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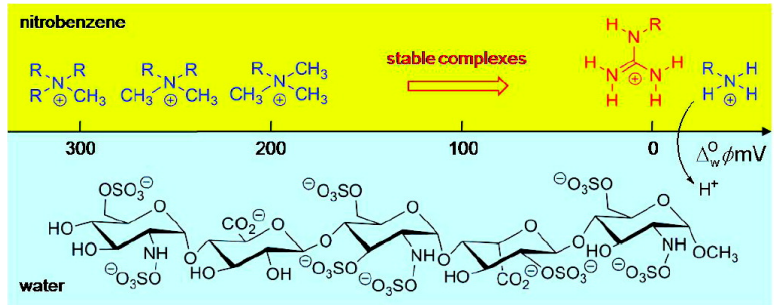

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Electrochemical Recognition of Synthetic Heparin Mimetic at Liquid/Liquid Microinterfaces

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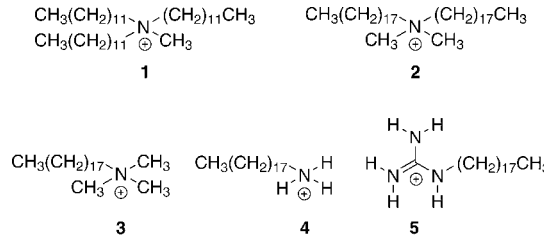
Abstract: Electrochemically controlled molecular recognition of a synthetic heparin mimetic, Arixtra, at nitrobenzene/water microinterfaces was investigated to obtain a greater understanding of interfacial recognition and sensing of heparin and its analogues with biomedical importance. In contrast to unfractionated heparin, this synthetic pentasaccharide that mimics the unique Antithrombin III binding domain of heparin possesses well-defined structure and ionic charge to enable quantitative interpretation of cyclic voltammetric/chronoamperometric responses based on the interfacial recognition at micropipet electrodes. Arixtra is electrochemically extracted from the water phase into the bulk nitrobenzene phase containing highly lipophilic ionophores, methyltridodecylammonium or dimethyldioctadecylammonium. Numerical analysis of the kinetically controlled cyclic voltammograms demonstrates for the first time that formal potentials and standard rate constants of polyion transfer at liquid/liquid interfaces are ionophore dependent. Moreover, octadecylammonium and octadecylguanidinium are introduced as new, simple ionophores to model recognition sites of heparin-binding proteins at liquid/liquid interfaces. In comparison to octadecyltrimethylammonium, the best ionophore for heparin recognition at liquid/liquid interfaces reported so far, these new ionophores dramatically facilitate Arixtra adsorption at the interfaces. With a saline solution at physiological pH, an Arixtra molecule is selectively and cooperatively bound to 5 molecules of the guanidinium ionophore, suggesting hydrogen-bond-directed interactions of each guanidinium with a few of 10 negatively charged sulfo or carboxyl groups of Arixtra at the interfaces.

Introduction

Molecular recognition of heparin and low-molecular-weight heparin (LMWH) is a crucial step in chemical sensing of these polyanionic carbohydrates[1] with broad biomedical importance as an anticoagulant, an antineoplastic, and beyond.[2] Potentiometric heparin-sensitive electrodes were developed by employing a liquid membrane doped with chloride salts of lipophilic quaternary ammonium ions such as ionophore **1** (Scheme 1).[3] Heparin and LMWH have been considered to be extracted from an aqueous sample into the liquid membrane to be ion-paired with the positively charged ionophore. Overall anion-exchange extraction of heparin is thermodynamically favorable, resulting in a large change in the phase boundary potential at the liquid membrane/sample solution interface under nonequilibrium conditions.[3b] More sophisticated ionophores with either primary ammonium[4] or guanidinium[5] groups were synthesized for heparin recognition and assays in bulk water or blood serum.

Scheme 1. Structure of Ionophores **1–5**



These cationic sites strongly and selectively bind to oxoanionic groups not only by ion pairing but also by hydrogen bonding.[6] In fact, heparin-binding proteins utilize arginine- and lysine-enriched peptides as recognition sites based on guanidinium and primary ammonium ions, respectively.[7] A highly selective potentiometric sensor for heparin and LMWH was developed

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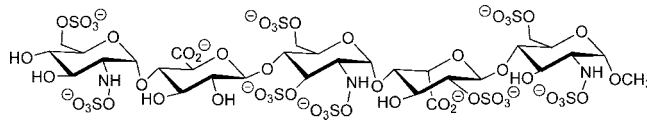
Scheme 2. Structure of Arixtra

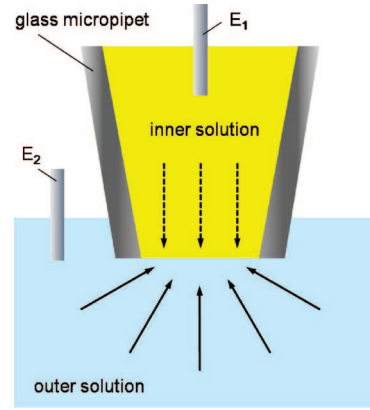


by using silicon field-effect transistors modified with heparin-binding proteins such as protamine and Antithrombin III.[8]

Recently, we[9] and others[10] have successfully developed novel heparin sensors by employing amperometry/voltammetry at interfaces between a heparin-containing aqueous phase and an ionophore-containing organic phase. With this electrochemical approach at liquid/liquid interfaces,[11] the phase boundary potential is controlled externally to selectively and reversibly drive interfacial complexation of heparin with positively charged ionophores such as **1**–**3**, which can be monitored as an ionic current response to heparin. Lowest detection limits of heparin reported so far (0.13 unit/mL in sheep blood plasma[9a] and 0.005 unit/mL in a saline solution[9b]) were obtained by stripping voltammetry based on adsorption of heparin as ionophore **3** complexes. The detection limits are lower than high-dose heparin (2.3–4.2 unit/mL) during cardiopulmonary bypass, vascular surgery, and angiographic/catheterization procedures[12] and are comparable to low-dose heparin (0.4–0.7 unit/mL) for thromboembolic disease.[13]

The recent amperometric/voltammetric studies also revealed that heparin recognition at liquid/liquid interfaces is poorly understood, thereby limiting further development of this promising sensor technology. The amperometric/voltammetric responses rise from heparin adsorption rather than from complete extraction of heparin into the organic phase.[9,10] The extraction process, however, has been considered as an origin of the nonequilibrium potentiometric heparin responses,[3b,c] which can be also affected by simultaneous interfacial adsorption.[14] Heparin adsorption is facilitated more by an ionophore with a less bulky ammonium group in the order of **3** > **2** > **1** as expected for the strength of the ion pairing.[9a] In contrast, a much larger potentiometric response was obtained with ionophore **1** than with ionophore **2** or hexadecyltrimethylammonium, an analogue of ionophore **3**.[3d] Moreover, facilitated heparin adsorption is so slow that a resulting voltammogram is electrochemically irreversible either at micro-[9a] or macro-interfaces,[9b] whereas a nernstian process has been assumed to explain voltammetric[10] and nonequilibrium potentiometric[3b,15] heparin responses at macrointerfaces. A major obstacle to a better understanding of heparin recognition at liquid/liquid interfaces is polydispersity of unfractionated heparin with molecular weight in the range of 5000–40 000,[7] which hinders quantitative interpretation of the electrochemical responses.

Scheme 3. Diagram of an Electrochemical Cell for Voltammetry/Amperometry at a Micropipet Electrode[a]



[a] The solid and dotted arrows represent non-linear and linear diffusion of species in the outer and inner solutions, respectively. E_1 and E_2 are counter/reference electrodes in the respective phases (see Experimental Section for details).

Here we report on electrochemically controlled molecular recognition of a synthetic heparin mimetic, Arixtra[16] (also known as fondaparinux sodium; Scheme 2), at nitrobenzene/water microinterfaces to obtain a greater understanding of interfacial heparin recognition. This synthetic LMWH mimics a pentasaccharide that serves as the unique Antithrombin III binding domain of heparin to inhibit blood coagulation. Despite its wide use as a FDA-approved anticoagulant drug for prophylaxis of deep vein thrombosis, there have been only a few studies of monitoring Arixtra in a saline solution[8] and human blood samples.[17] Importantly, structurally well-defined Arixtra enables quantitative assessment of its electrochemical recognition by employing cyclic voltammetry/amperometry at the micrometer-sized interface formed at the tip of a glass micropipet electrode (Scheme 3).[18,19] In addition to quaternary ammonium ionophores **1**–**3**, octadecylammonium **4** and octadecylguanidinium **5** are characterized as new, simple ionophores

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that model recognition sites of heparin-binding proteins at the interfaces. In fact, Arixtra-Antithrombin III binding is mediated by ammonium and guanidinium groups of lysine and arginine residues of the protein.[20] Also, interfacial interactions of proteins with heparin-like linear, highly charged polysaccharides, glycosaminoglycans, linked to cell membranes regulate intracellular communication.[7b]

Experimental Section

Chemicals. Tetradodecylammonium (TDDA) bromide, methyltridodecylammonium iodide, octadecyltrimethylammonium bromide, nitrobenzene (>99%), chlorotrimethylsilane (99%), and tetraethylammonium (TEA) hydroxide (20 wt % in water) were obtained from Aldrich (Milwaukee, WI). Dimethyldioctadecylammonium chloride was from Tokyo Kasei Kogyo (Tokyo, Japan). Octadecylamine hydrochloride was from Alfa Aesar (Ward Hill, MA). Tris(hydroxylmethyl) aminomethane (Tris Base, 99.9%), and *p*-toluenesulfonate monohydrate (98.5%) were from Sigma (St. Louis, MO). Arixtra (2.5 mg/0.5 mL and 7.5 mg/0.6 mL) was purchased from GlaxoSmithKline (Research Triangle Park, NC) as a saline solution for intravenous injection. Potassium tetrakis-(pentafluorophenyl)borate (KTFAB) was from Boulder Scientific Co. (Mead, CO). All reagents, except Arixtra, were used as received. All aqueous solutions were prepared with 18.3 MΩ cm^{-1} deionized water (Nanopure, Barnstead, Dubuque, IA).

Dialysis of Arixtra Solutions. Original Arixtra solutions were dialyzed against deionized water using a membrane with 500 molecular weight cutoff to remove sodium chloride because of serious chloride interference in some electrochemical experiments. Ready-to-use devices (DispoDialyzer, Spectrum Laboratories, Inc., Rancho Dominguez, CA) were used for the dialysis. An Arixtra concentration in the dialyzed solution was determined using a pipet electrode filled with a nitrobenzene solution of ionophore **2** after calibration with standard solutions obtained by diluting an original Arixtra solution.

Preparation of Ionophore−TFAB Salts. TFAB salts of quaternary ammonium ionophores **1–3** and a supporting electrolyte TDDA were prepared as reported previously.[9] TFAB salts of octadecylammonium **4** or octadecylguanidinium **5** were prepared by metathesis of KTFAB and octadecylguanidinium *p*-toluenensulfonate or octadecylamine hydrochloride in methanol. A dichloromethane solution of the mixture was washed several times with deionized water. The solvent was removed by rotary evaporator, and the product was dried further under vacuum. Octadecylguanidinium *p*-toluenensulfonate was synthesized and characterized as described elsewhere.[21]

Fabrication of Micropipet Electrodes. Micropipet electrodes were made from borosilicate glass capillaries (o.d./i.d. = 1.0 mm/0.58 mm, 10 cm in length) from Sutter Instrument Co. (Novato, CA) using laser-based pipet puller (model P-2000, Sutter Instrument).[9a,19] The inner and outer tip radii, a and r_g, and the inner and outer tip angles, θ_1 and θ_2, were determined as reported elsewhere.[19d] The inner or outer wall of each pipet was silanized with chlorotrimethylsilane so that either an organic or an aqueous solution, respectively, was injected into the pipet from the back using a 10-μL syringe.

Electrochemical Measurements. A computer-controlled CHI 660B electrochemical workstation equipped with CHI 200 picoampere booster and Faraday cage (CH instruments, Austin, TX) was used for all electrochemical measurements. The electrochemical cells employed are as follows:

Ag | AgCl | x μM Arixtra (aqueous buffer) || y mM ionophore−TFAB salt in 0.1 M TDDA-TFAB (nitrobenzene) | Ag.

Concentrations of Arixtra and ionophore salt as well as buffer compositions are given in corresponding figures and legends.

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Table 1. Ionophore-Dependent Parameters of Arixtra Extraction or Adsorption at Nitrobenzene/Water Microinterfaces

ionophore	$\Delta^o_w\phi_{1/2}V^a$	$\Delta^o_w\phi_pV^a$	$\Delta^o_w\phi^{0'}V^a$	k^0 cm/s	α	z_{eff}	z^b
1	0.33	0.30	0.31	1.4×10^{-3}	0.64	−7	−12
2	0.28	0.22	0.26	1.2×10^{-4}	0.78	−7	−10
3	0.24	0.21	−	−	−	−	−11
4	0.00	−0.07	−	−	−	−	−7
4[c]	0.00	−0.09	−	−	−	−	(−1.06)
5	0.04	−0.04	−	−	−	−	−4.8
5[c]	0.06	−0.06	−	−	−	−	(−1.02)

[a] Defined with respect to $\Delta^o_w\phi^{0'}$ of TEA^+ transfer. [b] Value in the parentheses is z/s. [c] Studied using water-filled pipets. Parameters in the other rows were obtained using organic-filled pipets.

The potential of a nitrobenzene phase was given with respect to a formal potential of TEA^+ transfer[19d] in Figures 1–4 and Figure S5 (see Supporting Information). A current carried by a negative charge from the aqueous phase to the organic phase was defined to be negative. Background-subtracted data are reported except in Figure S2 (see Supporting Information). A background current response was obtained using a cell without Arixtra or ionophre in the outer aqueous or organic phase, respectively. All electrochemical experiments were performed at 22 (±3) °C.

Results and Discussion

Electrochemical Characterization of Arixtra Recognition at Microinterfaces: Methodology. In this study, stability of Arixtra-ionophore complexes and potential-dependent dynamics of their formation are elucidated for various ionophores by cyclic voltammetry/amperometry at micropipet electrodes. Recently, we developed this unique electrochemical methodology into a powerful approach to study complicated transfers of polyions such as unfractionated heparin[9a] and protamine.[19] In addition to the complexation stability and dynamics, it is directly determined from a cyclic voltammogram (CV) whether Arixtra-ionophore complexes formed at the interface are extracted into the organic phase[19a] as defined by

$$A^z\,(\text{aqueous phase}) + s\text{LH}^+\,(\text{organic phase}) \rightleftharpoons A(\text{LH})_s^{(z+s)}\,(\text{interface}) \rightleftharpoons A(\text{LH})_s^{(z+s)}\,(\text{organic phase}) \quad (1)$$

where A^z, LH^+, and $A(LH)_s^{(z+s)}$ represent Arixtra, ionophore, and their complexes, respectively. Multiple charges transferred by each Arixtra molecule, z, and corresponding large stoichiometry of the complexes, s, are determined from limiting currents controlled by diffusion of Arixtra and ionophore to the microinterface, respectively.[19b,c] When the complexes are completely extracted into the bulk organic phase, all thermodynamic and kinetic parameters, that is, the formal ion-transfer potential, $\Delta^o_w\phi^{0'}$, the standard ion-transfer rate constant, k^0, and the transfer coefficient, α, can be determined from a single CV obtained under kinetic limitation.[19d] Numerical and analytical treatments of voltammetric/amperometric data are detailed in the Supporting Information, so only the outcomes are discussed in the following.

Quaternary Ammonium Ionophores 1–3. In contrast to unfractionated heparin,[9,10] Arixtra can be extracted into an organic phase containing highly lipophilic quaternary ammonium ionophore **1** or **2** as demonstrated by cyclic voltammetry at organic-filled pipets (Figure 1a and b, respectively). Arixtra extraction is confirmed by a peak current on the reverse potential sweep, which is smaller than a limiting current of a sigmoidal forward wave. The smaller peak current corresponds to linear diffusion of extracted Arixtra-ionophore complexes in the inner nitrobenzene phase. Arixtra extraction is thermody-

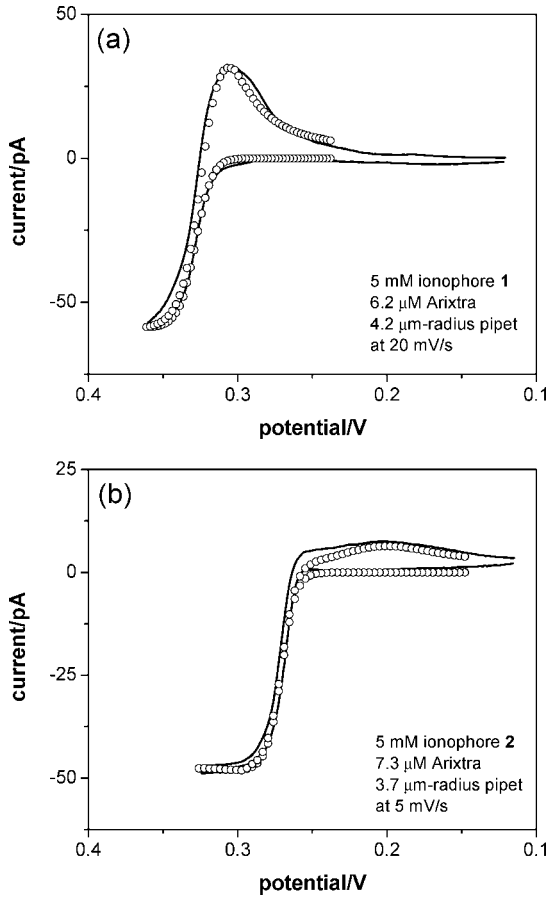

Figure 1. Background-subtracted CVs (solid lines) of Arixtra extraction facilitated by ionophores (a) **1** and (b) **2** at organic-filled pipets in contact with dialyzed Arixtra samples diluted with 0.1 M NaH_2PO_4/Na_2HPO_4 at pH 7.1. The open circles represent simulated CVs with parameters listed in Tables 1 and S1 (see Supporting Information).

namically more favorable with ionophore **2** than with ionophore **1**. Both a half-wave potential, $\Delta^o_w\phi_{1/2}$, and a peak potential, $\Delta^o_w\phi_p$, are more negative with ionophore **2** (Table 1). This result indicates that Arixtra is more stabilized in the nitrobenzene phase by ionophore **2** with more methyl groups attached to the nitrogen's positive charge, which is more accessible for ion pairing with Arixtra's negative charges. On the other hand, Arixtra extraction is faster with ionophore **1** than with ionophore **2**. The separation between $\Delta^o_w\phi_{1/2}$ and $\Delta^o_w\phi_p$ is much narrower with ionophore **1** than with ionophore **2**. Correspondingly, a peak current is much larger with ionophore **1** than with ionophore **2**.

Structurally well-defined Arixtra enables us to quantitatively assess the apparent thermodynamic and kinetic effects of ionophore structure on Arixtra extraction. The transient CVs based on kinetically limited Arixtra extraction fit well with simulated CVs (Figure 1a and b; see Supporting Information for details of the simulation), where the facilitated Arixtra transfer in the presence of excess ionophore was simplified to a first-order process

$$A^z \text{ (outer aqueous phase)} \rightleftharpoons A^z\text{(inner organic phase as ionophore complexes)} \quad (2)$$

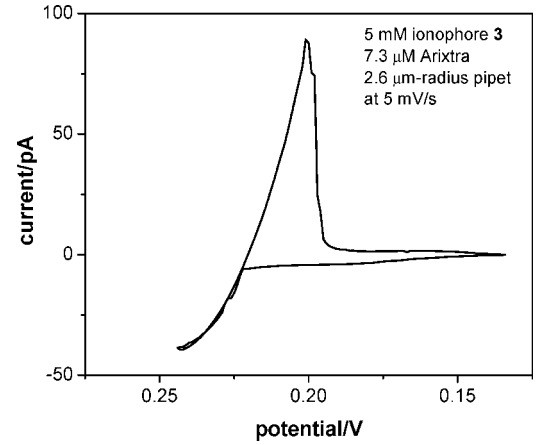

Figure 2. Background-subtracted CV of Arixtra adsorption facilitated by ionophore **3** at an organic-filled pipet with $r_g/a = 1.1$ in contact with a dialyzed Arixtra sample diluted with 0.1 M NaH_2PO_4/Na_2HPO_4 at pH 7.1.

The numerical analysis gives all parameters (Table 1) in the heterogeneous ion-transfer rate constants given by the Butler–Volmer-type relation as[11c,19c,d,22]

$$k_f = k^0 \exp[-\alpha z_{eff} F(\Delta^o_w\phi - \Delta^o_w\phi^{0'})/RT] \quad (3)$$

$$k_b = k^0 \exp[(1-\alpha) z_{eff} F(\Delta^o_w\phi - \Delta^o_w\phi^{0'})/RT] \quad (4)$$

where k_f and k_b are heterogeneous rate constants of the forward and backward ion transfer, respectively, in eq 2, z_{eff} is an Arixtra's charge that effectively contributes to transfer kinetics, and $\Delta^o_w\phi$ is the Galvani potential difference between the organic and aqueous phases.

A ~50 mV difference between $\Delta^o_w\phi^{0'}$ values for ionophores **1** and **2** corresponds to a significantly large difference of 48 kJ/mol in a Gibbs free energy of ion transfer given by $-zF\Delta^o_w\phi^{0'}$,[11c] because of a large actual charge of ~−10 transferred by each Arixtra molecule (see below). The k^0 values demonstrate that Arixtra extraction is intrinsically faster with ionophore **1** than with ionophore **2** by an order of magnitude, corresponding to quasi-reversible and irreversible Arixtra transfer with the respective ionophores. Since the α and z_{eff} values are similar, the kinetic effect is not due to different transfer mechanisms. Interestingly, the z_{eff} value of −7 confirms that multiple charges of an Arixtra molecule are transferred simultaneously across the interface. In fact, large potential dependence of Arixtra-transfer rates as governed by z_{eff} and α (eqs 3 and 4) results in the sigmoidal forward wave (Figures 1a and b) that is much steeper than that observed with conventional systems based on nernstian transfer of a monovalent ion at micropipet electrodes.[18] This simultaneous transfer of multiple charges across the interface is a unique electrochemical property of polyion transfer at liquid/liquid interfaces[9,19] and is not observed in redox reactions of multiple, independent redox centers with identical formal potentials at metal/liquid interfaces.[23]

Arixtra extraction facilitated by ionophore **1** or **2** is not based on a simple mechanism. The α values of 0.64 and 0.78 obtained

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with the respective ionophores are larger than normal values in the range of 0.4−0.6.[24] Moreover, the z_{eff} value of −7 is smaller than a total charge number of an Arixtra molecule expected from its 8 sulfo and 2 carboxyl groups (Scheme 2). Actual charges carried by each Arixtra molecule across the interface were determined from steady-state (Figure 1) and chronoamperometric (Figure S1, Supporting Information) diffusion-limited currents, thereby yielding $z = -12 \pm 1$ and -10 ± 1 for ionophores **1** and **2**, respectively (see Supporting Information). These z values, which are close to the number of negatively charged groups of an Arixtra molecule, are larger than the z_{eff} values. The different z and z_{eff} values indicate a multistep transfer mechanism as previously discussed for protamine transfer,[19c,d] although a z_{eff} value of Arixtra extraction is much closer to the corresponding z value in comparison to the case of facilitated protamine transfer with $z_{eff} = +2.9$ and $z = +20$.[19d] The deviation of α values from 0.5 suggest a double layer effect[25] on a potential-dependent step such as adsorption of charged Arixtra and ionophore molecules involved in the multistep mechanism.

In contrast to ionophores **1** and **2**, Arixtra-ionophore **3** complexes are adsorbed at nitrobenzene/water interfaces (Figure 2). A peak current on the reverse potential sweep, which is larger than a limiting current of a sigmoidal forward wave, corresponds to Arixtra desorption from the interface. This result indicates that a highly lipophilic ionophore is required for extraction of hydrophilic Arixtra into the nitrobenzene phase. On the other hand, Arixtra is more stabilized with ionophore **3** at the interface than with ionophore **1** or **2** in the bulk nitrobenzene phase as indicated by more negative $\Delta^o_w\phi_{1/2}$ and $\Delta^o_w\phi_p$ values with ionophore **3** (Table 1), which possesses a less bulky ammonium group to be ion-paired with Arixtra more strongly. A limiting current obtained with ionophore **3** is equivalent to a charge of -11 ± 1 carried by each Arixtra molecule (see Supporting Information) although the limiting current regime is narrowed by transfer of ionophore **3** from the nitrobenzene phase at more positive potentials than the switching potential. Despite the different interfacial behaviors of Arixtra, the charge transferred by each Arixtra molecule at pH 7.1 is nearly identical among quaternary-ammonium-based ionophores **1**–**3** and is consistent with the number of negatively charged groups of Arixtra.

Primary Ammonium Ionophore 4. Octadecylammonium **4** was employed as a new, simple ionophore to assess Arixtra-binding capability of a primary ammonium group at liquid/liquid interfaces. In comparison to quaternary ammonium ionophores **1**–**3**, ionophore **4** with a less bulky ammonium group is expected to bind more strongly to oxoanionic groups. In fact, a primary ammonium group serves as a major recognition site of not only proteins[7] but also several ionophores[4] to bind to heparin in bulk water at physiological pH. At liquid/liquid interfaces, however, deprotonation of a primary-ammonium-based ionophore, LH^+, is readily driven by the interfacial potential[26] to generate a neutral ionophore, L, with much weaker oxoanion-binding capability

$$LH^+(\text{organic phase}) \rightleftharpoons L\,(\text{organic phase}) + H^+\,(\text{aqueous phase}) \quad (5)$$

Preliminary experiments demonstrate that deprotonation of ionophore **4** is so favorable with an aqueous phase buffered at pH 7.1 that a large current response based on proton transfer (eq 5) overlaps with a current response to Arixtra (Figure S2, Supporting Information).

Arixtra adsorption facilitated by ionophore **4** was clearly observed by using an aqueous solution at pH 5 (Figure 3a), where ionophore deprotonation is suppressed.[26] Arixtra adsorption was dramatically facilitated by ionophore **4**, resulting in $\Delta^o_w\phi_{1/2}$ and $\Delta^o_w\phi_p$ values that are ∼250 mV more negative than those with ionophore **3** (Table 1). Although ionophore **3** is the best ionophore for heparin recognition at liquid/liquid interfaces reported so far,[9] $\Delta^o_w\phi_{1/2}$ and $\Delta^o_w\phi_p$ values with ionophore **3** are only <100 mV more negative in comparison to those with ionophore **1** or **2**. These results confirm the expectation that a primary ammonium group is a much stronger binding site for Arixtra than a quaternary ammonium group. On the other hand, large separation between $\Delta^o_w\phi_{1/2}$ and $\Delta^o_w\phi_p$ with ionophore **4** indicates sluggish Arixtra adsorption and desorption, resulting in the electrochemically irreversible CV. Also, a narrow anodic limit of the potential window is set by transfer of ionophore **4** from the nitrobenzene phase.

A current response to Arixtra with ionophore **4** was found to be rather small as represented by a small limiting current, which corresponds to $z = -7 \pm 1$ (see Supporting Information). This z value is significantly smaller than the z values of ∼−10 determined with ionophores **1**–**3** at pH 7.1. The smaller charge may be due to lower pH used for the characterization of ionophore **4**, where Arixtra may be partially protonated to carry the smaller charge across the interface. Alternatively, the charge may be carried by ionophore **4** rather than by Arixtra, indicating that ∼7 ionophore molecules are transferred across the interface to cooperatively bind to an Arixtra molecule at the aqueous side of the interface. Such a mechanism was proposed in voltammetric studies of DNA adsorption facilitated by cationic intercalators[27] and also by ionophore **2**.[28] This mechanism is highly likely with ionophore **5** (see below).

Overall stability of Arixtra-ionophore complexes depends not only on strength of interactions between each ionophore molecule and Arixtra, but also on stoichiometry, s, defined by eq 1. The complexation stoichiometry was determined for ionophore **4** using a water-filled pipet (Figure 3b),[19b,c] where limiting currents controlled by diffusion of ionophore **4** in the outer organic phase correspond to a z/s value of -1.06 ± 0.08 (see Supporting Information). With a z value of −7 determined using an organic-filled pipet, this z/s value indicates that 7 ionophore molecules are involved in interfacial complexation with each Arixtra molecule at either the nitrobenzene or aqueous side of the interface. In fact, the large complexation stoichiometry results in the sigmoidal forward wave that is not point symmetric with respect to the half-wave potential (Figure 3b).[9a] Importantly, these 1:7 Arixtra-ionophore **4** complexes are formed selectively in the presence of ∼40 mM Cl^-, which is a major interfering anion in blood samples.[9a]

Guanidinium Ionophore 5. Octadecylguanidinium **5** was synthesized and characterized as a new heparin ionophore to model arginine-enriched recognition sites of heparin-binding proteins[7] at liquid/liquid interfaces. In contrast to ionophore **4**, ionophore **5** with a less acidic guanidinium group facilitates Arixtra adsorption even at pH 7.1 without ionophore deprotonation (Figure 4a). In comparison to ionophore **3**, $\Delta^o_w\phi_{1/2}$ and

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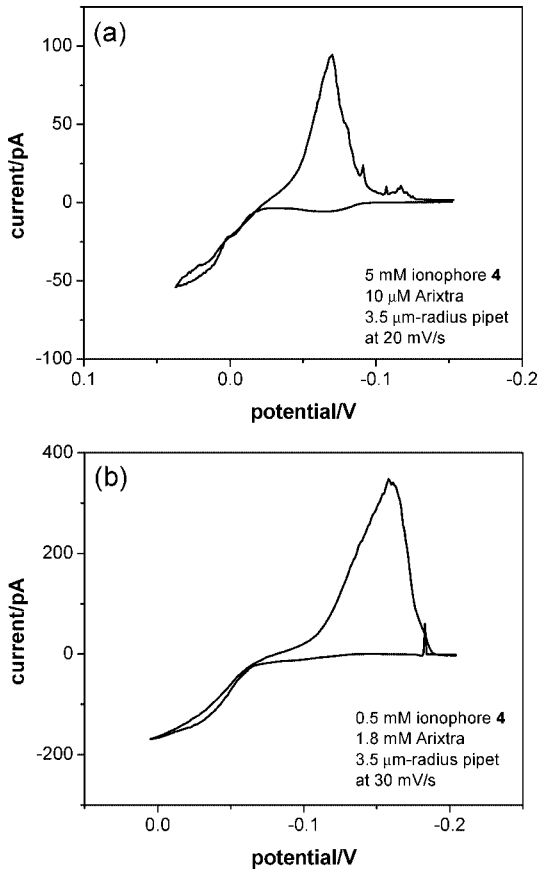

Figure 3. Background-subtracted CVs of Arixtra adsorption facilitated by ionophore **4** at (a) an organic- and (b) a water-filled pipet with $r_g/a = 1.3$. The aqueous solutions were prepared by diluting (a) a dialyzed and (b) an original Arixtra sample with 0.1 M acetic acid/sodium acetate at pH 5.0. The aqueous solution in (b) also contains ~40 mM NaCl.

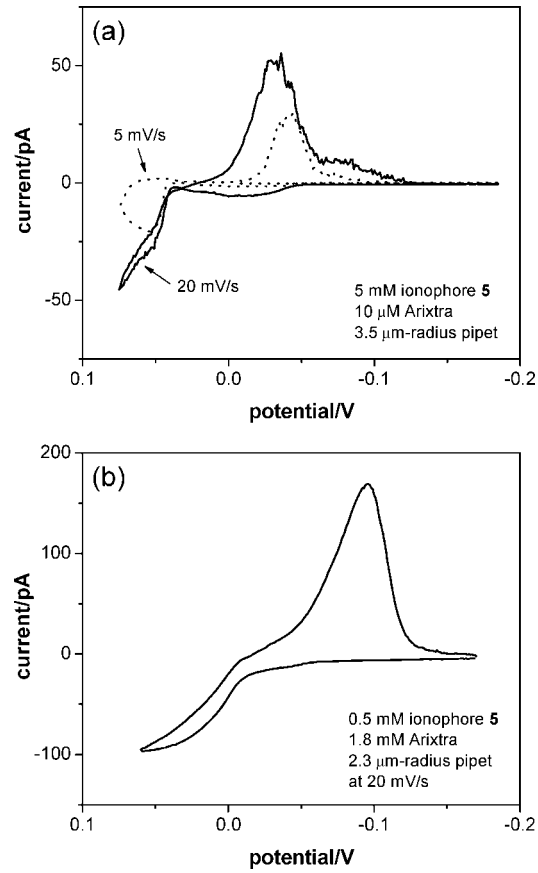

Figure 4. Background-subtracted CVs of Arixtra adsorption facilitated by ionophore **5** at (a) an organic- and (b) a water-filled pipet with $r_g/a = 1.3$. The aqueous solutions were prepared by diluting (a) a dialyzed Arixtra sample with 0.12 M NaCl and 0.01 M Tris/HCl at pH 7.1 and (b) an original Arixtra sample with 0.1 M Tris/acetate acid at pH 7.1. The aqueous solution in (b) also contains ~40 mM NaCl.

$\Delta^o_w\phi_p$ values with ionophore **5** are at least 200 mV more negative (Table 1), confirming much stronger binding of a guanidinium group to Arixtra. At the same time, slower Arixtra adsorption facilitated by ionophore **5** is electrochemically irreversible as indicated by large separation between $\Delta^o_w\phi_{1/2}$ and $\Delta^o_w\phi_p$. Importantly, the CV with ionophore **5** is well-defined despite the presence of 0.12 M NaCl in the aqueous phase. An increased current response around the switching potential is not due to Cl^- transfer but due to transfer of ionophore **5** from the nitrobenzene phase. These results indicate that the guanidinium unit is more selective to Arixtra against Cl^- than quaternary-ammonium-based ionophores.[9a]

The high selectivity of ionophore **5** for hydrophilic Arixtra is remarkable because potentiometric anion selectivity of liquid membranes based on simple alkylguanidinium ionophores follow Hofmeister series for small monovalent and divalent anions,[29] which is solely based on anion lipophilicity as a measure of a free energy required for anion extraction from the water phase into the membrane phase.[30] The high Arixtra selectivity is likely due to the requirement of less dehydration of Arixtra for complexation at the interface than for extraction into the bulk nitrobenzene phase. Also, complexation stoichiometry discussed in the following suggests that a guanidinium group of ionophore **5** is attracted electrostatically to multiple negative charges of a polyanionic Arixtra molecule, thereby forming more stable complexes with Arixtra than with such a small anion as Cl^-. On the other hand, Arixtra-ionophore **5** complexes are less stable than Arixtra-ionophore **4** complexes as indicated by more negative $\Delta^o_w\phi_{1/2}$ and $\Delta^o_w\phi_p$ values with ionophore **4**. The lesser degree of stability of ionophore **5** complexes is at least partially due to smaller complexation stoichiometry (see below).

Stoichiometry of Arixtra-ionophore **5** complexes was found to be smaller than the corresponding value of ~7 for ionophore **4** complexes. A charge involved in adsorption of each Arixtra molecule with ionophore **5** is only -4.8 ± 0.8 as determined from limiting currents at organic-filled pipets (Figure 4a; see Supporting Information). Since an Arixtra molecule carries a charge of ~ -10 across the interface at pH 7.1, this smaller charge indicates that ~5 molecules of ionophore **5** are transferred across the interface to form a complex with an Arixtra molecule at the aqueous side of the interface. This complexation stoichiometry was further confirmed using a water-filled pipet (Figure 4b), where a z/s value of -1.02 ± 0.07 was obtained from the limiting current (see Supporting Information). The z and z/s values confirm the formation of 1:5 Arixtra-ionophore **5** complexes at the interfaces. Participation of multiple ionophore molecules in the complexation process is supported by the shape of the sigmoidal forward wave without point symmetry with respect to a half-wave potential (Figure 4b).[9a]

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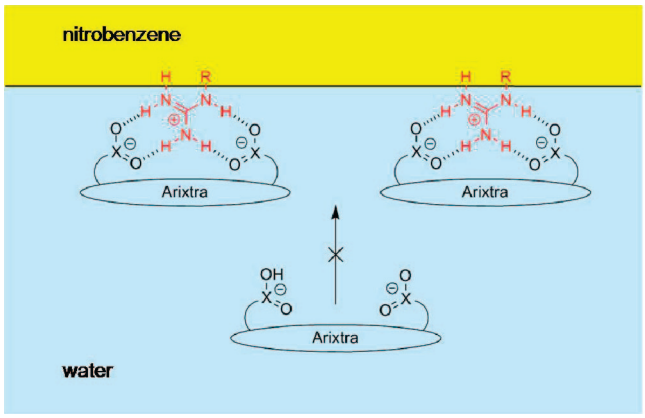

Figure 5. Scheme of 2:1 oxoanion-guanidinium binding in 1:5 Arixtra-ionophore **5** complexes and a resulting blocking effect on access of free Arixtra to the interface. XO_2^- represents a negatively charged carboxyl or sulfo group of Arixtra.

The formation of 1:5 Arixtra-ionophore **5** complexes is likely due to a multiple-hydrogen-bonding capability of a guanidinium group to simultaneously interact with two or three oxoanionic groups.[31] Figure 5 shows a possible mode of 2:1 oxoanion-ionophore **5** binding in the interfacial Arixtra complexes, where overall 10 oxoanionic groups of a Arixtra molecule are available for binding to 5 molecules of ionophore **5**. Such a binding mode was also found in a crystal structure of Arixtra-Antithrombin III complexes, where a guanidinium unit of an arginine residue of Antithrombin III interacts with a *N*-sulfo and a carboxyl group of adjacent saccharide units of Arixtra.[20] Notably, the 1:5 Arixtra-ionophore **5** complexes possess an overall charge of −5 (eq 1). The formation of highly negatively charged complexes is supported by a "blocking effect[32]" observed as a decay of a limiting current in the potential region of >0.05 V using an organic-filled pipet at slow scan rates (dotted line in Figure 4a). At a slower scan rate, more Arixtra-ionophore complexes with negative charges are adsorbed at the interface to block the access of polyanionic Arixtra molecules to the interface (Figure 5), thereby resulting in the decay of a current response. Such a blocking double-layer effect was not observed at a water-filled pipet, where aqueous Arixtra concentration is much higher and is not depleted. No blocking effect of Arixtra adsorption with ionophore **3** or **4** suggests that Arixtra complexes of the respective ionophores without or less hydrogen-bond donors are less negatively charged, while stoichiometry and concomitantly charge of Arixtra complexes were not obtainable for ionophores **1**–**3** using a water-filled pipet because of their weak binding to Arixtra (see Supporting Information).

Conclusions

This electrochemical study of a synthetic heparin mimetic, Arixtra, with well-defined structure and ionic charge revealed importance of anion-binding capability, lipophilicity, and acidity of ionophores for electrochemical heparin recognition at liquid/liquid interfaces. This work is the first to demonstrate that formal potentials and standard heterogeneous rate constants of polyion transfer depend on ionophores. The k^0 values in the range of $\sim 10^{-3}$–10^{-4} cm/s determined for Arixtra extraction by using highly lipophilic ionophores **1** and **2** indicate that the facilitated Arixtra transfer between bulk solutions can be nearly nernstian at the corresponding macrointerfaces because of relatively slow diffusion of Arixtra and its ionophore complexes with diffusion coefficients in the range of 1–4 × 10^{-6} cm^2/s. A comparison of these results with those of our previous studies of unfractionated heparin with molecular weight in the range of 5000–40 000[9] suggests that only such a small heparin molecule as Arixtra with molecular weight of 1498 can be extracted rapidly by the simple quaternary ammonium ionophores. Importantly, it can be determined unambiguously by voltammetry not by traditional potentiometry whether heparin-ionophore complexes are extracted or adsorbed and also how fast this interfacial process occurs.

A comparison of CVs based on Arixtra adsorption facilitated by ionophores **3**–**5** demonstrates for the first time that primary-ammonium and guanidinium groups serve as very strong and selective heparin-binding sites at liquid/liquid interfaces in comparison to quaternary-ammonium groups, which were used exclusively in traditional potentiometric[3,15,33] and more advanced amperometric/voltammetric[9,10] sensing of heparin at the interfaces. A highly stable Arixtra complex involves several molecules of ionophore **4** or **5**, which cooperatively bind to an Arixtra molecule at the interfaces. Despite similar anion-binding capability, a less acidic guanidinium group is required for interfacial heparin recognition at physiological pH. At the same time, ionophores **3**–**5** with an octadecyl group are not lipophilic enough to extract Arixtra or avoid potential-driven ionophore transfer from the organic phase, thereby narrowing the potential window at the nitrobenzene/water interface.

In comparison to the potentiometric counterpart, our amperometric/voltammetric approach enables more effective characterization and sensing applications of ionophores for electrochemical heparin recognition at liquid/liquid interfaces. In fact, high Arixtra selectivity against chloride is problematic in nonequilibrium potentiometry,[3,15,33] where simultaneous transfer of a polyion and its coions is required for a significant potentiometric response to the polyion. Moreover, by unique analogy between ionically polarized biological membranes and liquid/liquid interfaces,[34] the interfacial heparin recognition is envisioned as a model of protein-glycosaminoglycan interactions on cell membranes, which is important in intercellular communication.[7b]

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Supporting Information Available: Numerical simulation of cyclic voltammograms, determinations of transferred charges and complexation stoichiometry, and cyclic voltammetry of proton transfer and ionophore-free Arixtra transfer. This material is available free of charge via the Internet at http://pubs.acs.org.

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