular form (Perma Pure) that is placed inside a porous stainless steel cylinder. The Nafion tubing serves as a container for the stationary phase, and the stainless steel cylinder both prevents the deformation of the Nafion tubing under the high pressure of chromatographic flow and acts as the auxiliary electrode in a three-electrode electrochemical cell. The Nafion tubing, which was received in its acidic form, was pretreated by immersion into a boiling solution of neat ethanol for 10 min and then into a boiling aqueous solution (1 M $LiClO_4$) for 10 min. The length and inner diameter of the stainless steel column were 9.2 cm and 0.32 cm, respectively.

Uncoated PGC spheres (Hypersil, Runcorn, UK) with a diameter of ~7 μm were used as the conductive stationary phase. The PGC spheres were first dispersed in a dibromomethane/acetonitrile (10/7, v/v) mixture to form slurry and then packed into the EMLC column at 8000 psi by using a Shandon HPLC packing pump (Pittsburgh, PA, USA). The solvent used for packing was neat acetonitrile for ~2 hrs followed by deionized water for 1 hr. Characterizations using X-ray photoelectron spectroscopy have shown that PGC is devoid of any detectable oxygen-containing functional groups (estimated detection limit: 0.2 atomic %) [17].

Instrumentation. The chromatographic experiments were performed using an HP 1050 series chromatograph equipped with a solvent cabinet, pumping system, and diode-array-detector (Hewlett-Packard, Santa Clara, CA, USA). The pH determinations were carried out using an Orion model 520A pH meter (Boston, MA, USA). The electrode was

calibrated with pH 4.00, 7.00, and 10.00 standard buffer solutions; all values of pH, regardless of solution composition, are reported with respect to this calibration. The voltage applied to the column was controlled by an Amel Instruments potentiostat-galvanostat model 2055-2056 (Milano, Italy).

Chromatographic Operation. After packing, the EMLC column was equilibrated with degassed mobile phase at a flow rate of 0.50 mL/min until a stable baseline of detector response was obtained. UV detection at 210 nm was used. Two different mixed mobile phase compositions were used. The first mixed mobile phase (i.e., Mobile Phase 1) consisted of an aqueous buffer-acetonitrile (87:13, v/v), and was used for the catecholamine separations (**1-5**). The second mixed mobile phase (i.e., Mobile Phase 2) consisted of an aqueous buffer-acetonitrile (75:25, v/v), and was used for the separation of the catecholamine-related compounds (**1, 6-9**) to avoid unnecessary long elution time by using Mobile Phase 1. For buffers with a pH greater than 4, the pH was adjusted as needed by adding sodium hydroxide to an aqueous solution of sodium dihydrogen phosphate (0.05 M). For buffers with a pH less than 4, the pH was adjusted by adding orthophosphoric acid to the sodium dihydrogen phosphate solution. After adjusting to the target pH, the buffer was filtered through a 0.20 μm PTFE filter. All potentials are reported with respect to Ag/AgCl/sat'd NaCl reference electrode. All analyte solutions were prepared in water with a concentration of ~50 ppm. Injection volumes were 5 μL. The dead volume of the column was 0.61 mL, as determined by an injection of water. Capacity factors (k') were calculated from the expression: $k'=(t_r-t_0)/t_0$ where t_r is the analyte retention time, and t_0 is the column hold-up time, which was taken as the retention time of the solvent injection peak. All reported values of k' are an

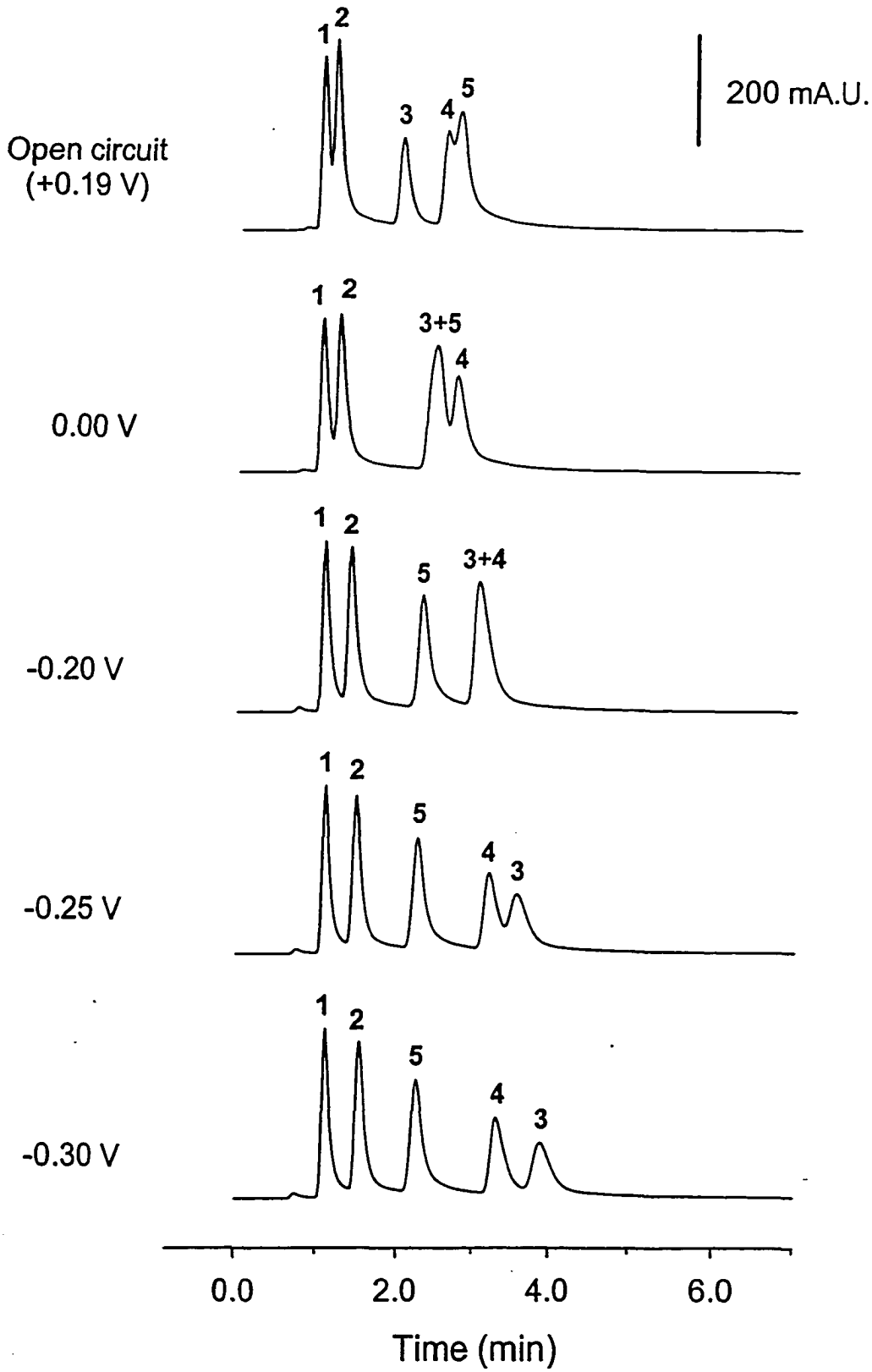
average of three or more replicate runs, and the range of values are roughly that of the size of the data points presented in several of the following figures.

Results and Discussion

A. Separation of Catecholamines at Open Circuit. The chromatograms in Figure 1 present the separations of a mixture of **1-5** at the open circuit potential (+0.19 V) as well as at several values of applied potential (E_{appl}) lower than the open circuit potential using Mobile Phase 1. The use of this mobile phase represents a compromise of two factors: 1) The stability of catecholamine in acidic media (i.e., catecholamines are notoriously unstable at high pH values) [26]; 2) The pH required for deprotonation of the carboxylic acid group in DOPA (i.e., carboxylate form of DOPA has a high retention sensitivity to changes in E_{appl} (see below)). Based on pK_a values of these compounds (Chart 1) and observed pH-dependence of the UV spectra and data, the amino groups of all five compounds are protonated, whereas the carboxylic acid group of DOPA is deprotonated in Mobile Phase 1.

At the open circuit potential, the mixture completely elutes within 4 min. The elution order, in terms of increasing retention time, is **1<2<3<4<5**. The separation, however, is only partially effective in resolving **1** and **2** and much less so for **4** and **5**. Furthermore, the correlation between structure and retention suggests that difference in the retention of **1-3** raises from hydrophobicity considerations, and is consistent with the strongly hydrophobic character of the PGC surface[27]. Interestingly, the presence of the carboxylic acid group in DOPA, which is absent in DA, increases the retention of DOPA with respect to DA. Compared to alkyl chain based stationary phases, the retention of PGC is unique due to its

Figure 1. Separations using EMLC of a mixture of NE (1), E (2), IP (3), DA (4), and DOPA (5) at a PGC stationary phase as a function of E_{appl}: open circuit potential (i.e., +0.19 V), 0.00 V, -0.20 V, -0.25 V, -0.30 V. All values of E_{appl} are given with respect to a Ag/AgCl/sat'd NaCl electrode. The mobile phase (Mobile Phase 1) was composed of 87% 0.05 M phosphate buffer (pH 3.3) and 13% acetonitrile. The flow rate was 0.5 mL/min and the detection wavelength was 210 nm.


1
2
3
4
5
200 mA.U.
Open circuit
(+0.19 V)
1
2
3+5
4
0.00 V
1
2
5
3+4
-0.20 V
1
2
5
4
3
-0.25 V
1
2
5
4
3
-0.30 V
0.0
2.0
4.0
6.0
Time (min)

delocalized electrons, which undergo donor-acceptor interactions [28, 29]. Thus, we attribute this phenomenon to the strong donor-acceptor interaction between charged carboxylic group and PGC surface.

In general, PGC is recognized as possessing donor-acceptor sensitivity superimposed upon classical reversed phase characteristics. Thus, the observed elution order for the analytes at the open circuit potential reflects the combined effect of hydrophobic and donor-acceptor interactions. The relative importance of the two interactions is briefly examined in a subsequent section.

B. Variations in Retention with Applied Voltage. Our earlier investigations of EMLC-based separations at PGC have revealed that the effect of E_{appl} on the retention for a variety of analyte types originates primarily by manipulating the ability of the carbon surface to participate in donor-acceptor interactions [30]; effects from changes in solvophobicity were also observed, but of less significance. It is therefore highly likely that changes in E_{appl} will alter the retention of **1-5**.

In attempts to improve the separation of **1-5** using PGC, different values of E_{appl}, extending from +0.30 V to –0.95 V, were used to manipulate retention. Representative chromatograms using Mobile Phase 1 are presented in Figure 1 in which the separations of a mixture of **1-5** are shown for several different values of E_{appl}: open circuit (+0.19 V), 0.00 V, -0.20 V, -0.25 V, and -0.30 V. These chromatograms show that the alteration of E_{appl} has a marked effect on the retention for all five compounds. For example, at –0.30 V, the retention of **1-4** increases with respect to that at the open circuit potential. The retention dependence of **5**, in contrast, is opposite to that of **1-4**, decreasing as E_{appl} moves negative of the open circuit

potential. Moreover, the effect of alterations in E_{appl} on the extent of the change in retention differs for each compound. As examples, the retention of **4** increases from 2.8 min at the open circuit potential to 3.3 min at –0.30 V; **3** undergoes an even greater relative change, increasing from 2.1 min at the open circuit potential to 3.9 min at –0.30 V. This difference results in a reversal of the elution order for the two compounds. That is, **3** is retained less strongly than **4** at the more positive values of E_{appl}, **3** and **4** co-elute at –0.20 V, and **3** is more strongly retained than **4** at the more negative values of E_{appl}. Though not shown, we note that the separation at +0.30 V resulted in the extensive overlap of **1-3** and the co-elution of **4** and **5**.

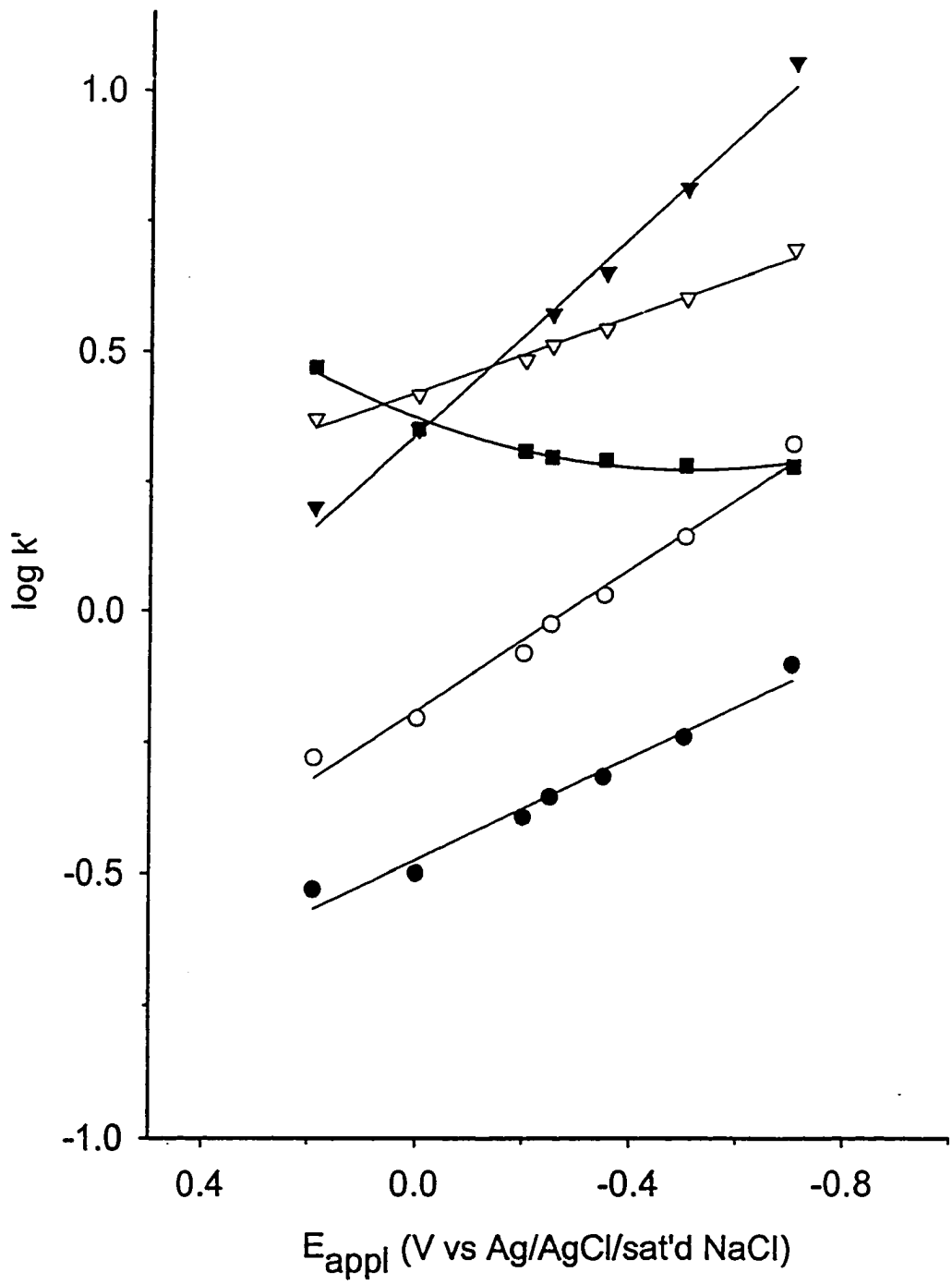
Interestingly, the presences of the carboxylic acid group in **5** results in exhibiting a clear decrease in retention from 3.1 min at the open circuit potential to 2.2 min at –0.30 V; at –0.30 V, **5** elutes before **3** and **4**. These differences in retention sensitivity to changes in E_{apple} translate to a dependence of the elution order on E_{appl}, which allows the baseline resolution of all five compounds at -0.30 V at a total elution time only slightly larger than that at the open circuit potential.

As a rough assessment of the effectiveness of EMLC in separating **1-5**, it is worthwhile to compare the results in Figure 1 to those obtained using conventional isocratic approaches. To this end, we have systematically changed the relative composition of the mixed mobile phase used for the separations in Figure 1 to determine the composition requisite to separate fully all of the components in the mixture. We have found that a more hydrophilic mobile phase (i.e., 92.5% water (0.1 M NaH_2PO_4) and 7.5% acetonitrile) was needed to obtain a baseline separation of all five components in the mixture. However, the

elution time for this isocratic separation was ~20 min, whereas that for the EMLC-based separation at –0.30 V was only ~4 min. This comparison shows an instance in which an EMLC-based separation is more effective than those obtained by using conventional solvent strategies, and that the optimization of the separation is fairly facile.

A more detailed summary of the dependencies of the retention of the catecholamines on E_{appl} is presented in Figure 2 through plots of log k' vs. E_{appl}, where k' is the capacity factor for each analyte at a given E_{appl}. These plots, then, reflect the sensitivity of retention to changes in E_{appl}. The plots extend from + 0.30 to – 0.95 V. These limits reflect concerns about 1) the possible oxidation of both the catechol functionalities and the PGC at more anodic values of E_{appl} and 2) theincreased residual current in the column at more cathodic values of E_{appl}. The latter is a possible issue in view of controlling the potential applied to the column. The retention of **1-4** exhibits a linear dependence on E_{appl}, whereas that of **5** shows a parabolically-shaped dependence. Thus, the retention of each compound is modified to differing extents by changes in E_{appl}, yielding the crossovers in the elution order evident in Figure 1. In addition, the dependence of the sensitivities for **1-4** exhibits an increase in retention as E_{appl} becomes more negative, while the dependence of **5** undergoes a decrease in retention. These trends are consistent with our earlier mechanistic analysis in that the donor-acceptor interactions between analytes and the PGC surface play a central role in the observed retention dependence [19, 20]. In other words, the more negative the applied potential, the greater donor character of PGC surface. Therefore, when E_{appl} moves negatively, the electrostatic attraction between the positively charged amino group on **1-4** and negatively charged PGC surface dominates the dependence of retention.

Figure 2. Plot of log k' vs. E_{appl} for the separation of the mixture of NE (**1**, ●), E (**2**, ○), IP (**3**, ▼), DA (**4**, ▽), and DOPA (**5**, ■) from Figure 1 using Mobile Phase 1.

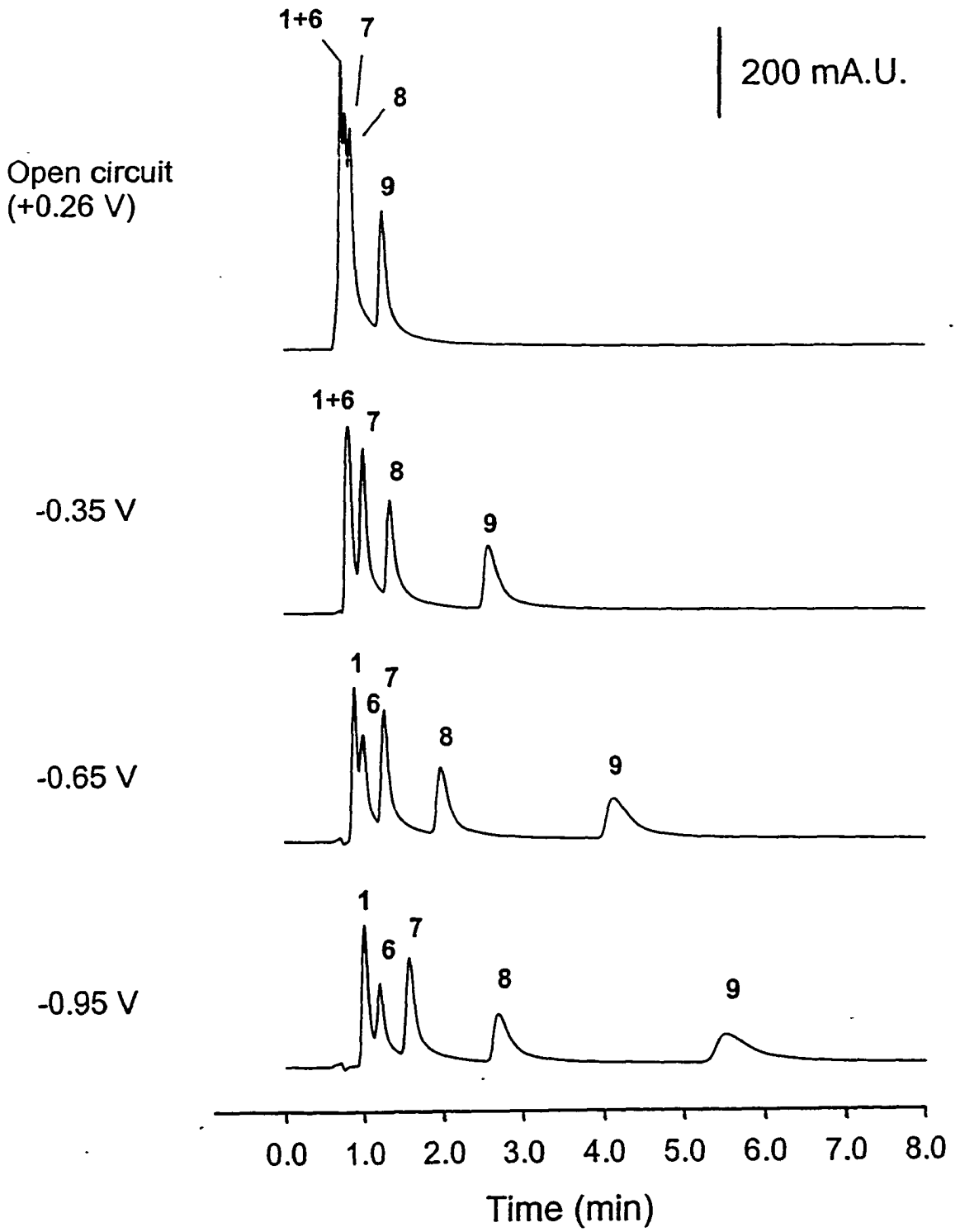

1.0
0.5
0.0
-0.5
-1.0
log k'
0.4
0.0
-0.4
-0.8
E_appl (V vs Ag/AgCl/sat'd NaCl)

On the other hand, the dependence of the retention for **5** on E_{appl} shows a nonlinear decrease, which points to a more convoluted set of interactions between the analyte and PGC surface. Albeit speculative at this time, we believe that this dependence reflects the contributions of the interactions of the negatively charged carboxylate group and the positively charged amino group with the PGC surface. At the more positive values of E_{appl}, the interaction between PGC and the negatively charged carboxylate group tends to dominate the dependence. In contrast, the interaction between PGC and positively charged amine group begins to counteract the interaction between PGC and carboxylate group as E_{appl} moves more negatively.

C. Effect of Substituents on EMLC-Based Alterations of the Retention. In this section, the effect of substituent on the retention is examined in a bit more detail. The intent is to begin to facilitate an understanding of the various factors that govern the EMLC-based separation of such materials. Five compounds that are structurally similar to the catecholamines examined in Figure 1 were employed, with the only structural differences in the ionic and/or hydrophobic nature of the substituents. The intent is to explore qualitatively how the different substituents on the aromatic ring and side chain affect retention through both donor-acceptor and solvophobicity considerations.

The chromatograms in Figure 3 illustrate the separations of **1** and **6-9** obtained at various values of E_{appl} at a constant mobile phase composition of pH 3.5. At the open circuit potential +0.26 V, the observed elution order of **1** and **6-9** is in accordance with their differences in hydrophobicity, paralleling the arguments applied to describe the elution order of **1-5** at the open circuit potential in Figure 1. Furthermore, the elution bands of **1** and **6-8**

Figure 3. Separations using EMLC of a mixture of NE (1), OCT (6), NMN (7), TYM (8), and MT (9) at a PGC stationary phase as a function of E_{appl}: open circuit potential (i.e. +0.26 V), -0.35 V, -0.65 V, -0.95 V. All values of E_{appl} are given with respect to a Ag/AgCl/sat'd NaCl electrode. The mobile phase (Mobile Phase 2) was composed of 75% 0.05 M phosphate buffer (pH 3.5) and 25% acetonitrile. The flow rate was 0.5 mL/min and the detection wavelength was 210 nm.

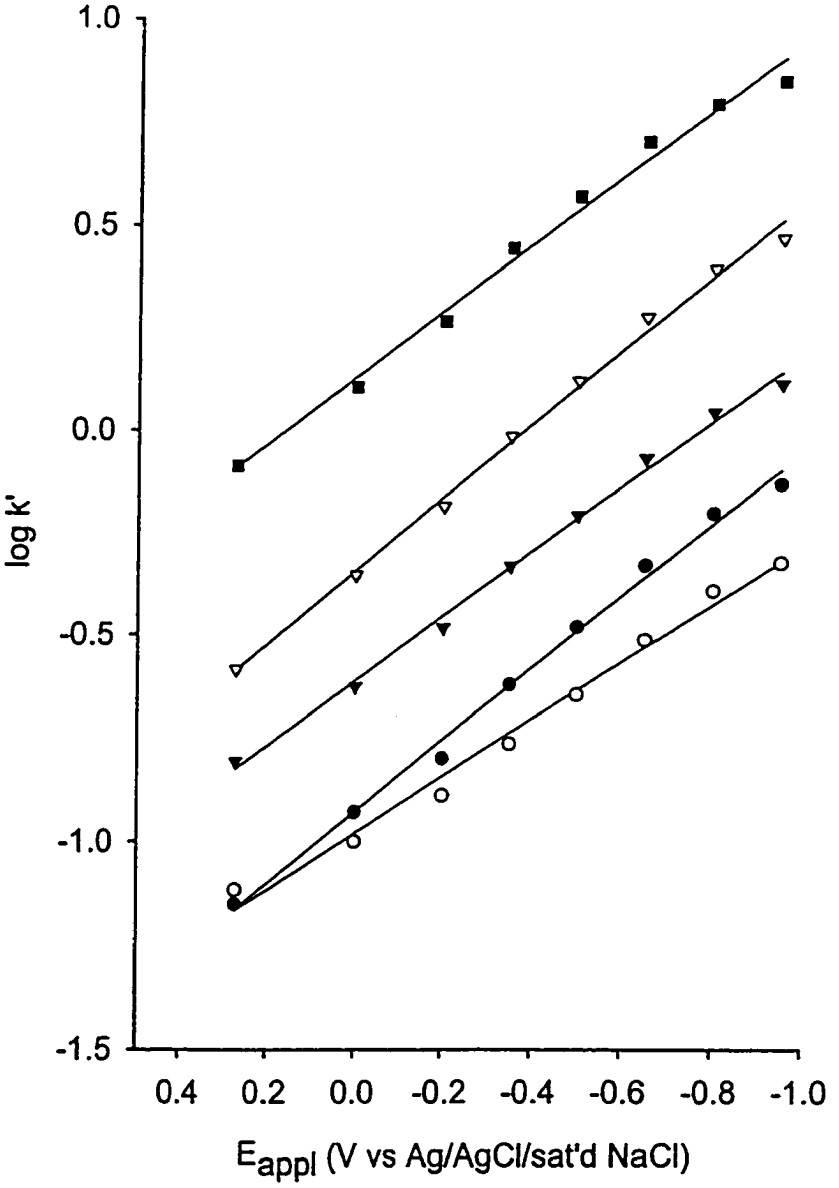

1+6
7
8
9
200 mA.U.
Open circuit
(+0.26 V)
1+6
7
8
9
-0.35 V
1
6
7
8
9
-0.65 V
1
6
7
8
9
-0.95 V
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
Time (min)

are strongly overlapped at the open circuit potential, whereas a well-resolved separation of all compounds is obtained at –0.95 V with the exception of **1** and **6** which are not fully resolved.

We note that **1** and **6** co-elute and were inseparable over the entire range of mobile phase compositions used earlier to evaluate the effectiveness of an isocratic separation, and can only be resolved by the alteration of E_{appl}. Furthermore, the plots of log k' versus E_{appl} shown in Figure 4 are nearly linear, but with different slopes (i.e., sensitivities to changes in E_{appl}.). The order of retention sensitivity with respect to the changes of E_{appl}, in terms of increasing slope, is **8>6>9>7>1**. It is evident, in view of the structural differences in these compounds, that when E_{appl} moves negatively, the replacement of a hydrogen by an electron-rich methoxyl and/or hydroxyl group induces an decrease in the sensitivity of retention (i.e., **6** vs. **7**, **8** vs. **9**, **8** vs. **6**, and **6** vs. **1**, etc.). The same effect is observed when a methoxyl group is replaced by a more polar hydroxyl group (i.e., **7** vs. **1**).

On the other hand, the attachment of a hydroxyl group to the α-carbon atom decreases the sensitivity to retention to a degree that is significantly less than the effect of the methoxyl group on the aromatic ring (i.e., **6** vs. **9**). These observations are likely due to the effect of an electron-rich substituent (e.g. a hydroxyl or methoxyl group) which decreases the overall interaction with PGC. We also note that the substituents on the aromatic ring (e.g., electron donor/withdrawing strengths) probably alters the ability of the aromatic ring to function as a π-donor. As found in an earlier study [20], the interaction between the π-systems of aromatic rings and PGC can play an important role in affecting the sensitivity of an EMLC-based retention process.

Figure 4. Plot of log k' vs. E_{appl} for the separation of the mixture of NE (**1**, ○), OCT (**6**, ●), NMN (**7**, ▼), TYM (**8**, ▽), and MT (**9**, ■) from Figure 3 using Mobile Phase 2.

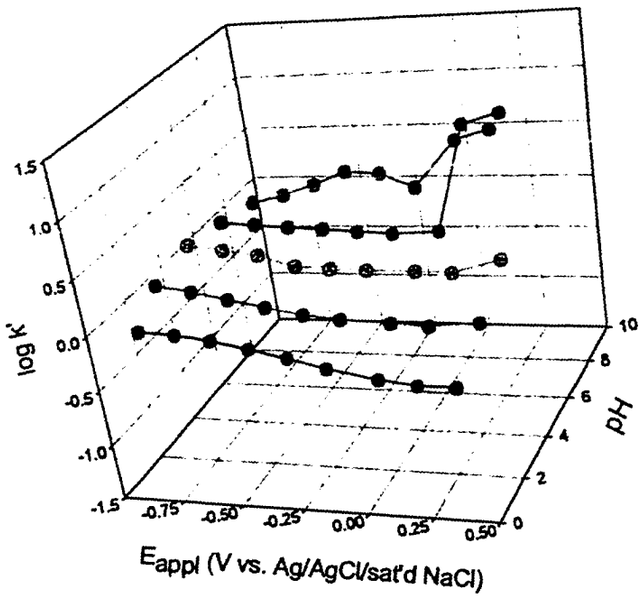

1.0
0.5
0.0
-0.5
-1.0
-1.5
log k'
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
E_appl (V vs Ag/AgCl/sat'd NaCl)

D. Influence of pH on Chromatographic Performance. Since the degree of dissociation of these compounds depends on the pK_a values of the ionizable groups and the pH of the eluent, the influence of differences in the pH (pH 1.7-9.2) of the mobile phase on the separation of a mixture of **1-5** was systematically investigated at various values of E_{appl}. We note that attempts to perform separations at higher pH were not successful because of the ease in which these compounds oxidize upon deprotonation. For instance, chromatograms obtained at pH 11 exhibited a clear decrease in the absorbance of the elution bands for each of the five compounds. Furthermore, multiple elution bands were often observed for an injection of a single analyte, which we view as diagnostic of the electrochemical conversion of the analyte.

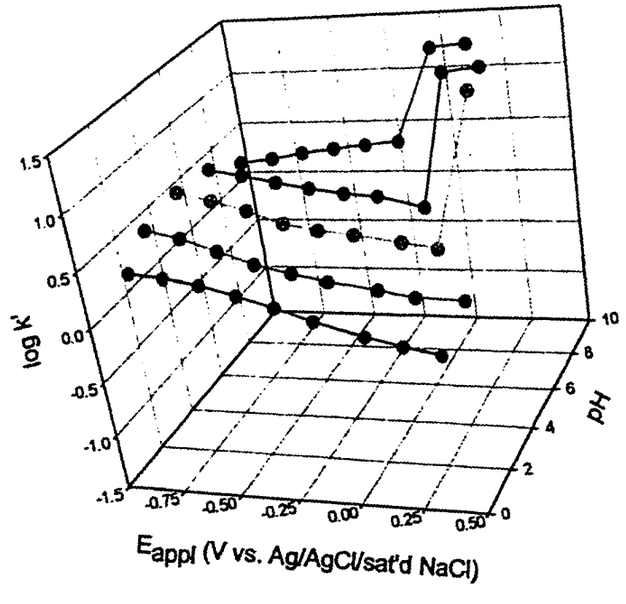
The retention behavior of **1-5** as a function of mobile phase pH is summarized in Figures 5A-E by using three-dimensional plots of log k' vs. E_{appl} at different pH values. For the purpose of clarity, the x-axis for E_{appl} and the y-axis for pH values in Figure 5E are arranged differently. These data demonstrate that changes in the pH of the mobile phase have a diverse range of effects on the retention of **1-5**. These dependencies are qualitatively consistent with predictions based on the degree of analyte ionization at a given pH. There are two factors that are influenced by pH. The first is, by suppressing the degree of analyte ionization, the solvophobic interactions between the analyte and PGC stationary phase are enhanced due to the increased hydrophobicity of the analyte. This change results in an increase in analyte retention. The second is, by suppressing the degree of ionization, donor-acceptor interactions between the analyte and PGC are weakened because of the decrease in the electron donor/acceptor strength of the analyte. The consequence is that the retention of

Figure 5. Plots of log k' vs. E_{appl} at different mobile phase pH values: 1.7, 3.3, 5.1, 7.2, 9.2 for: (A) NE, (B) E, (C) IP, (D) DA, (E) DOPA.

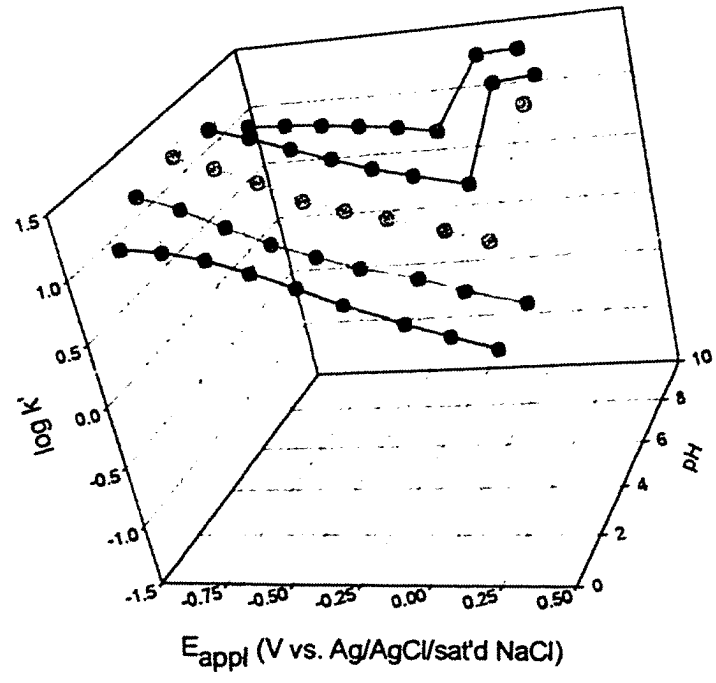
A


log k'
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-0.75
-0.50
-0.25
0.00
0.25
0.50
pH
0
2
4
6
8
10
Eappl (V vs. Ag/AgCl/sat'd NaCl)

B


log k'
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-0.75
-0.50
-0.25
0.00
0.25
0.50
pH
0
2
4
6
8
10
Eappl (V vs. Ag/AgCl/sat'd NaCl)

C



D

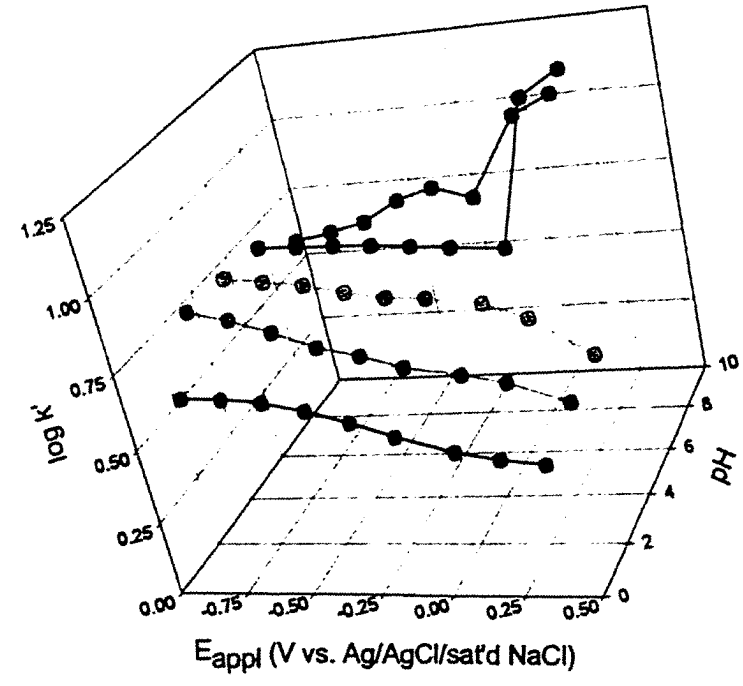


Figure 5 (cont'd)

E

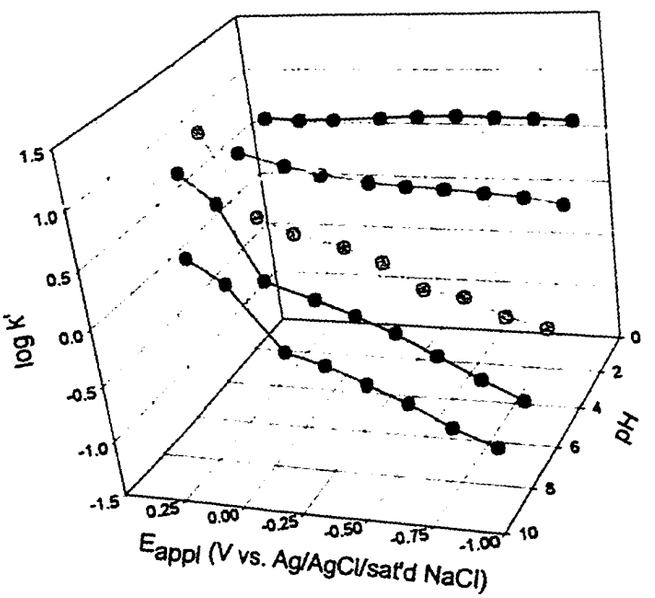


Figure 5 (cont'd)

an analyte either increases or decreases, depending on the ionization and the value of E_{appl} with respect to the potential of zero charge.

From the plots in each of the figures, it is evident that, when the pH<8, changes in pH target the retention dependence of the carboxylic acid group(i.e., **5**) without substantially affecting that of the amines (i.e., **1-4**) or catechol groups. As E_{appl} moves negatively, the relative retention of **1-4** tends to increase in a manner consistent with the ionization dependence of the acidic moiety. However, when the pH moves to higher values, the relative retention of **1-4** increases more strongly than expected at the more positive values of E_{appl}. This observation can be readily explained by the electrochemical redox characteristics of the catecholamines. Catechol functionalities are electroactive over a wide potential range, and are oxidized by a series of pH-dependent steps [26]. Assuming a Nernstian dependence, a 60-mV shift in the half-wave potential is expected for the two-electron electrooxidation of a catecholamine upon a unit change in pH; i.e., the half-wave potential shifts negatively as the mobile phase becomes more alkaline. Thus, catecholamines are more easily oxidized at higher pH values. This situation is further complicated by the fact that the quinone product from the oxidation is not stable, and can change to its indole form via a cyclization reaction [31].

The indole form of the catecholamines tends to be retained more strongly than its amine precursor at more positive values of E_{appl} due to the decrease in electron acceptor strength that results from the coupling of the amino group with the aromatic ring. This situation is evident from abrupt change in k' at the positive extremes of E_{appl}. At pH 9.2, the retention of **1-4** is distinctly different than that observed at the other pH values. Since the

amino groups are deprotonated at pH 9.2, each of the analytes are transformed from their monocationic to neutral form, which greatly decreases their electrostatic interactions with PGC.

The pH dependence of the retention of **5** shows an even more complex pattern, as shown in Figure 5E. The decrease in pH lengthens the retention of **5** at the open circuit potential. We ascribe this dependence to the presence of the different ionizable forms of **5** as supported by the acid dissociation constants in Chart 1. At a pH of ~2, the amino group of **5** is fully protonated, but the carboxylic acid moiety remains protonated. Thus, the protonated amino group plays an important role in interacting with PGC as an electron acceptor. As the mobile phase pH increases, the carboxylic acid group slowly dissociates, and the ionized carboxylic group begins to interact strongly with PGC as an electron donor. In general, **5** is effectively a cation at a pH < 2, a zwitterion between a pH of 3 to 8, and an anion at a pH > 9. At a pH below 2, the electron donor strength of the carboxylic acid group is weakened because its dissociation is suppressd. This effect is countered by the electron acceptor strength of protonated amino group, which is reflected overall by the comparative insensitivity of the log k'-E_{appl} plot shown in Figure 5E. We also note the possibility of internal hydrogen bonding between carboxylic group and amino group may result in the formation of a neutral molecular. When pH is greater than 3, the degree of dissociation of carboxylic group increases, which gives rise to an increase of interaction between carboxylic group and PGC surface. Consequently, the retention sensitivity increases due to the importance of electrostatic interactions as E_{appl} moves negatively.

Conclusions

In this paper, the application of EMLC as a technique for the analysis and separation of catecholamines and related compounds has been demonstrated. Results indicate that the retention of these analytes can be markedly and effectively manipulated through alterations in $E_{appl.}$ These changes are realized through the dependence of the donor-acceptor interactions between the analytes and PGC on $E_{appl.}$ This interaction, modulated by the influence of other substituents (e.g., electron donor/withdrawing groups) on retention, then gives rise to dependencies that result in a reversal in elution order.

In general, alterations in E_{appl} can enhance the resolution and efficiency of a separation in two ways. First, the selectivity of PGC to differences in functional groups can be optimized by fine tuning $E_{appl.}$ Second, the elution bands can be effectively distributed by rearranging the elution order. The separations described herein demonstrate an important attribute of this new technique in that the analytical figures of merit for a separation (e.g., resolution and retention time) can be readily manipulated by changes in $E_{appl.}$

Based on our findings, we believe EMLC can be easily implemented in many clinical applications as well as for basic neurosciences research. We are presently exploiting a applications of EMLC to the separation of samples of biomedical importance.

Acknowledgments

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CHAPTER 4. SEPARATIONS OF BIOGENIC AMINE NEUROTRANSMITTER BY ELECTROCHEMICALLY MODULATED LIQUID CHROMATOGRAPHY (EMLC): II. INDOLEAMINES AND STRUCTURALLYR ELATED COMPOUNDS

A paper to be submitted to *Journal of Chromatography*

Bin Lin, Hajime Takano, and Marc D. Porter

Abstract

Electrochemically-modulated liquid chromatography (EMLC) has been applied to the separation of a mixture of structurally-similar indole derivatives (i.e., 5-hydroxytryptophan, tryptophan, serotonin, tryptamine, 5-hydroxyindoleacetic acid, indoleacetic acid) using a porous graphitic carbon stationary phase. Changes in the potential (E_{appl}) applied to the stationary phase exert a pronounced effect on the retention of all six compounds, with the mixture fully resolved at negative values of E_{appl}. The observed dependencies of retention have the unusual effect of stretching both ends of the chromatogram. That is, the retention for some of the indole derivatives increases as E_{appl} moves negatively, whereas that for some of the other indole derivatives decreases. The combined weight of these dependencies results in the ability to achieve a fully resolved separation of the mixture, while dramatically decreasing the overall elution time. Structural correlations in terms of the influence of the changes in E_{appl} on the extent of the interactions between these analytes and the PGC stationary phase are qualitatively examined.

Introduction

Biogenic amines, their precursors, and their metabolites have a wide range of physiological significance [1-4]. The strong structural similarity of these compounds,

however, continues to challenge the analytical chemist in devising separation methods that allow the determination of these compounds in the presence of related materials. At present, there are many fairly elaborate high-performance liquid chromatography (HPLC) approaches utilized for the analysis of substances of neurochemical interest (e.g., catecholamines and indoleamines in brain tissue [5-7], plasma or serum [8, 9], urine [10], and cerebrospinal fluid [11]). These precursors and metabolites in cerebrospinal fluid (CSF) are diagnostic of many of the processes in brain neurochemistry. Their analysis via HPLC is therefore an important component in the operation of many pharmaceutical analytical laboratories [11, 12], relying on a finely-tuned balance between organic modifier, ion pairing reagent, temperature, buffer pH, and stationary phase. Optimization of such separations can therefore be fairly laborious.

As part of ongoing research in our laboratory, we have begun to focus on the development of simple, flexible, and easy-to-use separation methodologies for the determination of both catecholamine and indoleamine neurotransmitters. In pursuing these and other objectives, we have developed a new liquid chromatographic method termed electrochemically modulated liquid chromatography (EMLC) [14-17]. In EMLC, the potential applied (E_{appl}) to a conductive stationary phase (i.e., porous graphitic carbon (PGC)) serves as the principle parameter for manipulating analyte retention. As reported in our earlier EMLC investigations using PGC, alterations in E_{appl} have a strong influence on the retention of several types of analytes, including aromatic sulfonates, corticosteroids, benzodiazepines and catecholamines [14-17, 18].

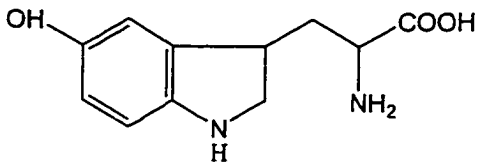
To evaluate the applicability of EMLC to the separation of catecholamine and indoleamine neurotransmitters, we have assessed the extent by which changes in E_{appl} can be used to manipulate the separation of both classes of compounds. This paper builds on our

previous investigation on the separation of catecholamines [18] by exploring the feasibility of the separation of a mixture of structurally-similar indole derivatives (i.e., 5-hydroxytryptophan (HTP), tryptophan (TRP), serotonin (5-HT), tryptamine (TT), 5-hydroxyindoleacetic acid (5-HIAA), and indoleacetic acid (IAA), see Chart 1). Of these, TRP is a precursor of 5-HT, while the major brain metabolite of 5-HT is 5-HIAA.

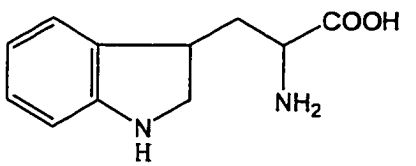
As shown in our previous investigations [16], the low selectivity of PGC toward differences in functional groups [19] can be overcome by the effects of E_{appl} on the retention characteristics of this type of stationary phase. In the following sections, we apply EMLC to the separation of a mixture of indole derivatives at a PGC stationary phase, further demonstrating the attributes (i.e., efficiency and ease of optimization) of this new chromatographic technique. We also present the results of an investigation of the dependencies of the retention of these indole derivatives on the pH of the mobile phase.

Experimental Section

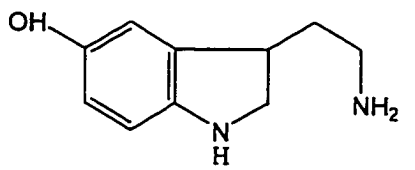
Reagents and Chemicals. Chart 1 shows the chemical structures, numerical labels and acid dissociation constants [20] for each of the indole derivatives. 5-HT was purchased from Sigma (St. Louis, MO, USA). HTP, TRP, TT, 5-HIAA, and IAA were obtained from Aldrich (Milwaukee, WI, USA). Acetonitrile (HPLC grade) was purchased from Fisher. Sodium dihydrogen phosphate, orthophosphoric acid, sodium hydroxide, and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Dibromomethane was from Eastman Kodak (Rochester, NY, USA). All chemicals were used as received. All aqueous solutions were prepared with water that was obtained from a Millipore Milli-Q purification system (Bedford, MA, USA).



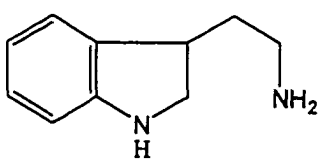
1. 5-Hydroxytryptophan (HTP)
   pK_{a1} = 2.51
   pK_{a2} = 9.49
   pK_{a3} = 10.54



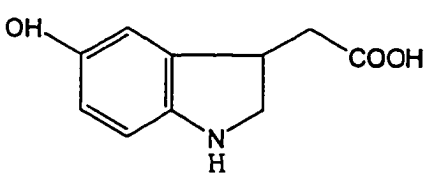
2. Tryptophan (TRP)
   pK_{a1}= 2.35
   pK_{a2} = 9.33



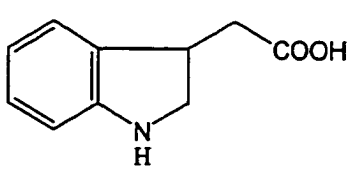
3. Serotonin (5-HT)
   pK_{a1} = 9.86
   pK_{a2} = 10.73



4. Tryptamine (TT)
   pK_{a1} =9.91



5. 5-Hydroxyindoleacetic acid (5-HIAA)
   pK_{a1} = 3.15
   pK_{a2} = 10.94



6. Indoleacetic acid (IAA)
   pK_a=3.38

Chart 1. Structures and numerical labels for the indole derivatives.

Chromatographic Column Construction. The general design of the EMLC column has been described elsewhere [21]. Briefly, the column consists of a Nafion cation-exchange membrane in tubular form (Perma Pure) that is placed inside a porous stainless steel cylinder. The Nafion tubing serves as a container for the stationary phase, and the stainless steel cylinder both prevents the deformation of the Nafion tubing under the high pressure of chromatographic flow and acts as the auxiliary electrode in a three-electrode electrochemical cell. The Nafion tubing, which was received in its acidic form, was pretreated by immersion into a boiling solution of neat ethanol for 10 min and then into a boiling aqueous solution (1 M $LiClO_4$) for 10 min. The length and inner diameter of the stainless steel column were 9.2 cm and 0.32 cm, respectively.

The conductive stationary phase consisted of uncoated PGC spheres (Hypersil, Runcorn, UK), with a diameter of ~7 μm. The PGC spheres were first dispersed in a dibromomethane/acetonitrile (10/7, v/v) mixture to form a slurry, and then packed into the EMLC column at 8000 psi using neat acetonitrile for ~30 min and then an acetonitrile solution (0.1 M $LiClO_4$) for ~12 hrs. Characterizations using X-ray photoelectron spectroscopy have shown that PGC is devoid of any detectable oxygen-containing functional groups (estimated detection limit: 0.2 atomic %) [14].

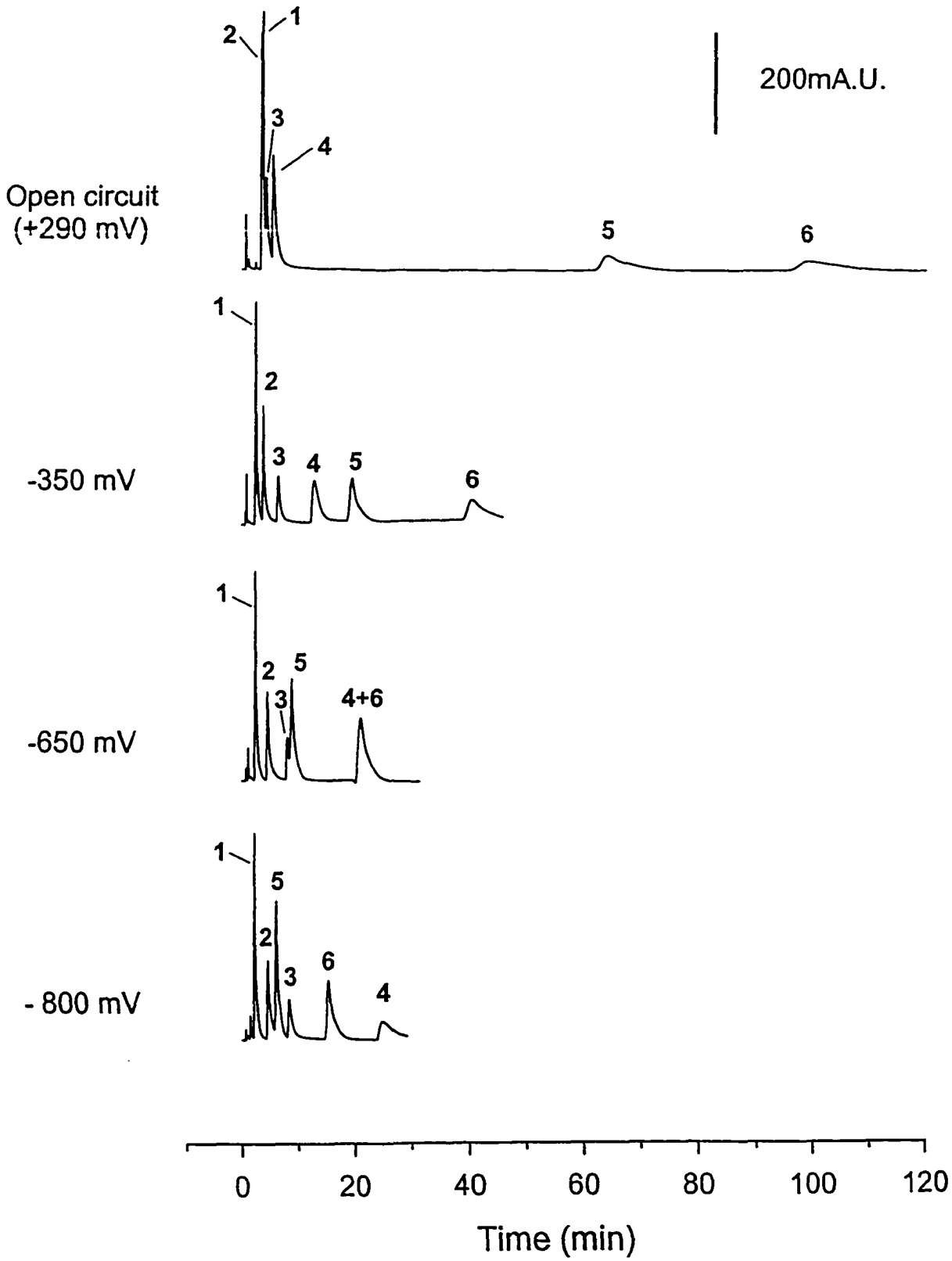
Instrumentation. The chromatographic experiments were performed using an HP 1050 series chromatograph equipped with a solvent cabinet, pumping system, and diode array detector (Hewlett-Packard, Santa Clara, CA, USA). The pH determinations were carried out using an Orion model 520 A pH meter (Boston, MA, USA). The voltage applied to the column was controlled by an Amel Instruments potentiostat-galvanostat model 2055-2056 (Milano, Italy).

Mode of Operation. After packing, the EMLC column was equilibrated with degassed mobile phase at a flow rate of 0.50 mL/min until a stable detector response was obtained. UV detection at 210 nm was used. The mobile phase consisted of an aqueous buffer-acetonitrile (75:25, v/v). For buffers with a pH greater than 4, the pH was adjusted as needed by adding sodium hydroxide to an aqueous solution of sodium dihydrogen phosphate (0.05 M). For buffers with a pH less than 4, the pH was adjusted by adding orthophosphoric acid to the sodium dihydrogen phosphate (0.1 M) solution. All potentials are reported with respect to Ag/AgCl/sat'd NaCl electrode. All analyte solutions were prepared in water with a concentration of ~50 ppm. Injection volumes were 5 μL. The dead volume of the column was 0.61 mL, as determined by an injection of water. Capacity factors (k') were calculated from the expression: $k'=(t_r-t_0)/t_0$ where t_r is the analyte retention time, and t_0 is the column hold-up time, which was taken as the "retention time" of the solvent injection peak. All of the reported values of k' are an average of at least three replicate runs, and the range of values are roughly that of the size of the data points presented in several of the following figures.

Results and Discussion

Separation of Indole Derivatives at Open Circuit. The chromatograms in Figure 1 present the separations of a mixture of **1-6** as a function of E_{appl}, including that at the open circuit potential (OCP), using an EMLC column with mobile phase of pH 3.3. The choice of this mobile phase reflects our interest in the eventual simultaneous determination of both indoleamines and catecholamines, and this mobile phase was found to be optimal for the separation of a mixture of several catecholamines [18]. Based on the pK_a values of these

Figure 1. Separations using EMLC of a mixture of HTP (1), TRP (2), 5-HT (3), TT (4), 5-HIAA (5) and IAA (6) at a PGC stationary phase as a function of E_{appl}: open circuit potential (i.e. +290 mV), -350 mV, -650 mV, -800 mV. All applied voltages are given with respect to a Ag/AgCl/sat'd NaCl electrode. The mobile phase (pH 3.3) was composed of 75% 0.05 M phosphate buffer and 25% acetonitrile. The flow rate was 0.5 mL/min and the detection wavelength was 210 nm.


200mA.U.
Open circuit
(+290 mV)
1
2
3
4
5
6
-350 mV
1
2
3
4
5
6
-650 mV
1
2
3
4+6
5
- 800 mV
1
5
2
3
6
4
0
20
40
60
80
100
120
Time (min)

compounds (Chart 1) and an analysis of the pH-dependent spectra, all are present in their zwitterionic forms at pH 3.3.

The separation at the OCP (+290 mV) serves as a reference point for an assessment of the effects of changes in E_{appl} on the separation. At the OCP, the overall elution time for all six compounds is ~110 min, which is unacceptably long. Furthermore, the separation is only partially effective in resolving **3** and **4**, while the elution bands for **1**, **2** and **3** are strongly overlapped.

The OCP separation in Figure 1 also provides evidence to develop a rough perspective of the types of interactions operative in the retention process. For example, the elution order indicates that **5** and **6**, both of which lack of an amine group, are much more strongly retained than the other four analytes. We believe that the elution order for the two sets of compounds largely reflects the differences in the donor-acceptor interactions between these analytes and PGC. As summarized in earlier mechanistic studies of retention at PGC [14], the net donor-acceptor interaction is the sum of five components: the electrostatic interaction, the polarization interaction, the exchange-repulsion interaction, the charge-transfer interaction, and a coupling interaction. Importantly, electrostatic interactions include those between permanent charges and dipoles, and have been shown to be the major factor affecting retention on PGC [22]. Thus, the differences in the retention of **1-4** with respect to **5** and **6** is in all likelihood due to the presence of the positively charge amine group on only **1-4**. In other words, since the OCP is positive of the likely value for the potential of zero charge for PGC in the electrolytic mobile phase (e.g. ~-0.15 V [, the positively charged amino group and positively charged PGC surface results in a decrease in retention of **1-4** with respect to **5** and **6**.

The other interesting feature of the open circuit separation is the elution order within each of the two sets of compounds. For compounds with and without hydroxyl substituents, **2** is more strongly retained than **1**, **4** is more strongly retained than **3**, and **6** is more strongly retained than **5**. We ascribe this ordering to two possible factors. The first factor is the solvophobic effect. That is, the compound that has a higher hydrophobicity tends to be retained longer on a nonpolar stationary phase like PGC. The second factor is the induction effect of hydroxyl substituents on the π-electron system on benzene ring. This effect decreases the π-electron density of the benzene ring, which weakens the extent of the interaction between the benzene ring and PGC. More importantly, the separation at the OCP demonstrates the difficulty in separating indole derivatives at PGC, a result that led us examine the applicability of EMLC to address this situation.

Separation of Indole Derivatives as a Function of Applied Potential. As discussed in our earlier EMLC investigations using PGC, we have found that the effects of E_{appl} on retention for several types of analytes arise notably from alterations in the donor-acceptor strength of the carbon surface [14]; solvophobicity and steric effects are also factors but of a lower significance. We therefore expect, in view of the discussion in the preceding section, that changes in E_{appl} will also have an impact on the retention of **1-6**, and Figure 1 shows that this appears to be the case.

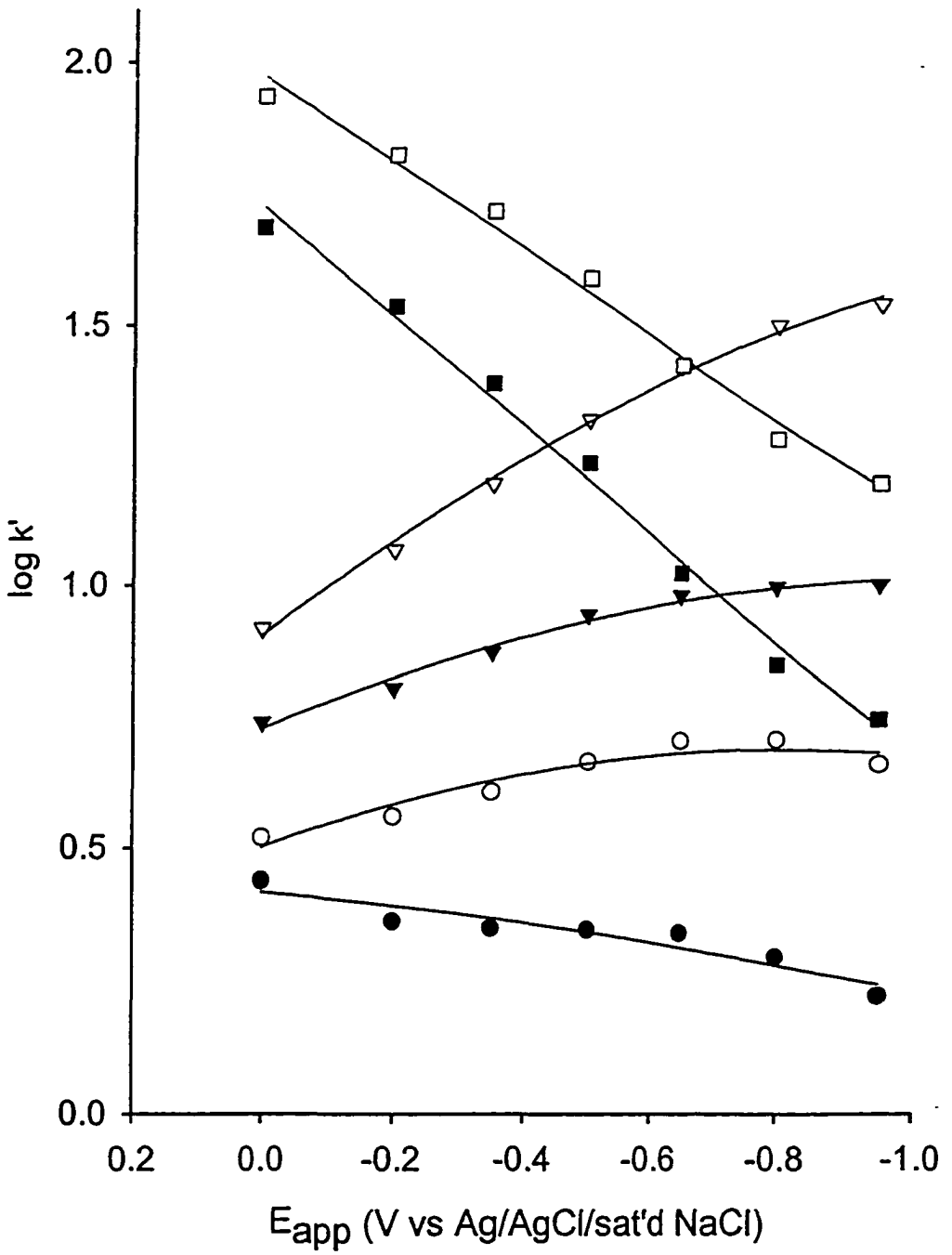
Alterations in E_{appl}, as evident in Figure 1, have a dramatic influence on the retention of all six compounds, with the dependencies of the compounds exhibiting some similarities and some differences. For example, as E_{appl} becomes more negative, the retention of **2-4** increases, whereas that of **1**, **5**, and **6** decreases. Moreover, the effect of E_{appl} on the extent of retention for the compounds is varied, which gives rise to large changes in selectivity. The

relative increase in retention (i.e., the sensitivity of retention to changes in E_{appl}) as E_{appl} moves from –0.35 V to –0.80 V is **4**>**3**>**2**; in contrast, the retention of **5** and **6** exhibits a decrease as E_{appl} becomes more negative. Interestingly, **5** and **6** undergo a dramatic decrease in retention with respect to that at open circuit. In fact, **5** and **6** show the largest sensitivity to E_{appl} among all the compounds that we have tested to date while exploring the development of EMLC. As a consequence of this marked sensitivity, the elution of **5** and **6** undergoes respective decreases of ~60 min and 120 min at the OCP to 8 min and 18 min at –800 mV. Moreover, **5** and **6** elute before **4** at –800 mV.

The differences in the retention dependencies of **1-6** have an unusual effect on the separation. That is, as E_{appl} shifts negatively, the early portion of the chromatogram is elongated, while the latter portion of the chromatogram is contracted. Thus, all six components of the mixture are fully resolved at –350 mV with a total elution time of ~45 min, and reasonably well-resolved at –800 mV with a retention time of ~25 min (although **2** and **5** are not fully resolved). With respect to the separation at the OCP, the decreases in the total elution of the mixture at –300 mV and –800 mV are more than 50 % and 75%, respectively.

Insights into the Mechanism for EMLC-Based Alterations of the Retention of 1-6. In this section, we briefly discuss the possible factors that contribute to the retention process in the separation of **1-6** via EMLC. For this purpose, Figure 2 presents a summary of the separations in Figure 1 through plots of log k' vs. E_{appl}, where k' is the capacity factor of each analyte. The plots for all six compounds exhibit different sensitivities to changes in E_{appl}, yielding the cross-overs in elution order noted in the last section.

Figure 2. Plot of log k' vs. E_{appl} for the separation of the mixture of HTP (1, ●), TRP (2, ○), 5-HT (3, ▼), TT (4, ▽), 5-HIAA (5, ■) and IAA (6, □) from Figure 1 at mobile phase pH of 3.3.


2.0
1.5
1.0
0.5
0.0
log k'
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Eapp (V vs Ag/AgCl/sat'd NaCl)

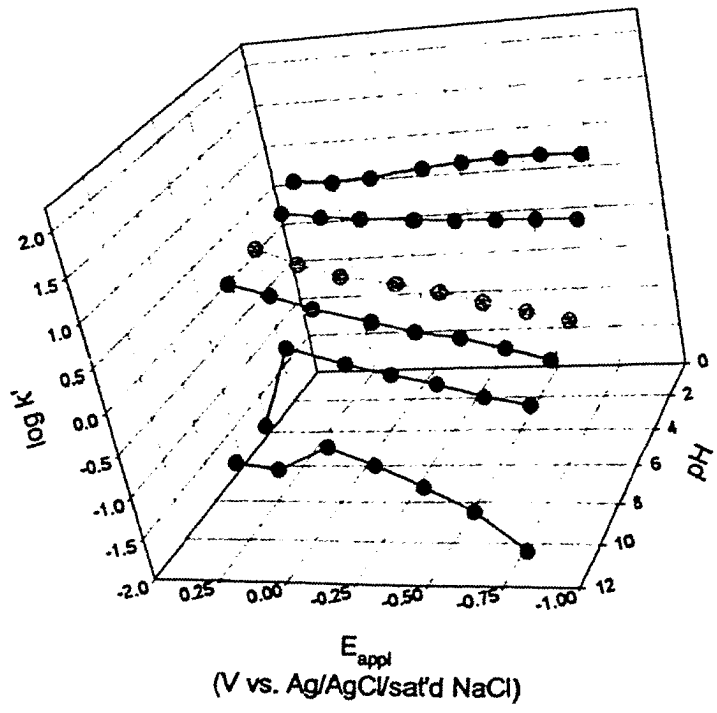
Examining these findings in total, we can begin to develop rough insights into the dependencies observed in Figure 2 through considerations of the structural similarities and differences of **1-6**. For example, **1-4**, when grouped together in terms of their structural similarities, are stronger electron acceptors than the group composed of **5-6**. This situation, which is reflected by the notable difference in the retention behavior for the two groups, arises from the presence of the amine substituent on **1-4** with respect to **5** and **6**. That is, PGC becomes a stronger donor as E_{appl} moves negatively, which increases the strength of the interaction with acceptor-type analytes (e.g., **1-4**) and decreases the extent of the interaction with donor-type analytes (e.g., **5** and **6**) [14]. It is this situation that results in the retention of the two groups shifting in opposite directions as E_{appl} move negatively.

D. Influence of pH on Chromatographic Performance. In this section, the influence of differences in the pH (pH 1.7-11.1) of the mobile phase on the separation of a mixture of **1-6** was systematically investigated at various values of E_{appl}. Such an investigation would help gain a more clear understanding of the retention characteristics of these compounds, which have ionizable substituents. The retention behavior of **1-6** with different mobile phase pH is detailed in Figures 3A-F through three-dimensional plots of log k' vs. E_{appl} at different pH values. For the purpose of clarity, the y-axis for pH values is arranged differently in Figures 3C and 3D.

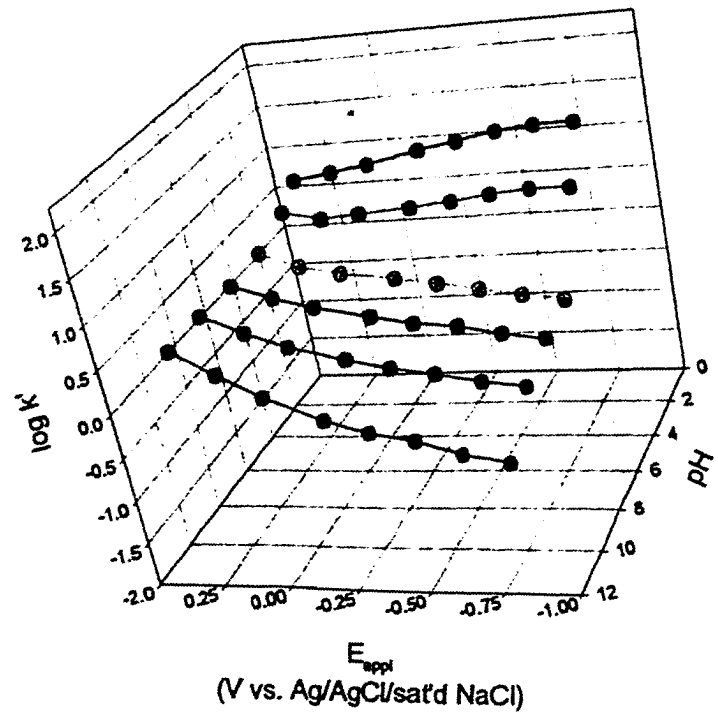
The plots demonstrate that changes in the pH of the mobile phase have a marked effect on the retention of **1-6**. Two general statements can be made. Firstly, by suppressing the degree of dissociation, the solvophobic interaction between the analyte and PGC stationary phase is enhanced due to the increased hydrophobicity for the analyte, which yields an increase in retention. Thus, the acidic compounds (e.g., **5** and **6**) are retained longer

Figure 3. Plots of log k' vs. E_{appl} at different mobile phase pH values: 1.7, 3.3, 5.1, 7.2, 9.2, and 11.1 for: (A) HTP, (B) TRP, (C) 5-HT, (D) TT, (E) 5-HIAA, (F) IAA.

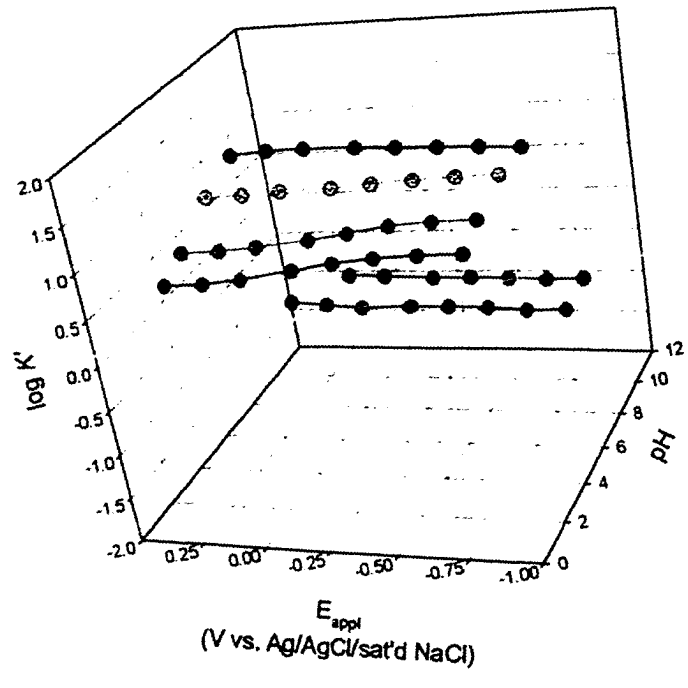
A


log k'
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
0.25
0.00
-0.25
-0.50
-0.75
-1.00
0
2
4
6
8
10
12
pH
E_{appl}
(V vs. Ag/AgCl/sat'd NaCl)

B


log k'
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
0.25
0.00
-0.25
-0.50
-0.75
-1.00
0
2
4
6
8
10
12
pH
E_{appl}
(V vs. Ag/AgCl/sat'd NaCl)

C



D

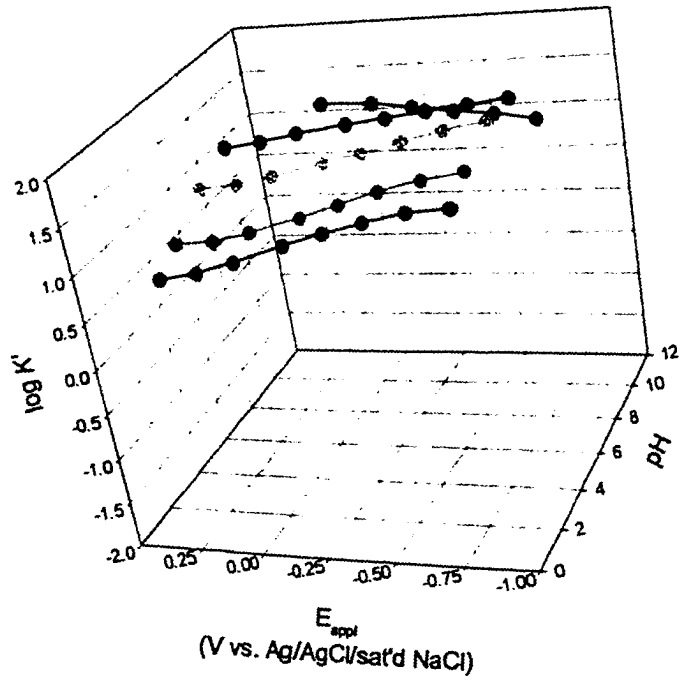
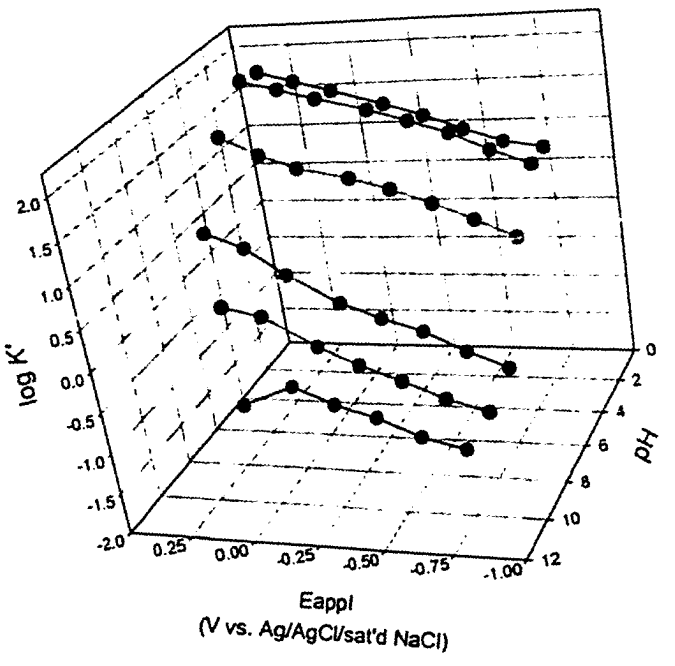


Figure 3 (cont'd)

E



F

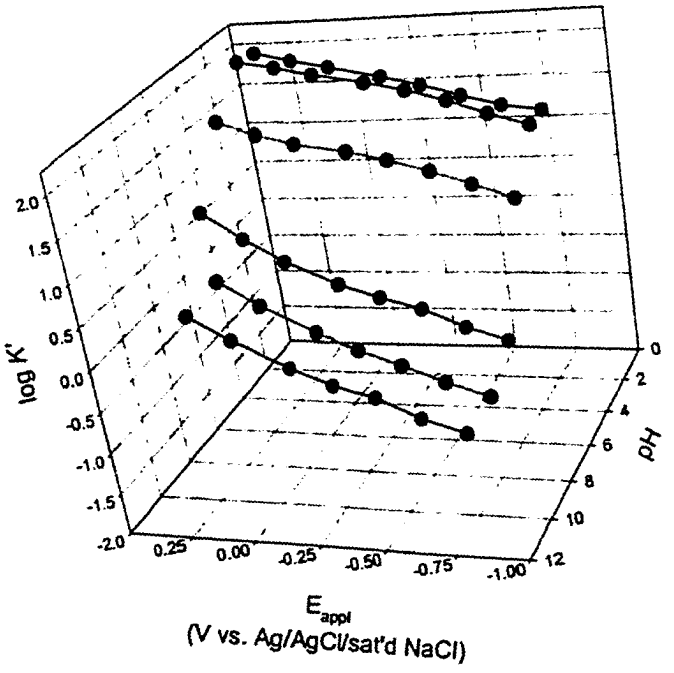


Figure 3 (cont'd)

at lower pH values, while the opposite applies to the amine-containing compounds (e.g., **3** and **4**). In contrast, **1** and **2**, which have both acidic and basic groups, show relative flat changes in retention with concave shape. This situation is probably due to the initial deprotonation of the carboxylic acid group to form the more polar zwitterion followed by deprotonation of the amine group to yield the amino-carboxylate anion (see Chart 1).

Secondly, the degree of dissociation affects the extent of the donor-acceptor interaction between the analyte and PGC. At a pH of ~2, the amino groups of **1** and **2** are fully protonated, but the ionization of the carboxylic acid groups is suppressed. Thus, the protonated amino group plays an important role in interacting with PGC and acts as an electron acceptor. In contrast, the electron donor strength of the carboxylic acid group is weakened because its dissociation is suppressed. This effect appears to be countered by the electron acceptor strength of protonated amino group, as suggested by the comparative insensitivity of the log k'- E_{appl} plots in Figures 3A and B. As pH increases, the carboxylic acid group slowly dissociates, and the ionized carboxylic group begins to interact more strongly with PGC as an electron donor. The combined effect of these two factors therefore results in a convoluted dependence.

From these plots, it is evident that, when the pH is less than 8, changes in pH targets the retention behavior of the compounds having carboxylic acid group (i.e., **1**, **2**, **5**, **6**) without substantially affecting that of the other compounds (i.e., **3**, **4**). As E_{appl} moves negatively, the relative retention of **3**, **4** tends to increase in a consistent manner at the various pH values within this range. This trend can be rationalized by the fact that there is no dissociation or protonation for **3** and within this pH range.

Conclusions

In this paper, we have demonstrated the feasibility of using EMLC for optimizing the separation of a mixture of indole derivatives at a PGC stationary phase. It has been shown that, by the alteration of E_{appl}, the separation pattern of this class of compounds can be dramatically manipulated That is, the retention of some of the indole derivatives increases as E_{appl} becomes more negative, whereas that of other indole derivatives decreases. These changes are realized through the dependence of the donor-acceptor interactions between the analytes and PGC on E_{appl}. We found this technique is particularly useful for the separation of a collection of analytes that have opposite charge. That is, the alteration of E_{appl} changes the retention of analytes with differing charge in opposite directions, which can enhance the overall separation efficiency. Also, this technique is highly sensitive to the presence of a carboxylic acid group, which is a common component in biomolecules, implying the enormous potential for the application of EMLC to biological samples.

Studies to further our insights into the retention mechanism are being pursued. Since EMLC has been successfully demonstrated for the manipulation of separation of catecholamines in our previous study [18], and indoleamines in this study, we predict that this technique has an unexplored potential for permitting simultaneous determination of both catecholamines and indoles in the routine analysis of real biological matrices. These experiments are being pursued.

Acknowledgments

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GENERAL CONCLUSIONS

Three years ago, when I started to dive into the EMLC project, I was full of doubt and suspicions on its prospects as a separation tool, like many people. The common question that comes from those both inside and outside our group was: Are your EMLC-based separations better than the same separation obtained using a silica C18 column? The fact is that, from the published and unpublished results of EMLC-based separations, its overall performance cannot compete with that silica-based column. Its future improvement on performance is also limited by the upper bound of using carbon-based stationary phase. So why EMLC?

Besides the advantages that have been cited in literatures, such as controllable stationary phase composition, etc, I have found the other merits that still might not be realized by the outsiders.

The conventional way for separation is to optimize the mobile phase by changing the composition of mobile phase. The EMLC way for optimizing the separation is to change the potential applied on stationary phase. If you have both working experience on conventional LC and EMLC, you will definitely have a feeling that working on EMLC is much more "comfortable". The tedious trial and error approach, which involves preparing mobile phase solution repetitively, is replaced by the simple switch of applied potential. From developer and vendor's viewpoint, one aspect that is desirable for a well designed and potentially commercial success instrument would be the automation of the entire system, which creates a comfortable working environment for the users. The trend of automation is "let computer do everything". After comparing the control mechanism of conventional LC and EMLC, which one is easy to achieve automation? The answer is obvious.

While the overall performance is still relatively lower than silica based column, the EMLC has its unique characteristics that is superior than silica based column. For certain classes of compounds, the elution profile can be handily rearranged through the manipulation of applied potential. By changing the elution order, the elution bands can be relocated at will to where they should be. Furthermore, by combining both manipulation of stationary and mobile phase compositions, the optimization of separation can be achieved at a degree that silica column cannot reach.

Then, here comes the question about how to exploit EMLC's merits mentioned above in spite of the low performance compared to current silica-based columns. The answer lies in the development strategy of EMLC. It is indisputable that development goal of EMLC is to push it to the market, commercialize it, and make it acceptance by the chromatographic community. The first strategy is based on the motivation of developing EMLC as a stand-alone separation tool, or say, as an alternative or substituent of silica column. The major limitation of the EMLC for the separation of complex samples rests with relatively low chromatographic efficiencies of EMLC. To achieve this goal, the performance of current EMLC column design has to be improved greatly so that it is comparable to silica column. This work is underway in our group. But its future is still unpredictable at this time.

At its present state of development, does the EMLC technique have any commercial value? The answer really depends on what you expect from EMLC. If you expect it as a stand-alone tool as described in first strategy, it is apparently not much qualified for marketing at this stage. But if you expect EMLC to complement conventional silica column rather than be its competitor, here comes the chance. As mentioned earlier, EMLC has its strong and weak points compared to conventional LC. It is very selective, sensitive and

easily applied for some important classes of compounds whereas its efficiency is relatively low. This determines that current EMLC is best used for the separation of less complex samples.

Multidimensional column chromatography (sometimes called column switching, multiphase, multicolumn, or coupled column chromatography) is a powerful technique for the separation of complex samples. In this approach, some sorts of fraction collection from one chromatographic column are selectively transferred to one or more secondary columns for further separation. The use of different columns gives different selectivity and retention so that solutes unseparated on the primary column can be further resolved on the secondary column. From the practical point of view, our strategy is to combine conventional LC and EMLC effectively. Conventional LC works as a primary column to achieve a partial separation of complex samples into less complicated mixtures which the EMLC can more easily handle, and the EMLC serves as a secondary column to finish resolving peaks that the conventional LC was unable to fully separate. This approach brightens the perspective of application of EMLC technique in the analysis of real world sample matrices.

In Chapters 3 and 4, the potential has been demonstrated for the application of EMLC as a technique for the analysis and separation of catecholamines and related compounds. Results indicate that the retention of these analytes can be markedly and effectively manipulated through alterations in the value of $E_{appl.}$ These changes are realized through the dependence of the donor-acceptor interactions between the analytes and PGC on $E_{appl.}$ This interaction, modulated by the diverse range and extent of the influence of other substituents on retention (e.g., electron donor/withdrawing strengths), then gives rise to the observed sensitivities and elution order.

In summary, alteration in E_{appl} to a PGC stationary phase could enhance resolution and efficiency of separation in two ways. First, the selectivity of PGC stationary phases to differences in functional groups can be optimized through fine adjustment of relative retention. Second, elution bands can be well distributed at rational time interval through rearranging the elution order. The separations in our study demonstrate an important attribute of this new technique in that the analytical figures of merit for a separation (e.g., resolution and retention time) can be readily manipulated by changes in E_{appl}.

We found EMLC technique is particularly useful for the separation of a collection of analytes that have charges of opposite sign such as ionizable compounds having amino and/or carboxylic group, since the alteration of E_{appl} would change the retention of analytes with differing charge in opposite direction, which facilitate the reorder of elution and, furthermore, the separation efficiency. Also, this technique is highly sensitive to the presence of carboxylic acid groups, which is very common in biological molecule, implying an enormous potential for application of EMLC on biological sample. Since EMLC has been successfully demonstrated for the manipulation of separation of catecholamines and indoleamines in our study, we can predict that this technique has a greater unexplored potential for permitting simultaneous determination of both classes of catecholamines and indoles in the routine analysis of real biological matrices, which would give rise to more efficient neurochemical examination of these often interrelated biogenic amine system. In our experience, EMLC is particularly advantageous for the separation of compounds containing carboxylic group and/or amino group. Although the technique described herein was developed for the analysis of indoleamine and catecholamine neurotransmitter but the methodology has universal application.

In spite of our progress in the development and application of EMLC, there continues to be a general lack of understanding of how this system works at a molecular level. Additional insights into the retention mechanism are needed to place the performance of this technique on a firm fundamental footing. Furthermore, by developing the rigorous treatment of EMLC, the theoretical groundwork can serve as a basis for expanding the scope of EMLC to a tool for certain physicochemical measurements, particularly for characterizing the electrical properties of compounds.

This dissertation also has described two applications of the electrochemical oxidation of amines for the modification of glassy carbon surfaces. Our long-range strategy is to develop the ability to manipulate surface architectures and control the interfacial properties of carbon-based materials, with specific interests in preparing new phases for EMLC.

In Chapter 1, the method for the fabrication of electroactive surfaces on glassy carbon was investigated. It was found that electrooxidation of the amine group in dopamine can only occur when the amine group is deprotonated. The instability of the oxidized form of catechol functionality in basic solutions also causes deactivation of dopamine modified surface. This latter problem was overcome by forming a cobalt catechol complex to prevent the oxidation of catechol functionality while electrolyzing the amine group during immobilization. Since the coordination conditions for metal ion binding to catechol is generally favored in basic solution, the application of these tethered catechol systems for the separation of trace metals demands an improvement in their stability of these systems. Inclusion of substituents at various ring positions may reduce/eliminate the instability through both steric and electronic effects. The immobilization of other redox couple that are not of the quinone type are also of potential value and should be investigated.

Chapter 2 has demonstrated the ability to immobilize secondary and tertiary amines by using solvents that are poor nucleophiles. It was found that there is a competition between the surface and solution reactions of the amine cation radicals produced by the one-electron oxidation of amines. Higher surface coverages are favored when choosing a solution environment that was less reactive to the generated amine cation radicals. It was also found that the surface coverage is closely related to the presence of surface oxygen functionalities. There is an optimal O/C ratio for achieving the highest coverage. Ratios higher or lower than this optimal value cause a decrease in coverage. The work in this paper also demonstrated that the alkylamine modified GC surface is very hydrophobic and very stable in strong acid and strong base, providing an intriguing prospect for applying this technique to create carbon-based reversed phases for LC separations.

As noted, our long range strategy is to develop the ability to manipulate surface architectures and control the interfacial properties of carbon particles that can be used as packing materials for LC. From preliminary tests, we found it is much more challenging to coat carbon particles than a planar carbon electrode. The major challenges include:

(1) translating carbon particles to a working electrode with good conductivity and good contact with the electrolyte solution to each particle;

(2) designing a new version of EMLC column without reactive metal parts connected to the working electrode part to avoid problems because of the high potential for oxidation of amine;

(3) overcoming the high surface concentration of proton caused by oxidation of the ethanol solvent, trace water, metal parts in the column, and the initial oxidation of the amine, due to the large area to volume ratio for carbon packing materials in EMLC column;

(4) pretreating the carbon particles because of much lower oxygen contents on PGC and GC particles than that on GCE surface. We believe this O/C ratio is lower than the optimal O/C ratio mention above which cause low surface coverage. In future work, we will attempt to increase appropriately the oxygen content on carbon particle surface by using various pretreatment techniques, including electrochemical pretreatment and radio frequency plasma pretreatment, or by using a mixture solvent of ethanol and water for immobilization. Other methods for modification of carbon particles without using EMLC column should also be considered.