ELSEVIER

Analytica Chimica Acta 411 (2000) 163–173

ANALYTICA CHIMICA ACTA

www.elsevier.com/locate/aca

Development of an ion-channel sensor for heparin detection

Victor P.Y. Gadzekpo[1], Philippe Bühlmann, Kang Ping Xiao[2], Hiroshi Aoki, Yoshio Umezawa*

Department of Chemistry, School of Science, The University of Tokyo, 7-3-1 Hongo, Tokyo 113-0033, Japan

Received 6 September 1999; received in revised form 28 December 1999; accepted 21 January 2000

Abstract

The ion-channel sensor technique was used to determine heparin concentrations in artificial and horse serum with cyclic voltammetry. The sensor is based on self-assembled monolayers (SAMs) of thioctic acid on which protamine is attached as a receptor to control the rate of $[Mo(CN)_8]^{4-}$ oxidation or $[Fe(CN)_6]^{3-}$ reduction in the presence of heparin. The analyte, heparin, with its negative charges, neutralizes the positive charges on the protamine receptor and at high heparin concentrations provides the electrode surface with an excess of negative charge, thereby repulsing the marker ions from the electrode surface. This decreases the redox currents and makes them a function of the analyte concentration. In artificial serum, a linear concentration range of 0.05–1.5 μg/ml was obtained for the heparin response at a scan rate of 10.24 V/s when $[Mo(CN)_8]^{4-}$ was used as marker. Repeated measurements of heparin in artificial and horse serum gave average heparin concentrations of 1.30 and 1.56 μg/ml, respectively, compared to 1.25 μg/ml heparin that was introduced into the serum. Measurements of heparin in horse serum using a fresh electrode for each sample, however, gave an average heparin concentration of 1.21 μg/ml with a standard deviation of 0.026 μg/ml.

Keywords: Ion-channel sensor; Voltammetry; Self-assembled monolayer; Polyion; Heparin; Protamine; Thioctic acid

1. Introduction

Since the introduction of ion-channel or ion-channel–mimetic sensors in 1987 [1], research efforts have been directed at the use of this technique for the detection of various ions and molecules [2]. For example, they have been used for the detection of hydrogen ions [3–6], metal cations [1,7–10], nucleotides [11–14], inorganic anions [15], antibodies [16,17], and dopamine [18]. The principle of this technique is based on binding of the analytes to receptors at an electrode surface, which controls the reduction or oxidation of electroactive ions or molecules, often referred to as markers. This control is either due to physical exclusion [1] or electrostatic attraction or repulsion [1,11,12] between the receptor/analyte complexes and the electroactive markers. Because the working principle of these sensors is similar to that of ion-channel proteins in biomembranes, these sensors have been called 'ion-channel sensors' or 'ion-channel–mimetic sensors' [2,19,20]. Ion-channel sensors offer the advantages of robustness, ease of electrode preparation and regeneration, relatively fast analysis time, ease of automation and reasonable instrumentation cost. These advantages

* Corresponding author. Fax: +81-3-5841-8349.
E-mail address: umezawa@chem.s.u-tokyo.ac.jp (Y. Umezawa)
[1] On sabbatical leave from the Department of Chemistry, University of Cape Coast, Ghana.
[2] Research Fellow of the Japan Society for the Promotion of Science (JSPS). Present address: Department of Chemistry, Michigan State University, East Lansing, USA.

0003-2670/00/$ – see front matter
PII: S0003-2670(00)00740-6

are paramount for techniques in routine analysis. Recently, we have reported that an ion-channel sensor based on electrodes chemically modified with self-assembled monolayers (SAMs) of thioctic acid can detect protamine, a polycation, at concentrations as low as 0.5 μg/ml when $[Ru(NH3)_6]^{3+}$ is used as a marker ion [21]. The detection of polyions with ion-channel sensors is advantageous since the large charge numbers on polyions (about −70 for heparin) result in strong binding to receptors and, therefore, allow highly sensitive detection of the polyions. The detection of protamine with an ion-channel sensor has encouraged us to further investigate the use of ion-channel sensors for the detection of heparin, a polyanion and an important biopolymer.

Heparin is extensively used as an anticoagulant in many clinical procedures for the prevention of blood clotting, especially during open heart surgery [22,23]. The normal heparin concentration during these procedures is between 2 and 8 U/ml, which corresponds to 0.8–3.2 μM or 12–48 μg/ml [24]. In the treatment of post-operative thrombosis and embolism, however, the therapeutic concentration range of heparin is 0.2–0.7 U/ml [25]. This implies that any method for heparin analysis should be able to detect heparin at concentrations as low as 0.2 U/ml. Heparin has a molecular weight ranging from 5,000 to 30,000 with an average molecular weight of 15,000. At physiological pH, heparin is ionized and becomes negatively charged due to complete ionization of sulphate ($ROSO_3^-$ and $RNHSO_3^-$) and carboxylate ($RCOO^-$) groups [26]. The method commonly used for heparin determination in blood samples is the activated clotting time (ACT) method or the activated partial thromboplastin time (APTT) method [27]. In these methods, the clotting time of the plasma sample is measured after an initiator of the clotting process has been added. The heparin activity is then determined from the delay in the appearance of a clot. Typical clotting times for normal plasma are in the order of 30–40 s. Even though these methods give accurate results and have been in use for quite a long time, it has been reported that the clotting times determined by these methods not only depend on the amount of heparin but also on the concentration of antithrombin III (ATIII) and other coagulation factors [28].

Research efforts have been directed at developing alternative methods of heparin analysis in recent times. A heparin responsive potentiometric sensor has been reported with a detection range of 1.0–9.8 U/ml [29–36]. This sensor employed tridodecylmethylammonium chloride (TDDMACl) as a sensing element incorporated in a polymeric liquid membrane. This polymeric membrane electrode has been used successfully to determine heparin concentrations in blood samples. van Kerkhof et al. have reported on a heparin sensor based on protamine as affinity ligand using an ion-sensitive field effect transistor (ISFET) [28,36]. An indirect ion-step method was employed in this technique to detect heparin. The detection range of this sensor was reported to be 0.1–2.0 U/ml. A surface plasmon resonance sensor based on protamine and polyethylene imine (PEI) as affinity ligand has also been reported for heparin measurements in blood plasma with a detection limit of 0.2 U/ml and a linear range of 0.2–2.0 U/ml [37–39].

In this paper we report the detection of heparin using ion-channel sensors based on electrodes chemically modified with SAMs. The sensor has been applied to the measurement of heparin in artificial and horse serum.

2. Experimental

2.1. Reagents

Thioctic acid (1,2-dithiolane-3-pentanoic acid), *N*,*N*′-dicyclohexyl carbodiimide, and protamine sulphate (salmine sulphate from salmon sperm with average M_r of 4,500) were obtained from Tokyo Chemical Industry (TCI), Tokyo, Japan. $[Ru(NH_3)_6]Cl_3$ and (2-aminoethyl)trimethyl ammonium chloride were purchased from Aldrich, Milwaukee, WI, USA, and triethylamine from Kokusan Chemical Works, Tokyo, Japan. Tris(hydroxymethyl)aminomethane (Tris), *N*-hydroxysuccinimide, albumin (bovine serum, low salt), $K_3[Fe(CN)_6]$, KCl, NaCl, $MgCl_2$, $CaCl_2$, KH_2PO_4, and $MgSO_4$ were obtained from WAKO Pure Chemical Industries, Osaka, Japan. $K_4[Mo(CN)_8]$ was prepared and purified according to Refs. [40,41]. Heparin was obtained as a donation from Daiichi Chemical, Tokyo, Japan, while 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide hydrochloride was obtained from Nacalai Tesque, Kyoto, Japan. γ-Globulin was obtained from Sigma

Chemical, St. Louis, MO, USA, and horse serum was obtained as a donation from Tissue Culture Biologicals, Tokyo, Japan. All chemicals were of the highest grade available and used without further purification. Buffer solutions of pH 7.4 contained 10 mM Tris. The pH values of the buffer solutions were adjusted with KOH or HCl. All solutions were prepared from deionized and charcoal-treated water (specific resistance >18 MΩ cm) prepared by passage through a Milli-Q cartridge filtering system (Millipore, Bedford, MA, USA).

2.2. Self-assembly of thioctic acid monolayers on Au electrodes

A gold electrode (Bioanalytical Systems, West Lafayette, IN, USA) was polished with wet 0.3 and 0.05 μm alumina slurry (Alpha Micropolish alumina No. 2 and Gamma Micropolish alumina No. 3, respectively; Buehler, Lake Bluff, IL, USA) on a felt pad for 10 min and rinsed several times with water and finally cleaned in a sonicator. The polished electrode was then dipped in 0.5 M KOH and the potential was cycled between 0 and −1.4 V until the cyclic voltammogram (CV) indicated a perfectly clean electrode surface. The electrode was then rinsed several times with water and finally with anhydrous ethanol. The electrode was then immersed in a 0.1% thioctic acid solution in ethanol for at least 24 h and rinsed several times with water.

2.3. Electrostatic attachment of protamine to self-assembled thioctic acid

Gold electrodes with the self-assembled thioctic acid were dipped in tris(hydroxymethyl)aminomethane (Tris) buffer/0.1 M KCl solution (pH 7.4) containing 55.6 μg/ml protamine. To ascertain that protamine adsorbed to the thioctic acid SAM, CVs of $[Ru(NH_3)_6]^{3+}$ were run in Tris/0.1 M KCl buffer solution of pH 7.4 between the potentials of +0.1 and −0.5 V. The decrease in oxidation and reduction peaks confirmed the protamine attachment.

2.4. Covalent attachment of protamine to self-assembled thioctic acid

The thioctic acid modified electrode was dipped into a 1:1 mixture of *N*-hydroxysuccinimide (0.1 M) and 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (0.4 M) for about 10 min. The electrode was washed with water and then dipped into a protamine solution (1 mM). After 20 min the electrode was washed and stored in water until use. To ascertain the protamine attachment, CVs were run as described for the case of the electrostatic attachment.

2.5. Preparation and self-assembly of *N*-(2-trimethylammoniumethyl) 1,2-dithiolane-3-pentanamide chloride

Thioctic acid (1 M, 2 ml) and *N,N′*-dicyclohexylcarbodiimide (1 M, 2 ml) were mixed and allowed to stand for about 10 min. Then, 2 ml of *N*-hydroxysuccinimide (1.0 M) was added and the solution stirred and allowed to stand again for about 10 min. A 1:1 mixture of triethylamine and (2-aminoethyl)trimethylammonium chloride was prepared, each with a concentration of 1.0 M. Then, 2 ml of this 1:1 mixture was added to the *N*-hydroxysuccinimide mixture and stirred for 1 month at room temperature (∼27°C). All the solutions were prepared in deuterated dimethyl sulfoxide (DMSO-d_6) and the extent of each reactions determined by comparing the intensities of the signals for $C\underline{H}_2COOH$ of thioctic acid at 2.10 ppm and for $C\underline{H}_2CONH$ of the product at 2.06 ppm. Formation of the product was further confirmed by FAB mass spectroscopy. Attempts to enhance the reaction rate with higher temperatures failed because this led to the formation of byproducts, apparently acylated dicyclohexylureas. At the end of the reaction, DMSO-d_6 was evaporated using a bulb-to-bulb tube oven and the resulting product dissolved in methanol at reflux temperature. Upon slowly cooling to 0°C and removal of a *N,N′*-dicyclohexylurea precipitate by filtration, the solvent was evaporated, giving a very hygroscopic crude product containing the desired receptor. It was dissolved in ethanol and directly used for self-assembly without further purification.

2.6. Artificial serum

Artificial serum was prepared by dissolving 4.037 g NaCl, 0.109 g KCl, 0.100 g KH_2PO_4, 0.185 g $CaCl_2{\cdot}2H_2O$, and 0.129 g $MgSO_4{\cdot}7H_2O$

in 500 ml water, as recommended [42]. This composition is typical for blood. γ-Globulin (3.51 g) and albumin (bovine serum, 6.0 g) were dissolved in 100 ml of the blood electrolyte solution. Tris(hydroxymethyl)aminomethane (0.12 g) was then added and the pH adjusted to 7.4. The solution so prepared in this work is referred to as artificial serum. It was stored at <0°C.

2.7. Electrochemical measurements

Cyclic voltammetry experiments were performed with a BAS CV-50W potentiostat/galvanostat (Bioanalytical Systems). All potentials were measured versus a Ag/AgCl reference electrode, and a platinum wire was used as an auxiliary electrode. All CVs were run after purging the sample solutions with nitrogen gas for 15 min. Solutions for cyclic voltammetry contained 0.1 M KCl and either 1.0 mM $K_3[Fe(CN)_6]$, 1.0 mM $[Ru(NH_3)_6]Cl_3$ or $K_4[Mo(CN)_8]$. They contained Tris buffer of pH 7.4, unless stated otherwise. The heparin stock solutions also contained Tris buffer and 0.1 M KCl. The concentration of heparin was varied by spiking small aliquots of 0.101 mg/ml heparin stock solutions into 10 ml of the KCl/marker solutions. Potential windows of +0.6 to −0.1 V, +0.1 to −0.5 V and +0.1 to +0.65 V were used for the cyclic voltammetry with $[Fe(CN)_6]^{3-}$, $[Ru(NH_3)_6]^{3+}$ and $[Mo(CN)_8)]^{4-}$, respectively.

2.8. Preparation of artificial and horse serum heparin samples

Heparin solutions were prepared by dilution of 50 μl of heparin stock solution (500 μg/ml) to 1 ml with the blood electrolyte solution. Five microliters of the thus prepared solution was pipetted into 5 ml of the artificial serum and horse serum, giving a heparin concentration of 27 μg/ml in both the horse and artificial serum. This concentration is equivalent to 4.2 U/ml heparin.

2.9. Determination of heparin in artificial and horse serum

Artificial or horse serum (500 μl) was pipetted into 9.5 ml of blood electrolyte solution containing 1.0 mM $[Mo(CN)_8]^{4-}$. CVs of $[Mo(CN)_8]^{4-}$ were run between the potentials of +0.1 and +0.65 V at a scan rate of 10.24 V/s. Heparin standard (0.101 mg/ml; 60 ml) prepared in blood electrolyte solution was added and CVs run again. The peak potential of the CV before the serum was added was used to assess the $[Mo(CN)_8]^{3-}$ reduction current. The concentration of heparin in the unknown serum was then calculated by the standard addition equation.

3. Results and discussion

3.1. Thioctic acid SAMs on gold electrode

Electrodes chemically modified with SAMs of thioctic acid have been reported as ion-channel sensors for the detection of protamine [21]. The negative charges on the surface of self-assembled thioctic acid monolayers caused protamine to bind electrostatically to the thioctic acid monolayers. This allowed negative marker ions in solution to approach the electrode surfaces or repulsed positive marker ions from them, thereby controlling the redox current of the marker ion. The protamine bound to the thioctic acid monolayer was not removed unless it was washed in 0.1 M KCl solution with its pH adjusted to 5.1 [21]. This suggested that protamine-modified electrodes could also be used as sensing elements. The major components of protamine contain approximately 20 arginine groups. Their guanidine groups provide protamine with a charge of about +20 per molecule and can repulse positively charged marker ions or attract negatively charged marker ions [43]. In the presence of a negatively charged analyte, the reduction or oxidation of the marker ions can be thus controlled according to the principle of ion-channel sensing, thereby allowing the detection of the negatively charged analytes. We have herein investigated monolayers of *N*-(2-trimethylammoniumethyl)-1,2-dithiolane-3-pentanamide chloride, and protamine electrostatically or covalently attached to thioctic acid monolayers as receptors for heparin detection (Fig. 1).

3.2. Heparin response with $[Mo(CN)_8]^{4-}$ as marker using electrodes with electrostatically attached protamine as receptor

The heparin responses of the ion-channel sensor based on electrostatically attached protamine as

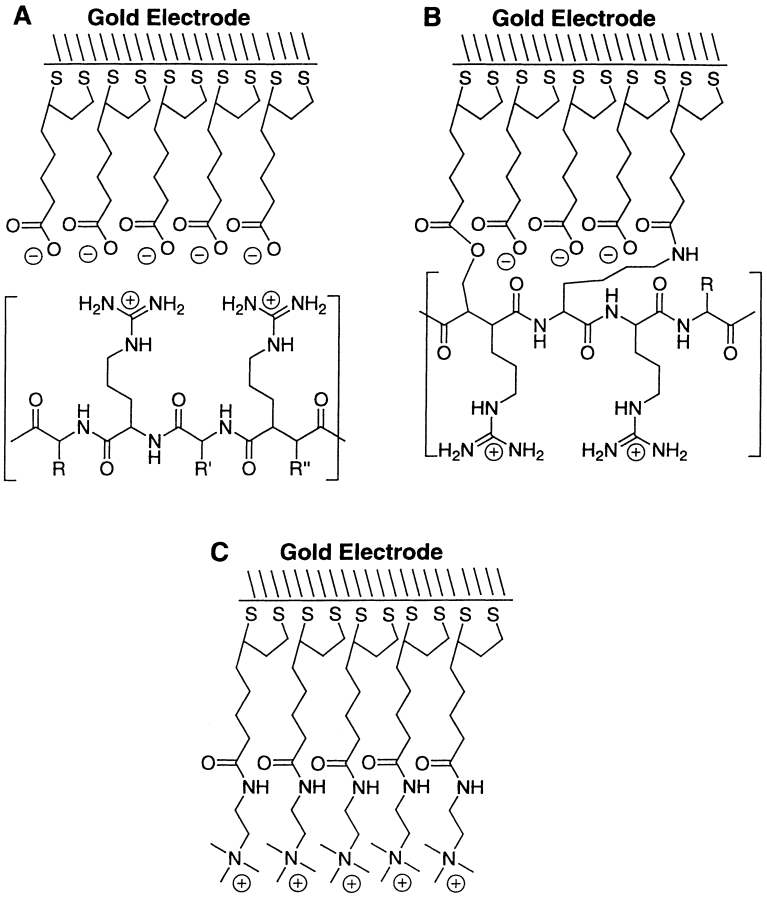

Fig. 1. Chemical structures of heparin receptors. (A) Protamine electrostatically attached to a SAM of thioctic acid. Protamines are a group of simple oligopeptides that contain arginine, alanine, and serine as major amino acids, as well as proline, valine, glycine, isolycine, cysteine, histidine, lysine, threonine, aspartic and glutamic acid [43]. (B) Protamine covalently attached to a SAM of thioctic acid. (C) Synthetic receptor with a quaternary ammonium group (*N*-(2-trimethylammoniumethyl)1,2-dithiolane-3-pentanamide chloride).

receptor (Fig. 1A) at pH 7.4 (Tris buffer in 0.1 M KCl) with $[Mo(CN)_8]^{4-}$ as marker are shown in Fig. 2. CVs were obtained at a scan rate of 0.1 V/s. A pH of 7.4 was used because it is close to the pH of blood. It can be seen that as the currents for the $[Mo(CN)_8]^{4-/3-}$ redox couple decrease, the separation between the peak potentials increases and the CVs become irreversible as aliquots of heparin are added to the buffer. These changes can be ascribed to binding of the negatively charged heparin to the positively charged electrostatically attached protamine. The presence of heparin on the electrode surface results in electrostatic repulsion of $[Mo(CN)_8]^{4-}$ and $[Mo(CN)_8]^{3-}$. The dependence of the sensor response on the heparin concentration is shown in Fig. 3. The reduction currents at +0.525, +0.506, +0.471 and +0.448 V in the backward scans of the CVs were used to assess the heparin responses at 0.1, 1.0, 5.12 and 10.24 V/s, respectively. These potentials are the peak potentials of the $[Mo(CN)_8]^{3-}$ reduction in the absence of heparin. At a scan rate of 0.1 V/s, the response range for heparin is from 1.0 to 3.0 μg/ml. Fig. 3 shows the response range for heparin from 1.0 to 3.0 μg/ml. We showed previously that increasing the scan rate of the CVs allows detection of the analyte at lower concentrations and also increases the linear concentration range [21]. This is also the case for this sensor, as shown in Fig. 3, where the current over the square root of the scan

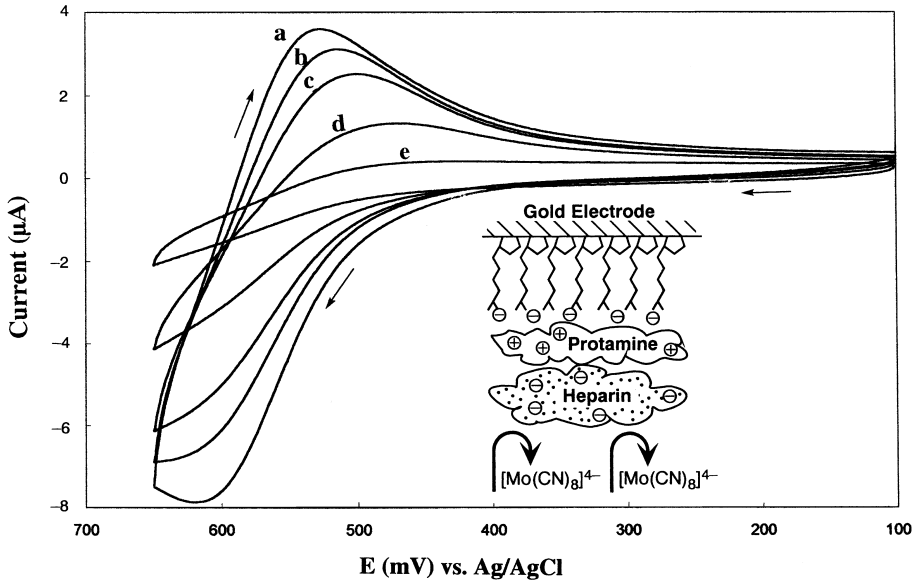

Fig. 2. Cyclic voltammograms of $[Mo(CN)_8]^{4-}$ (1 mM) in Tris buffer (pH 7.4)/0.1 M KCl with protamine electrostatically attached to SAMs of thioctic acid. Heparin concentrations: (a) 0.00, (b) 1.52, (c) 2.02, (d) 2.53, and (e) 4.04 μg/ml. Scan rate, 0.1 V/s.

rate is plotted against the heparin concentration. The dynamic range increased as the scan rate is increased. The linear concentration range for the scan rate of 1.0 V/s is from 1.0 to 2.5 μg/ml (0.17–0.42 U/ml) with a dynamic range of 0.6–3.0 μg/ml (0.1–0.5 U/ml). For the scan rate of 10.24 V/s, the dynamic range is wider (0.2 to 2.5 μg/ml, 0.03–0.42 U/ml) with a linear concentration range of 0.6–2.5 μg/ml (0.1–0.41 U/ml) for the scan rate of 10.24 V/s. These linear ranges are at similar but slightly lower concentrations than the linear response ranges that have been reported so far for heparin detection [28,30,39]. This may be due to the strong binding between electrostatically attached protamine and heparin on one hand and the high surface concentration of the receptor on the other hand. The stronger the binding and the higher the surface concentration of the receptor, the more sensitive is the response, resulting in responses at lower concentrations. These sensors are suitable for heparin determination in blood serum. The reproducibility of the heparin calibration curve using the same electrode at a scan rate of 1.0 V/s is shown in Fig. 4. In all, four measurements were taken for each heparin concentration, and averages are plotted with the standard deviations.

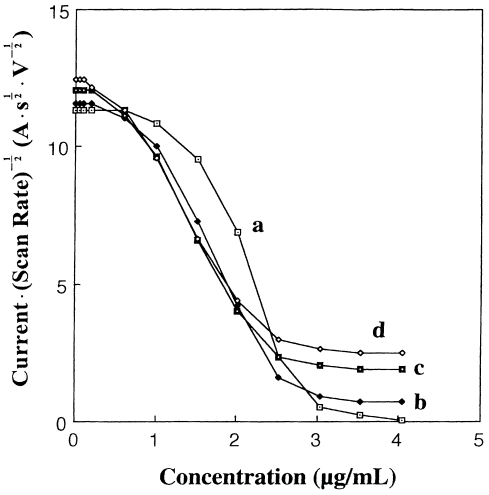

Fig. 3. Effect of scan rate on the dynamic range of heparin response (1.0 mM $[Mo(CN)_8]^{4-}$ as marker; in Tris buffer pH 7.4/0.1 M KCl). (a) 0.10, (b) 1.00, (c) 5.12, and (d) 10.24 V/s. For enhanced comparability, the *y*-axis represents the ratio of the oxidation currents at the peak potential in the backward scans and the square root of the scan rate.

After heparin detection, heparin was removed from the surface of the electrode by washing the electrode in 0.1 M KCl solution of pH 1.5 (The pH was adjusted by the addition of small aliquots of concentrated HCl to the KCl solution.). The washing was done by

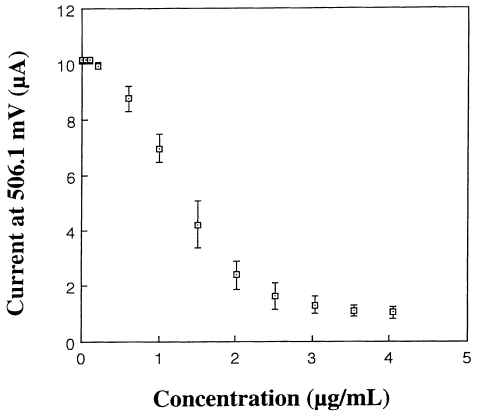

Fig. 4. Reproducibility of the heparin calibration curve at a scan rate of 1.00 V/s when using 1.0 mM $[Mo(CN)_8]^{4-}$ as marker in Tris buffer pH 7.4/0.1 M KCl. The current was measured in the backward scan at +506 mV versus Ag/AgCl.

shaking a test tube containing the washing solution and the electrode for about 2 min. This washing removed both protamine and heparin from the surface of the electrode. A CV of $[Ru(NH_3)_6]^{3+}$ was then run in Tris/0.1 M KCl buffer (pH 7.4) to ascertain that all heparin and protamine had been removed from the electrode surface. Then, the new CV was compared to that of $[Ru(NH_3)_6]^{3+}$ before the electrode was used for the heparin measurement. Protamine was again put back on the electrode by dipping the electrode in a 56 μg/ml protamine solution and a CV was run to ensure that protamine was indeed bound to the electrode. The 56 μg/ml of protamine was found to be the minimum concentration of protamine that gave no further significant decreases in the reduction current for $[Ru(NH_3)_6]^{3+}$ when the electrode was once more exposed to a protamine solution. Such electrodes can thus be used repeatedly by going through the washing and the protamine attachment process until the CV of the marker after protamine attachment is different from the CV right before the first electrode use.

3.3. Heparin response with $[Fe(CN)_6]^{3-}$ as marker using electrodes with electrostatically attached protamine as receptor

The responses of this ion-channel sensor to heparin at pH 7.4 (Tris buffer in 0.1 M KCl) with $[Fe(CN)_6]^{3-}$ as the marker ion are shown in Fig. 5. In the absence of heparin, a reversible CV is obtained. With the addition of small aliquots of heparin (1.01 μg/ml) to the sample solution, the peak current decreased and the CV becomes quasi-reversible. Further addition of heparin up to 3.03 μg/ml further reduces the reversibility of the CVs. The presence of heparin in the sample solution clearly inhibits the reduction of the negatively charged $[Fe(CN)_6]^{3-}$ at the electrode surface. This result can be explained by binding of heparin to the protamine electrostatically attached to the surface of the thioctic acid monolayer. Heparin with its negative charges binds to the protamine, reducing the positive excess charge on the monolayer, and at high heparin concentrations gives the electrode surface an excess negative charge. The reduction current depends on the heparin concentration at different scan rates, as shown in Fig. 6. The calibration curve at a scan rate of 51.2 V/s gave the most linear curve with a rather wide linear concentration range of 0.05–1.0 μg/ml. This result is comparable to that obtained with $[Mo(CN)_8]^{4-}$ as a marker ion. The linear concentration range, however, was at a lower heparin concentration with a shorter dynamic range for the sensor with $[Fe(CN)_6]^{3-}$ as marker. The response of the same electrode at a scan rate of 51.2 V/s gave results similar to those shown in Fig. 4 for $[Mo(CN)_8]^{4-}$. Half of the maximum response was reached at 0.6 mg/ml heparin.

3.4. Heparin response using electrodes with covalently attached protamine as receptor

Protamine was permanently attached to monolayers of self-assembled thioctic acid through covalent bonding of protamine to the carboxylic acid groups of the thioctic. This attachment likely mainly occurs by formation of amide type bonds to the guanidinium groups of heparin, but covalent attachment through the side chains of other amino acids appears possible, resulting, e.g., in formation of ester bonds (c.f. Fig. 1B). This covalently attached protamine was used as a receptor. The response of this ion-channel sensor to heparin with $[Fe(CN)_6]^{3-}$ as marker is similar to the other responses mentioned above. As aliquots of heparin are added to the buffer solution, the current of $[Fe(CN)_6]^{3-}$ reduction decreases, the reduction peak potential shifts to a more negative value and the CVs become irreversible. These changes can also be

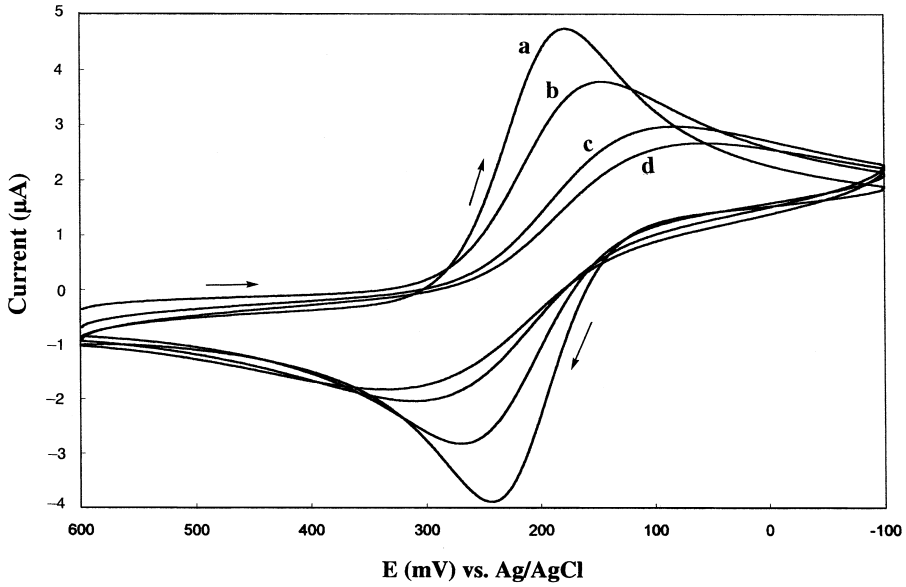

Fig. 5. Cyclic voltammograms of 1.0 mM $[Fe(CN)_6]^{3-}$ in Tris buffer pH 7.4/0.1 M KCl with protamine electrostatically attached to SAMs of thioctic acid. Scan rate, 0.1 V/s. Heparin concentrations: (a) 0.00, (b) 1.01, (c) 1.52, and (d) 3.03 μg/ml.

attributed to binding of the negatively charged heparin to the positively charged and covalently attached protamine. The dependence of the sensor response on the heparin concentration at pH 7.4 at different scan rates gave responses with a dynamic range of 0.05–0.5 μg/ml for all the scan rates investigated. The linear concentration range increased as the scan rate was increased. At a scan rate of 51.2 V/s, the linear concentration range was 0.05–0.3 μg/ml. This range is narrower than what was observed for the electrostatically attached receptor. This may be explained by the fact that the covalently attached protamine covers the electrode with a higher concentration, resulting in a more homogeneous coverage of the electrode and avoiding surface sites of low protamine concentration that can only be blocked at high heparin concentrations. After exposure of the sensor to heparin, however, it was difficult to remove heparin from the surface of the electrode. Using the same electrode with $[Mo(CN)_8]^{4-}$ as a marker, it responded to heparin with a dynamic range of 0.1–0.6 μg/ml for the scan rates of 5.12 and 10.24 V/s. The dynamic range for this sensor at the scan rate of 1.0 V/s is at 0.2–0.6 μg/ml, which is also at lower heparin concentrations as compared to the dynamic range of the sensors based on electrostatic attachment. This further suggests that covalently attached protamine has a higher surface concentration than electrostatically attached protamine. However, heparin could not be removed from the electrode surface, as it was also the case when using $[Fe(CN)_6]^{3-}$

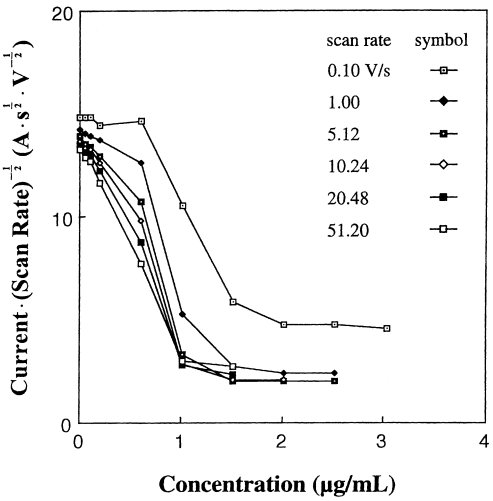

Fig. 6. Calibration curves of heparin response at different scan rates using protamine electrostatically attached to SAMs of thioctic acid in 1.0 mM $[Fe(CN)_6]^{3-}$ marker; Tris buffer pH 7.4/0.1 M KCl. For enhanced comparability, the *y*-axis represents the ratio of the measured currents and the square root of the scan rate.

as marker. Therefore, these sensors could not be used for repeated measurements of heparin.

3.5. Heparin response using a synthetic receptor with a trimethylammonium group

Trying to avoid the removal of the receptor together with heparin in the washing procedure (c.f. Section 3.2) and to avoid the problems encountered with the covalently attached protamine (c.f. Section 3.4.), we designed and synthesized a receptor with a disulfide group for the attachment to gold and a quaternary ammonium group for binding of heparin, *N*-(2-trimethylammoniumethyl)1,2-dithiolane-3-pentanamide chloride, (Fig. 1C). A receptor group with a quaternary ammonium group rather than a protonated amine was chosen because this avoids that the charge density of the monolayer depends on the pH of the sample solution. Evaluation of the desorption peak in the CVs by assuming desorption of two thiol groups per receptor shows that the surface density of this receptor was 2.94×10^{-10} mol/cm^2. Considering the typical surface density of 7.9×10^{-10} mol/cm^2 for alkanethiols [44] and the fact that with two sulfurs the footprint of this receptor must be expected to be larger than that of alkanethiols, this indicates formation of a fairly well packed monolayer of the quaternary ammonium receptor.

This receptor, when used with $[Ru(NH)_3]^{3+}$ as marker in 0.1 M KCl, repulsed the marker from approaching the electrode surface, causing a peak separation of 67, 123, 143 and 224 mV when the scan rates of 0.1, 5.12, 10.24 and 51.2 V/s were used, respectively. For heparin detection, the sensor was used with $[Mo(CN)_8]^{4-}$ as a marker. The response at a scan rate of 10.24 V/s gave the best response with a decrease of the total current of already 15% below 0.05 μg/ml. It is interesting to note that upon washing the sensor with water, the CV showed currents and peak potentials almost equal to the original CV before contact of the sensor with heparin. This implies the absence of heparin on the electrode surface and shows that this sensor can be more easily regenerated than the other ones reported here. However, the response at high heparin concentrations was not as sensitive as in the case of the sensors based on electrostatically attached protamine. At 1.0 μg/ml heparin, the sensor response was still nearly 75% of the response in absence of heparin. At present, this result cannot be satisfactorily explained and will require further exploration. Notwithstanding, these results indicate that artificial receptors for heparin appear to be promising for further lowering detection limits and improve the ease of regeneration of ion-channel sensor for heparin.

3.6. Heparin determination in artificial and horse serum

The sensor based on the electrostatic attachment of protamine (Fig. 1A) was used for the determination of heparin in both artificial and horse serum. Previous studies of ion-channel sensors for protamine revealed no significant interference from blood electrolytes such as sodium, potassium, calcium and magnesium [21]. In horse serum, while the response of the sensor was small at low potential scan rates, the analyte could be detected at a high CV scan rates [21]. Similarly, the present heparin sensor with $[Mo(CN)_8]^{4-}$ as marker responds to heparin in artificial serum with a linear concentration range of 0.05–1.5 μg/ml at a scan rate of 10.24 V/s. This marker was used because at moderately high scan rates the current response to heparin was linear even at lower heparin concentrations. The determination of heparin was, therefore, carried out at a scan rate of 10.24 V/s. The artificial serum contained albumin (6 g/100 ml) and γ-globulin (3.5 g/100 ml) in addition to the blood electrolytes sodium (140 mM), potassium (5.6 mM), calcium (2.5 mM) and magnesium (1.5 mM). This composition is typical for blood serum [42]. This shows that interference from blood electrolytes and these two macromolecules is minimal.

The method of standard addition was then used for the determination of heparin in diluted artificial and horse serum. Heparin was introduced into the serum sample by spiking with an aliquot of heparin stock solution. The artificial serum and the horse serum samples were both diluted 20 times, bringing the heparin concentration to 1.25 μg/ml (0.21 U/ml). In the artificial serum, an average result of 1.30 μg/ml was obtained for three measurements, using the same sensor repeatedly. This result is 4.0% higher than what had been actually added. In the horse serum, however, an average heparin concentration of 1.56 μg/ml was obtained when the sensor was used repeatedly, which is 24.5% higher than the true value. The higher devia-

tion in the analysis of horse serum may be due to the false positive and partly irreversible response to other macromolecules, resulting in a constant increase in the value in repetitive measurements. When a fresh electrode was used for each determination of heparin in horse serum, however, a heparin concentration of 1.21 μg/ml was obtained in three measurements, which is 2.9% lower than what was added. The standard deviation was 0.026 μg/ml. Further work will be required to allow the repeated use of these electrodes.

4. Conclusions

Ion-channel sensors have been used for the detection of heparin with $[Mo(CN)_8]^{4-}$ or $[Fe(CN)_6]^{3-}$ as electroactive markers. The detection is based on the attachment of protamine onto self-assembled monolayers of thioctic acid. The sensors have been used to determine heparin in both artificial and horse serum. Preliminary results with a quaternary ammonium receptor for heparin suggest that further improvements in detection limits and ease of electrode regeneration can be obtained by use of designed synthetic receptors. In summary, this work has shown that polyions with their characteristic high charge numbers, usually hydrophilic in nature and large in size, can be detected by using ion-channel sensors. These polyions with their multiple charges have the advantage of being strongly bound to receptor monolayers, which allows them to electrostatically control the access of marker ions to the electrode surface. This mode of sensing is a very general approach to sensing of polyions.

Acknowledgements

Y.U. acknowledges the Japan Society for the Promotion of Science (JSPS) for the Invitation Fellowship Programme for Research in Japan (long term) to V.P.Y.G. A JSPS fellowship to K.P.X. is also gratefully acknowledged. We thank Daiichi Chemical Company, Tokyo, Japan and Tissue Culture Biologicals, Tokyo, Japan for the kind donation of heparin and horse serum, respectively. This work was supported by Grant-in-Aid for Scientific Research for the Priority Areas of ‘Electrochemistry of Ordered Interfaces’ (No. 10131216).

References

[1] M. Sugawara, K. Kojima, H. Sazawa, Y. Umezawa, Anal. Chem. 59 (1987) 2842.
[2] P. Bühlmann, H. Aoki, K.P. Xiao, S. Amemiya, K. Tohda, Y. Umezawa, Electroanalysis 10 (1998) 1149.
[3] N. Nakashima, T. Taguchi, Colloids Surf. 103 (1995) 159.
[4] Q. Cheng, A. Brajter-Toth, Anal. Chem. 64 (1992) 1998.
[5] H.O. Finklea, in: A.J. Bard, I. Rubinstein (Eds.), Electroanalytical Chemistry, Vol. 19, Marcel Dekker, New York, 1996, p. 109.
[6] Y. Liu, M. Zhao, D.E. Bergbreiter, R.M. Crooks, J. Am. Chem. Soc. 119 (1997) 8720.
[7] M. Maeda, K. Nakano, S. Uchida, M. Takagi, Chem. Lett. (1994) 1805.
[8] K. Takahara, M. Aihara, Y. Miura, F. Tanaka, Bioelectrochem. Bioenerg. 39 (1996) 135.
[9] K. Yagi, S.B. Khoo, M. Sugawara, T. Sakaki, S. Shinkai, K. Odashima, Y. Umezawa, J. Electroanal. Chem. 401 (1996) 65.
[10] M. Takaya, P. Bühlmann, Y. Umezawa, Mikrochim. Acta 132 (1999) 55.
[11] M. Sugawara, M. Kataoka, K. Odashima, Y. Umezawa, Thin Solid Films 180 (1989) 129.
[12] S. Nagase, M. Kataoka, R. Naganawa, R. Komatsu, K. Odashima, Y. Umezawa, Anal. Chem. 62 (1990) 1252.
[13] Y. Katayama, Y. Ohuchi, M. Nakayama, M. Maeda, H. Higashi, Y. Kudo, Chem. Lett. (1997) 883.
[14] K. Tohda, S. Amemiya, T. Ohki, S. Nagahora, S. Tanaka, P. Bühlmann, Y. Umezawa, Isr. J. Chem. 37 (1997) 267.
[15] K.P. Xiao, P. Bühlmann, Y. Umezawa, Anal. Chem. 71 (1999) 1183.
[16] R. Blonder, E. Katz, Y. Cohen, N. Itzhak, A. Riklin, I. Willner, Anal. Chem. 68 (1996) 3151.
[17] A. Doron, E. Katz, G. Tao, I. Willner, Langmuir 13 (1997) 1783.
[18] A. Dalmia, C.C. Liu, R.F. Savinell, J. Electroanal. Chem. 430 (1997) 205.
[19] W. Kutner, J. Wang, M. L’Her, R.P. Buck, Pure Appl. Chem. 70 (1998) 1301.
[20] L. Luo, X. Yang, E. Wang, Anal. Lett. 32 (1999) 1271.
[21] V.P.Y. Gadzekpo, K.P. Xiao, H. Aoki, P. Bühlmann, Y. Umezawa, Anal. Chem. 71 (1999) 5109.
[22] B. Casu, in: F.A. Ofosu, I. Danishefsky, J. Hirsh (Eds.), Heparin and Related Polysaccharides, Structure and Activities, The New York Academy of Sciences, New York, 1989, p. 556.
[23] J. Hirsh, Nouv. Rev. Fr. Hematol. 26 (1984) 262.
[24] L.B. Jaques, Pharm. Rev. 31 (1980) 99.
[25] J. van Putten, M. van De Ruit, M. Beunis, H.C. Hemker, Clin. Chim. Acta 122 (1982) 261.
[26] R.J. Linhardt, Chem. Ind. 21 (1991) 45.
[27] S.B. Bull, W.M. Huse, F.S. Brauer, R.A. Korpman, J. Thorac. Cardiovasc. Surg. 69 (1975) 685.
[28] J.C. van Kerkhof, P. Bergveld, R. Schasfoort, Biosens. Bioelectron. 10 (1995) 269.
[29] S.C. Ma, V.C. Yang, M.E. Meyerhoff, Anal. Chem. 64 (1992) 697.

[30] S.C. Ma, V.C. Yang, B. Fu, M.E. Meyerhoff, Anal. Chem. 65 (1993) 2078.
[31] M.E. Meyerhoff, V.C. Yang, J.A. Wahr, L.M. Lee, J.H. Yun, B. Fu, E. Bakker, Clin. Chem. 41 (1995) 1355.
[32] M.E. Meyerhoff, B. Fu, E. Bakker, J.H. Yun, V.C. Yang, Anal. Chem. 68 (1996) 168A.
[33] B. Fu, E. Bakker, J.H. Yun, E. Wang, V.C. Yang, M.E. Meyerhoff, Electroanalysis 7 (1995) 823.
[34] S. Dai, J.M. Esson, O. Lutze, N. Ramamurthy, V.C. Yang, M.E. Meyerhoff, J. Pharm. Bio. Anal. 19 (1999) 1.
[35] S. Mathison, E. Bakker, J. Pharm. Bio. Anal. 19 (1999) 163.
[36] J.C. van Kerkhof, P. Bergveld, R. Schasfoort, Biosens. Bioelectron. 8 (1993) 463.
[37] K. Gaus, E.A.H. Hall, J. Colloid Interface Sci. 194 (1997) 364.
[38] K. Gaus, E.A.H. Hall, J. Colloid Interface Sci. 194 (1997) 373.
[39] K. Gaus, E.A.H. Hall, Biosens. Bioelectron. 13 (1998) 1307.
[40] A.A. Schilt, R.C. Taylor, J. Inorg. Nucl. Chem. 9 (1959) 211.
[41] J. van de Poel, H.M. Neumann, Inorg. Synth. 11 (1968) 53.
[42] G.D. Christian, in: Analytical Chemistry, 5th Edition, Wiley, New York. 1994, pp. 612 and 741.
[43] J.A. Hoffmann, R.E. Chance, M.G. Johnson, Protein Expression and Purification 1 (1990) 127.
[44] L. Strong, G.M. Whitesides, Langmuir 4 (1988) 546.