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Application of the 'gate effect' of a molecularly imprinted polymer grafted on an electrode for the real-time sensing of heparin in blood

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Heparin is the most important anticoagulant drug used during surgeries and extracorporeal therapies. Although the blood levels of heparin should be monitored continuously during the procedure to ensure the safety of the patient, there is currently no technique for measuring heparin in real time. This study describes the use of a molecularly imprinted polymer (MIP) as a recognition element in the development of a heparin sensor for real-time monitoring. An indium tin oxide (ITO) electrode grafted with a heparin-specific MIP was used as a working electrode to perform cyclic voltammetry of ferrocyanide. The anodic current was found to be dependent on heparin concentration, probably due to the "gate effect", which is a change in the accessibility of the MIP-modified electrode to ferrocyanide, triggered by specific interaction between MIP and heparin. The kinetics of heparin interaction with the MIP-grafted electrode was evaluated using potentiostatic chronoamperometry of ferrocyanide in an electrochemical flow cell. The response time to stepwise changes in heparin concentration between 0 and 0.04 units per mL was estimated at 20 s, which is remarkably shorter than that achieved using conventional methods for monitoring heparin. The MIP-grafted electrode demonstrated exceptional sensitivity and could detect heparin in whole blood samples (0–6 units per mL) diluted 100-fold with physiological saline containing ferrocyanide. Therefore, the MIP-grafted electrode is suitable for real-time monitoring of heparin in blood. Another advantage is that a very small volume of blood is needed, which is very important, especially when regular measurements are required.

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1 Introduction

In extracorporeal therapy (*e.g.*, haemodialysis, cardiopulmonary bypass), blood comes into contact with artificial materials in the blood vessels, creating a risk of clotting. Clotting can result in plugged tubes used for extracorporeal perfusion, or clogged blood capillaries. Clotting is prevented by the administration of anticoagulants. Heparin is the most widely used injectable anticoagulant and is metabolised rapidly by the human body. Controlling anticoagulation during a medical procedure is of paramount importance because either an overdose or an underdose of heparin can be fatal to the patient. The administered dose of heparin and its antidote, protamine sulphate, is usually determined by measuring the "activated clotting time", or ACT. However, ACT does not always depend on the heparin concentration.[1] A heparin-monitoring device (Hepcon®, Medtronic Co., Ltd., Minneapolis, MN) based on the measurement of ACT concentration by titration with protamine sulphate has been commercialised. Ohata *et al.* reported that the device is effective for optimising the post-operative neutralisation of heparin by protamine sulphate, and for reducing bleeding and inflammatory reactions after cardiopulmonary bypass surgery.[2] Although its effectiveness in monitoring heparin levels in blood has been proven, Hepcon® is not used widely because of its high cost and complicated operation. Therefore, a simple method for sensing heparin in blood is needed. Mayerhoff *et al.* have described heparin sensors based on potentiometry using an ion-selective membrane.[3,4] However, since the heparin level in blood during extracorporeal circulation changes less than 10-fold, the sensitivity of potentiometric measurements (<60 mV per 10-fold change in concentration) is insufficient for monitoring the heparin level in blood. In addition, the selectivity of the method was inadequate. In this study, we propose a new sensor for the quantification of heparin based on a heparin-specific molecularly imprinted polymer (MIP).

MIPs are synthetic polymers that contain specific binding sites formed by imprinting the target molecule, referred to as the template, during the polymerisation process. A MIP layer on an electrode can be prepared using a simple and economical "tailor-made" procedure. In order to use MIPs as molecular recognition elements in chemical sensors, the transduction of specific interactions between the MIP and the template into an

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electric signal must be characterised. The "gate effect", which refers to the change in solute diffusive permeability in the MIP layer resulting from specific interactions with the template, could be used as a mechanism for signal transduction.[5–7] The redox current at the MIP-grafted electrode is sensitive to the presence of the template because binding of the template by the template-specific polymeric layer changes the accessibility of the electrode to the redox species due to the gate effect. Therefore, the template can be detected using simple amperometric analysis by monitoring the change in the redox current at the MIP-grafted electrode. As reported earlier, the gate effect phenomenon has been used to measure the concentration of theophylline,[5] a derivative of phenylalanine,[6] and glucose.[7] It was found that an ample effect can be generated by an amount of adsorbed template as small as 0.01 wt% of MIP.[8,9] Thus, due to the gate effect, a custom-made MIP-grafted electrode can work as a highly sensitive sensor.

In the present study, an amperometric sensor based on the gate effect produced by a heparin-specific imprinted polymer was fabricated and tested.

2 Materials and methods

2.1 Chemicals

3-Aminopropyltrimethoxysilane was purchased from Tokyo Kasei Co. Ltd. (Tokyo, Japan). The solvents toluene, ethanol and *N*,*N*-dimethylformamide (DMF) were purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (water-soluble carbodiimide; WSC) was purchased from Dojindo Laboratories (Kumamoto, Japan). Sodium heparin (unfractionated; 130 units per mg; from porcine intestinal mucosa), (2-(methacryloxy)-ethyl)trimethylammonium chloride acrylamide (METMAC), acrylamide, *N*,*N*-methylenebisacrylamide (MBAA), potassium ferrocyanide, potassium nitrate and sodium chondroitin C (from shark cartilage) were purchased from Wako Pure Chemical Industry Co. Ltd. (Osaka, Japan). Sodium dermatan sulphate (from cockscomb, MW: *ca.* 90 000) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Indium-tin oxide positioned on a glass plate (IN-100, 10 ohm cm^{-2}) was purchased from Furuuchi Chemical Co. (Tokyo, Japan).

2.2 Introduction of the photoinitiator on the electrode surface

A MIP specific for heparin was grafted onto an ITO electrode using an immobilised photoactive initiator *via* living radical polymerisation.[10–12] The ITO-deposited glass plate was cut into 1 cm × 5 cm pieces. They were washed with potassium methoxide, following a previously described procedure.[5]

A solution of 3-aminopropyltrimethoxysilane (30% w/w) in toluene was dried using anhydrous magnesium sulphate. The washed ITO was heated to 80 °C in this anhydrous solution for 4 h in order to introduce the amino group onto the ITO surface. The aminated ITO was ultrasonicated in methanol and water for 5 min each in order to remove silica grains produced during the silanisation reaction.

The aminated ITO was soaked in DMF containing 0.2 M WSC and 0.1 M 4-chloromethyl benzoic acid for 24 h in order to introduce the chloromethylphenyl group onto the ITO surface. The ITO was then immersed in a 0.3 M ethanolic solution of sodium diethyldithiocarbamate for 24 h in order to introduce the diethyldithiocarbamate benzyl group, used as an initiator for radical polymerisation. This reaction was confirmed by the crystallisation of sodium chloride in the solution. The treated ITO was washed with distilled water and methanol, dried under a nitrogen stream, and stored in a desiccator.

2.3 Grafting of MIP

Sodium heparin (80 mg; 10 400 units), METMAC (225 mg; 1.08 mmol), and acrylamide (250 mg; 3.52 mmol) were dissolved in 3 mL of distilled water. MBAA (250 mg; 1.62 mmol) was dissolved in 9 mL of DMF. The initiator-introduced ITO was immersed in this polymeric mixture in a quartz crystal tube and the solution was deoxygenated by bubbling with argon for 5 min. The ITO was irradiated with ultraviolet light ($\lambda = 254$ nm) using a germicidal UV lamp (GL6, 6 W, Panasonic Co., Ltd., Osaka, Japan). The treated ITO was ultrasonicated in distilled water for 1 h. Silicone resin (PRX305 Clear, Toray Dow Corning Silicone, Tokyo, Japan) was coated on the treated ITO electrode as an insulator in order to limit the effective electrode area to 1.0 cm^2. This modified ITO will be referred to hereafter as MIP–ITO. Another working electrode was prepared by the same procedure with the exception that the template (sodium heparin) was omitted during the grafting of the polymer; this ITO will hereafter be referred to as non-imprinted polymer–ITO (NIP–ITO). The elemental compositions of the surface of the treated and untreated ITO were analysed by X-ray photoelectron spectroscopy (XPS) using a Kratos Axis-Ultra DLD (Shimadzu Co., Ltd., Kyoto, Japan).

2.4 Cyclic voltammetry

Traditional cyclic voltammetry (CV) was performed on the modified ITO, used as the working electrode in a typical three-electrode cell with a 50 mL capacity. An untreated ITO electrode and an Ag/AgCl electrode (66-EE008, Cypress Systems Inc., Lawrence, KS, USA) were used as the counter electrode and reference electrode, respectively. The potential of the working electrode was scanned at a rate of 0.20 V s^{-1} using a potentiostat (PS-08, Toho Giken Co., Ltd., Tokyo, Japan). CV was conducted in a sample solution comprising 0.1 M aqueous potassium nitrate as the supporting electrolyte, 5 mM potassium ferrocyanide as a marker, and 0–40 units per mL (0–300 μg mL^{-1}) of sodium heparin as the analyte. The sensitivity of the MIP–ITO sensor surface for heparin was evaluated using the dependence of the anodic current of ferrocyanide on the heparin concentration. In order to evaluate the selectivity of the MIP–ITO sensor response, sodium chondroitin sulphate C and sodium dermatan sulphate were used as reference analytes.

2.5 Evaluation of the response time by potentiostatic chronoamperometry

The response time to the stepwise change in heparin concentration was evaluated by potentiostatic chronoamperometry

using an electrochemical flow cell and a valve for flow path switching, using the procedure described previously[7] with two modifications: the potential of the MIP–ITO was 0.40 V *versus* Ag/AgCl, and the sample solutions were delivered using a head tank instead of pumps at a flow rate of 2 mL min^{-1}.

2.6 Simulation of heparin sensing in whole blood

Sodium heparin was dissolved in whole bovine blood in the concentration range 0 to 50 μg mL^{-1} (0 to 7 units per mL). Three 50 μL aliquots of each blood sample were diluted with 5 mL of physiological saline solution (0.9 g dL^{-1} of sodium chloride) containing 5 mM potassium ferrocyanide. The MIP–ITO was installed as a working electrode for voltammetry in a miniature electrochemical cell (Plate Material Evaluating Cell, ALS Co., Ltd., Tokyo, Japan) with an inner volume of approximately 1.2 mL. The cell was filled with the diluted whole blood sample (approximately 1 mL), and then a platinum counter electrode and reference electrode 66-EE008 were inserted into the cell. Cyclic voltammetry was performed by scanning between 0.1 and 0.6 V at a rate of 0.20 V s^{-1}. The same procedure was performed after dissolving sodium chondroitin sulphate C or dermatan sulphate instead of sodium heparin in order to evaluate the selectivity of the MIP–ITO in diluted blood.

3 Results and discussion

3.1 Characterisation of the MIP–ITO surface

The elemental compositions of the surface of the treated and untreated ITOs are listed in Table 1.

A large peak for carbon was detected even for the untreated ITO, probably due to vapour from oil in the XPS vacuum pump being adsorbed onto the surface of the sample in the high vacuum environment. The surface concentration of carbon and nitrogen was significantly higher for the MIP–ITO compared to the untreated ITO, whereas indium and tin were lower. However, an indium peak was barely detected for the MIP–ITO. These results indicate that a large amount of polymer was grafted onto the ITO electrode in the presence of heparin as a template. The thickness of the grafted layer was approximately 5–7 nm, which corresponds to the limit of the depth detectable by XPS; this conclusion is based on the observation of a residual indium peak following the grafting treatment. In contrast, the amount of grafted polymer on NIP–ITO was smaller than that on MIP–ITO, judging from the more intense indium and tin peaks of NIP–ITO. However, some poly(acrylamide-*co*-METMAC-*co*-MBAA) was grafted onto NIP–ITO because a large nitrogen peak was evident, suggesting that poly(acrylamide-*co*-METMAC-*co*-MBAA) is originally hard to grow on the surface of the ITO, but the presence of heparin promotes the growth. Growth suppression may be due to electrostatic repulsion among the cationic trimethylammonium groups in METMAC, in which case neutralisation by anionic heparin would promote the growth of the grafted polymer. This may explain why the polymer layer on the MIP–ITO was thicker than that on the NIP–ITO.

A sulphur peak was detected clearly at the surface of the MIP–ITO, indicating the presence of some residual surface templates even after thorough washing. This residual heparin might be covalently bound onto the surface during radical polymerisation, or be encapsulated in highly crosslinked domains.

Table 1 Elemental compositions of the surface of the ITOs determined by XPS[a]

ITO	C(1s)	N(1s)	O(1s)	Si(2p)	In(3d)	Sn(3d)	S(2p)
Untreated	37.3	n.d.	37.8	3.2	20.4	1.4	n.d.
Aminated	20.7	n.d.	47.6	3.3	27.0	1.4	n.d.
Initiator-introduced	14.5	2.8	49.5	2.2	29.1	1.8	n.d.
NIP	30.9	5.6	41.1	4.5	16.5	1.2	n.d.
MIP	57.2	11.2	28.6	1.3	0.4	n.d.	1.4

[a] n.d.: peak was not detected.

3.2 Sensitivity and selectivity of the MIP–ITO surface to heparin

The cyclic voltammogram of potassium ferrocyanide on the surface of the MIP–ITO or NIP–ITO electrode in the presence and absence of heparin is shown in Fig. 1. The current density of ferrocyanide at the MIP–ITO electrode was significantly smaller than that at the NIP–ITO or untreated ITO electrode: the peak current densities in the absence of heparin were 0.91 ± 0.08, 1.38 ± 0.04 and 1.48 ± 0.02 mA cm^{-2}, respectively, $n = 3$ each. The peak potentials were very similar among the electrodes (0.32–0.33 V *versus* Ag/AgCl). 0.04 units per mL of heparin increased the current at the MIP–ITO surface, while 40 units per mL of heparin decreased the current. The peak potential did not change in the presence of heparin. The current density at the NIP–ITO electrode was also not dependent on the presence of heparin.

The relationship between the relative change in the peak anodic current of ferrocyanide at the MIP–ITO and NIP–ITO electrodes and the heparin concentration is shown in Fig. 2. The effect of different concentrations of sodium chondroitin sulphate C and sodium dermatan sulphate on changes in the peak value of the anodic current on the surface of the MIP–ITO is also shown in Fig. 2. The anodic current at the surface of the NIP–ITO electrode is not affected by the heparin concentration in the range tested (10^{-2} to 300 μg mL^{-1}, or 10^{-3} to 40 units per mL). This may be due to the small amount of the grafted polymeric layer or the lack of imprinted sites produced by the template molecules. In contrast, the anodic current at the surface of the MIP–ITO electrode increased as the concentration of heparin increased from 0 to 0.3 μg mL^{-1} (0–0.04 units per mL). The detection limit of heparin was 10 ng mL^{-1}. However, this limit decreased with an increase in the heparin concentration from 0.3 to 300 μg mL^{-1} (from 0.04 to 40 units per mL). The current through the MIP–ITO electrode was insensitive to any concentration of sodium chondroitin sulphate C or sodium dermatan sulphate, and to albumin or IgG in the same concentration range. These results indicate that the MIP–ITO electrode acts as designed as a selective heparin sensor.

Mayerhoff and Gemene recently developed a potentiometric heparin sensor using a chronopotentiometric method

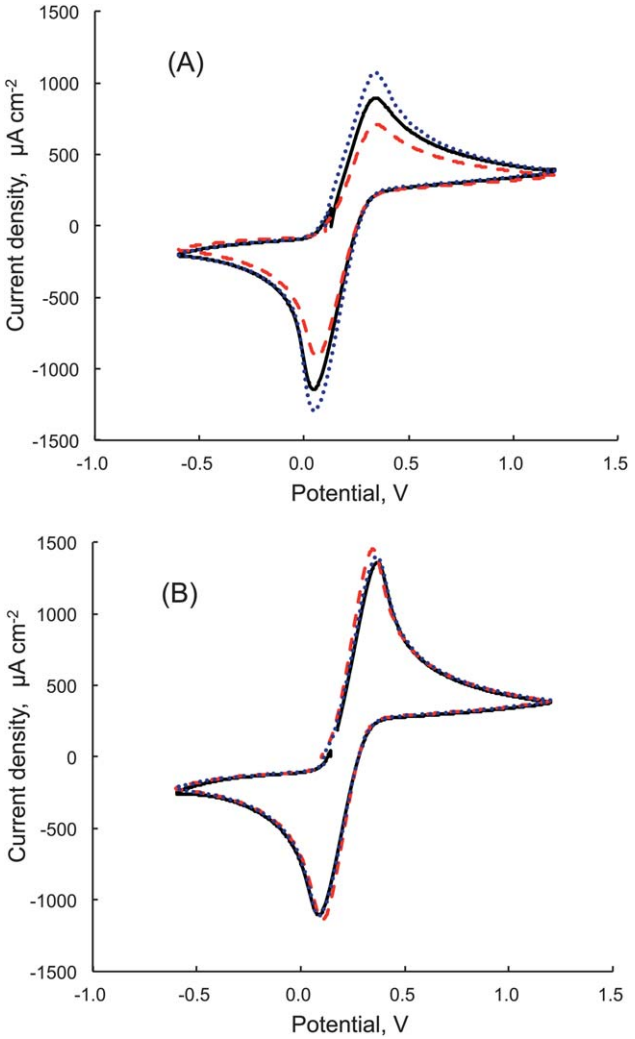

Fig. 1 Cyclic voltammograms of 5 mM potassium ferrocyanide on the surface of the (A) MIP-grafted electrode and (B) NIP-grafted electrode, with heparin concentrations of 0 units per mL (solid line), 0.04 units per mL (dotted line) and 40 units per mL (dashed line).

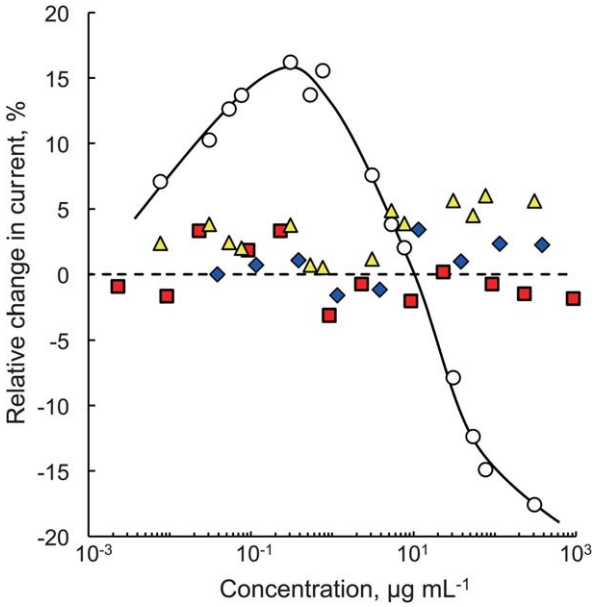

Fig. 2 Relationship between the relative change in the peak current of ferrocyanide at the surface of the MIP–ITO (circles) or NIP–ITO (triangles) electrode and heparin concentration, and the relationship between the change in the current at the MIP–ITO surface and the concentration of chondroitin sulphate C (squares) or dermatan sulphate (diamonds).

employing a polyion-selective membrane doped with tridodecylmethylammonium chloride.[4] However, the sensitivity of this sensor to chondroitin sulphate C was 0.5 times as high as its sensitivity to heparin. Thus, the selectivity of the MIP–ITO reported here is superior to the selectivity of the sensor developed by Meyerhoff. Liu *et al.* recently developed a potentiometric heparin sensor using a carbon electrode coated with a polymer film imprinted with heparin[13] with a detection limit of 10 ng mL^{-1}. Thus, the sensitivity of the MIP–ITO described here is similar to that of the MIP-coated electrode developed by Liu.[13]

The maximum heparin concentration in blood during cardiopulmonary bypass surgery is approximately 4 units per mL. Therefore, the concentration range at which heparin increases the current intensity (0–0.04 units per mL, or 0–0.3 μg mL^{-1}) is equivalent to its concentration in 100-fold diluted blood during a cardiopulmonary bypass procedure.

The peak potential of the cyclic voltammogram using the MIP–ITO was the same as those obtained using the NIP–ITO and untreated ITO, and was unaffected by heparin, as shown in Fig. 1. These results indicate that the current at the MIP–ITO surface is generated by direct electron transfer between the ferrocyanide anion and the substrate ITO electrode. This may suggest that the grafted MIP-layer has a porous structure. The change in the current density by heparin is probably due to a change in the accessibility of the ferrocyanide anion across the pore of the MIP-layer toward the ITO electrode, rather than a change in electron transfer between the ITO electrode and the redox marker.[14] If this is the case, then the porosity of the MIP-layer would control the accessibility of the ferrocyanide anion to the surface of the ITO and would depend on the specific interaction between the template and heparin. Unfortunately, we do not have direct evidence of the specific adsorption of heparin onto the heparin-imprinted polymer layer because the grafted layer is extremely thin (less than 10 nm in the dry state); consequently, the amount of adsorbed heparin would be too small for quantitative analysis. However, it is unlikely that the interaction between heparin and the ferrocyanide anion in the bulk solution affects accessibility to the electrode or the current, since the current at the NIP–ITO or untreated ITO was insensitive to heparin. It is also unlikely that non-specific polyion complexation between the anionic heparin and the cationic MIP-layer affects the current intensity, since the current was insensitive to the polyanions chondroitin sulphate C and dermatan sulphate, even at high concentration. It is therefore most likely that the specific interaction between heparin and the imprinted site on the MIP changes the porosity of the layer by a mechanism similar to "induced fit" in natural receptors and induces change in the diffusive current. We previously demonstrated that the porosity and permeability of MIP is sensitive to the amount of site-specifically adsorbed template by

using a molecularly imprinted 50 μm thick self-supported membrane.[8,9] A similar phenomenon could occur in the molecularly imprinted thin layer on the electrode.

The insensitivity of the MIP–ITO to sodium chondroitin sulphate C or dermatan sulphate shows that the MIP–ITO can discriminate heparin from its structural analogues, the linear polysaccharide sulphates, at a wide range of concentration. It is somewhat surprising that linear anionic polysaccharides are discriminated by MIP, which usually recognises the template by its 3-dimensional molecular structure. Heparin contains around 2.7 sulpho-groups per disaccharide, whereas chondroitin sulphate contains one sulpho-group per disaccharide,[15] as shown in Fig. 3. Also taking into account the difference in the number of sulpho-groups in the corresponding disaccharide molecules, the sensitivity of MIP to these three analogues is very different. The average molecular weight of heparin from porcine intestinal mucosa, chondroitin sulphate C from shark cartilage, and dermatan sulphate, are 15 000 (ref. 15) 20 000 (ref. 16) and 90 000 (nominal value from the supplier), respectively. Heparin is smaller than the two analogues. However, it is unlikely that the imprinted site discriminates the polysaccharide sulphate by size, because each polysaccharide has a wide molecular weight distribution (for example the distribution of the molecular weight of heparin is 5000 to 40 000 (ref. 17)). If the size were the distinguishing attribute, then part of the imprinted site would be sufficiently large to accept a small-sized portion of the chondroitin sulphate C or dermatan sulphate molecule.

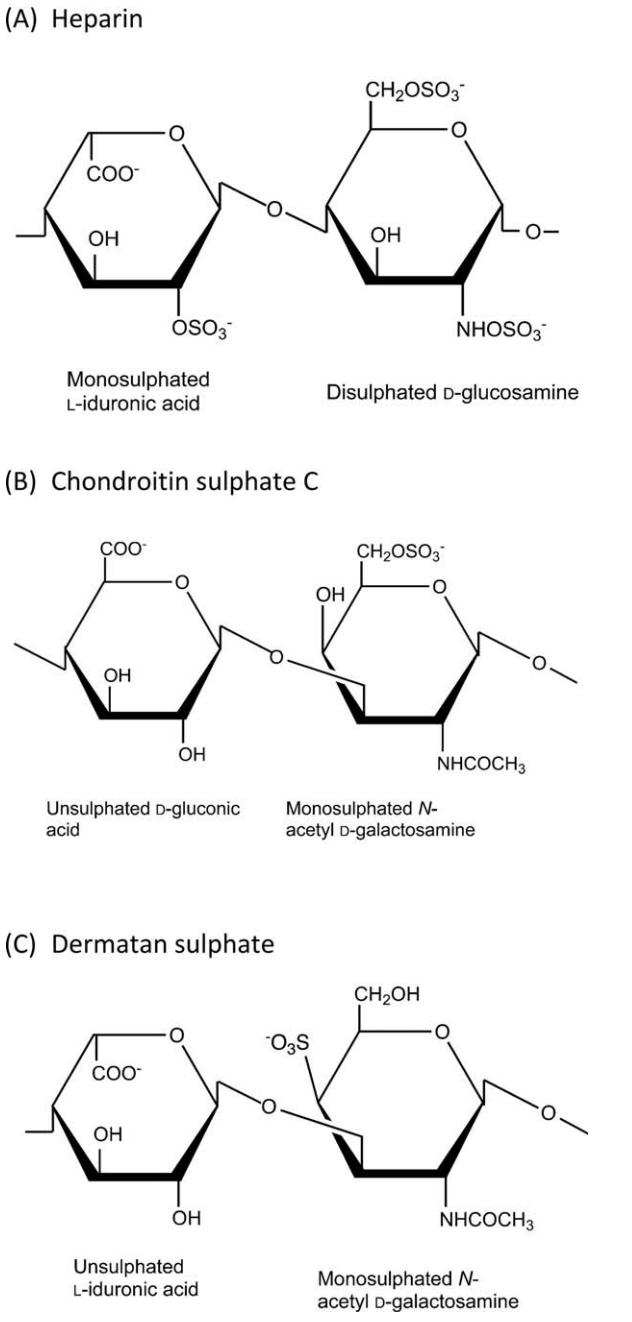

Fig. 3 Structures of major components of heparin, chondroitin sulphate C and dermatan sulphate.

The results indicate that the imprinted site on the MIP discriminates the structure of the polysaccharide sulphate components. The difference in the density and position of the sulpho-groups and, correspondingly, in the electrostatic charges of the analytes may be an important discriminating factor. The major components of heparin are monosulphated L-iduronic acid and disulphated D-glucosamine. However, components of chondroitin sulphate C include unsulphated D-glucuronic acid and monosulphated *N*-acetyl D-galactosamine; similarly, the components of dermatan sulphate C are unsulphated L-iduronic acid and monosulphated *N*-acetyl D-galactosamine. It appears that these differences allow MIP to recognise the template (heparin) and discriminate its analogues (chondroitin sulphate C or dermatan sulphate C) by changing the permeability of the MIP layer towards ferrocyanide.

These results suggest that the interaction between heparin and the imprinted site on MIP increases the porosity at concentration ranges below 0.04 units per mL (0.3 μg mL^{-1}) due to specific interaction between heparin and the imprinted sites. However the interaction decreases the porosity at concentrations above 0.04 units per mL. Unfortunately, we have insufficient information to explain why the relationship between the current density at the MIP-grafted electrode and the concentration of heparin is so complicated, although it might be related to the wide molecular weight distribution of heparin. If this is the case, then the imprinted sites on the MIP-layer of the electrode would also have a comparable distribution in size. At the lower concentration range, most of the heparin is believed to be bound in sites imprinted by high molecular weight heparin; this bound heparin attracts the cationic trimethyl ammonium group and expands the pores of the MIP layer. However, at higher concentrations, some of the adsorbed heparin molecules would extend from the binding site, blocking ferrocyanide access to the surface of the ITO.

3.3 Evaluation of the response time of the MIP-electrode to a stepwise change in heparin concentration

Fig. 4 shows the kinetics in the response of the current density during chronoamperometry to stepwise changes in heparin concentration.

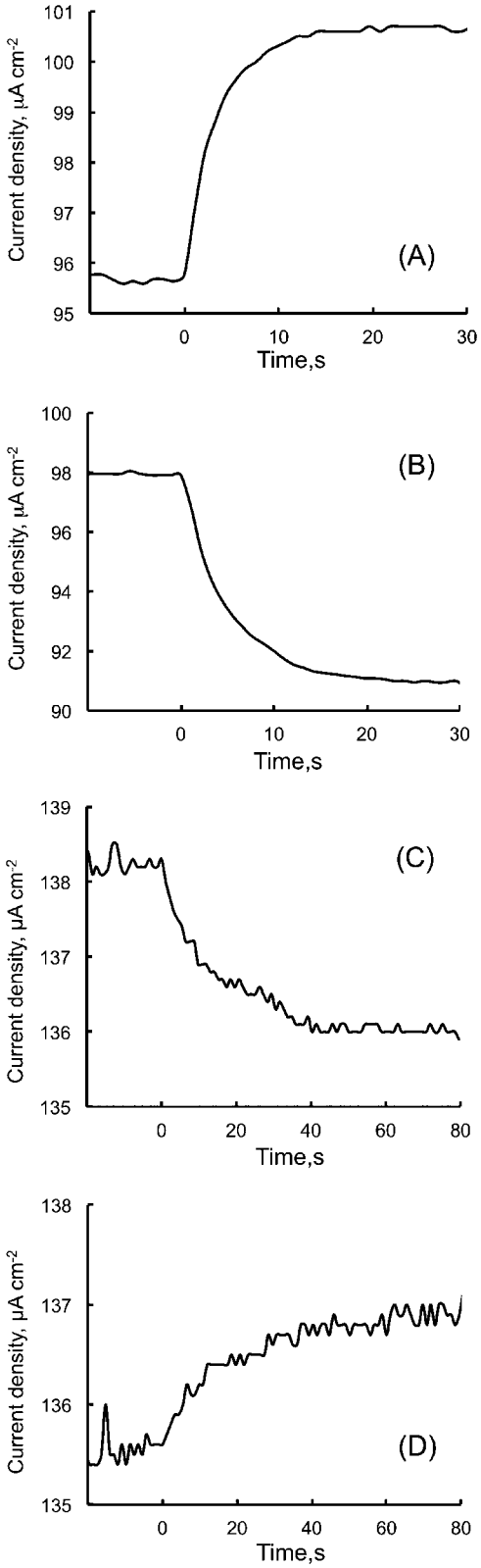

Fig. 4 Potentiostatic chronoamperograms of 5 mM potassium ferrocyanide at 0.40 V using a MIP–ITO electrode toward stepwise changes in heparin concentration: (A) 0 to 0.04 units per mL, (B) 0.04 to 0 units per mL, (C) 0 to 40 units per mL, and (D) 40 to 0 units per mL. (0 s on the abscissa indicates the time when the stepwise change was performed.)

As the concentration changes from 0 to 0.04 units per mL or *vice versa*, the current stabilises within 15–20 s. However, stabilisation took approximately 60 s after a change in heparin concentration from 0 to 40 units per mL or *vice versa*. If morphological changes in the MIP layer occur homogeneously, the change in the current would follow a first order time lag, as expressed in eqn (1).[18]

$$\frac{\Delta I(t)}{\Delta I_\infty} = 1 - \exp\left(-\frac{t}{\tau}\right), \quad (1)$$

where $\Delta I(t)$ is the change in the electric current at time (t) after the stepwise change in heparin concentration, ΔI_∞ is the saturated change in the electric current, and τ is the time constant. The relationship between $-\ln[1 - \Delta I(t)/\Delta I_\infty]$ and t is shown in Fig. 5. For a change in heparin concentration between 0 and 0.04 units per mL, the relationship shows high linearity. Therefore, the response to lower current intensity can be

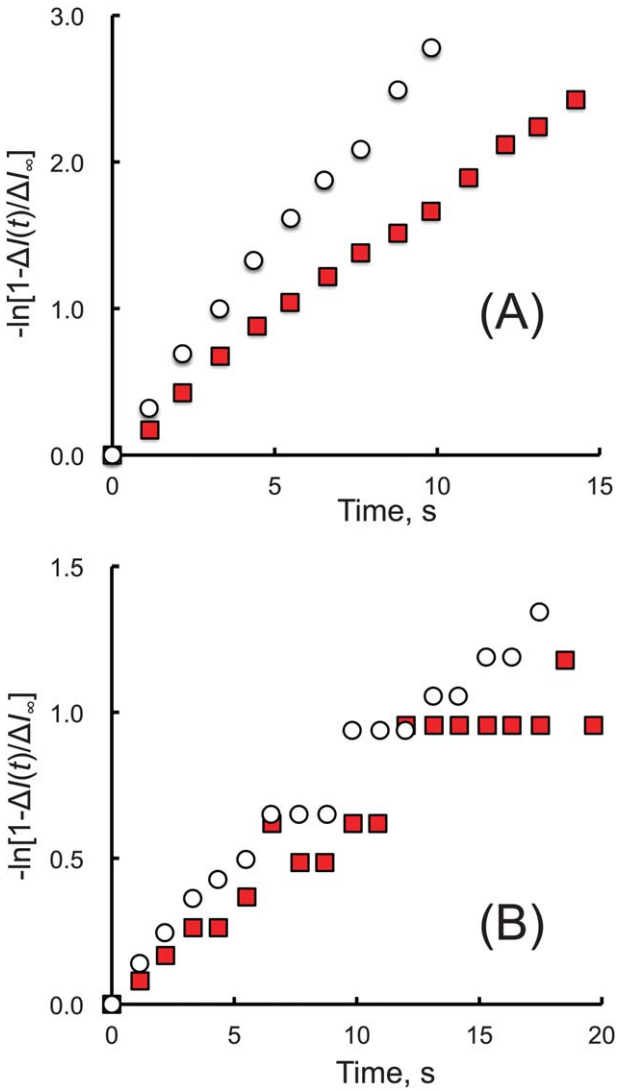

Fig. 5 Relationship between $-\ln(1 - \Delta I(t)/I_\infty)$ and time t upon stepwise changes in heparin concentration between (A) 0 and 0.04 units per mL and (B) 0 and 40 units per mL (increasing concentration is shown as circles; decreasing concentration as squares).

described as a first-order delay. This indicates that the specific interaction between heparin and the imprinted site increases the porosity of the MIP-layer monotonously at low heparin concentration (0–0.04 units per mL). The time constant τ was calculated to be 3.4 s for the increase in the electric current in the presence of heparin and 6.1 s for the decrease in current in the absence of heparin. Approximately 20–30 s was required to stabilize the current density after the stepwise change at low heparin concentration. A change in concentration between 0 and 40 units per mL provided insufficient linearity to calculate the time constant. During the change in heparin concentration from 0 to 40 units per mL, two effects (increasing the porosity of the MIP–ITO at the lower concentration (0–0.04 units per mL) and decreasing the porosity at the higher concentration (0.04–40 units per mL)) by the specific interaction would overlap. The poor linearity may reflect the overlapping of the two effects. Approximately 60–80 s are required to stabilize the current density after the stepwise change at high heparin concentration.

The results obtained by the electrochemical techniques, CV and chronoamperometry, show that the interaction between heparin and heparin-specific MIP can lead to an increase or a decrease in the current, depending on the concentration of heparin. The response at low heparin concentrations (0 and 0.04 units per mL) is very sensitive and fast, and is manifested as an increase in the electric current. High heparin concentrations (0–40 units per mL) also produce an electrochemical effect, which is manifested as a decrease in the current which takes long to stabilize. These results suggest that there are two mechanisms governing the interaction of heparin with the heparin-specific MIP, depending on the heparin concentration.

The response time in either case is remarkably shorter than that of ACT typically used during extracorporeal therapy with a heart–lung machine (usually 600 s). Hepcon®, used to measure the heparin level by measuring ACT, also requires about 600 s. Our results indicate that the control and adjustment of anticoagulants during surgery could be dramatically improved by using MIP-grafted electrodes for monitoring heparin.

The response time of the MIP-grafted electrode described here is significantly shorter than that of the MIP-deposited electrode developed by Liu (approximately 300 s). This difference in response time is probably due to the thin grafted MIP-layer on our electrode (<10 nm) compared to the thickness of the deposited film of MIP described in Liu's work (~5 μm).[13]

In order to use our heparin-imprinted sensor for practical applications such as real-time monitoring of heparin levels in blood, the response time should be as short as possible. Quick response and the desirable range of heparin concentrations could be assured by 100-fold dilution of whole blood samples with physiological saline solution containing the redox marker, ferrocyanide.

3.4 Simulation of the monitoring of heparin in a blood sample

The heparin sensor must be able to determine the concentration of heparin in whole blood rather than in plasma, since there is no time to centrifuge blood samples during surgical procedures involving extracorporeal bypass. We thus evaluated the MIP–ITO as a sensor for determining heparin levels in whole blood. Three aliquots (50 μL) of whole blood containing heparin, chondroitin sulphate C or dermatan sulphate were diluted with 5 mL physiological saline solution containing 5 mM potassium ferrocyanide. Cyclic voltammetry was performed using the diluted blood sample as a test solution to characterise the sensitivity of the MIP grafted electrode to heparin in whole blood. Fig. 6 shows the relationship between the change in the anodic peak current density in the diluted sample and the concentration of polysaccharide sulphate (heparin, chondroitin sulphate C or dermatan sulphate).

The current density increased with an increase in heparin concentration in the blood sample from 0 to 0.5 μg mL^{-1} or 0 to 0.06 units per mL. This result is consistent with the result shown in Fig. 2. Since the concentration range of heparin in the whole blood sample before dilution would be 0 to 6 units per mL, the dynamic range covers the range of heparin concentration in blood during cardiopulmonary bypass (0–4 units per mL).

The current was insensitive to the same range of concentration of chondroitin sulphate C or dermatan sulphate, indicating that MIP retains its sensitivity in diluted blood and that MIP-grafted electrodes are suitable for monitoring heparin in blood after 100-fold dilution of the blood sample with physiological saline solution containing a redox marker. For comparison, ACT cannot be measured using diluted blood, and Hepcon® requires a minimum of 1.5 mL blood sample for heparin concentration determination.[19] Such a large amount of blood needed for analysis is detrimental to the patient, especially infants. Thus, the application of the MIP-grafted electrode for measuring the heparin concentration in blood is very

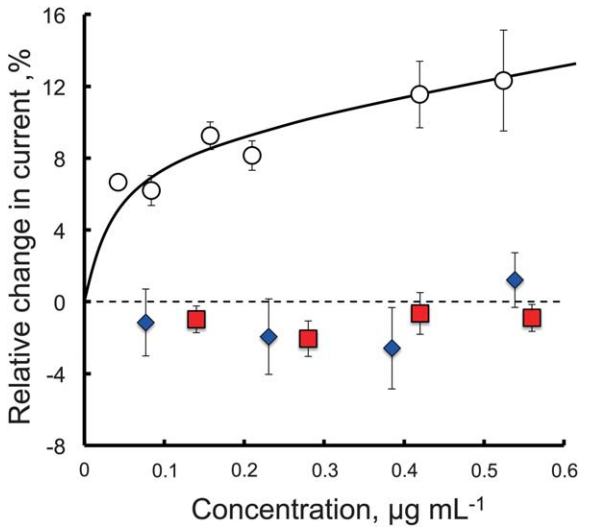

Fig. 6 Relative change in the anodic current of ferrocyanide obtained using cyclic voltammetry with a MIP–ITO electrode following changes in the concentration of heparin (circles), chondroitin sulphate C (squares), or dermatan sulphate (diamonds) in the test solution. The test solution was prepared by diluting 50 μL of a whole blood sample with 5 mL of physiological saline solution containing 5 mM potassium ferrocyanide. The error bars show the standard deviation among data points obtained using three aliquots of whole blood samples.

attractive because it reduces the volume of blood required for the analysis from millilitres to microlitres, since 1 mL of 100-fold diluted blood is sufficient for electrochemical analysis. Dilution of the blood also helps extend the use of the electrode by minimising fouling with serum proteins.

Although blood contains ascorbic acid (20–80 μM) and uric acid (0.2–0.5 mM) which would be oxidized at the anode and can generate current, this anodic current would be too small to interfere the measurement of heparin as long as the blood sample is diluted 100-fold with 5 mM ferrocyanide solution, as described in our protocol. Indeed, no remarkable redox current of these pre-existing species was detected in cyclic voltammetry with MIP–ITO using 100-fold diluted blood.

4 Conclusions

We demonstrate that an electrode grafted with a heparin-imprinted polymer can detect heparin with high sensitivity, high selectivity, and rapid response. Selectivity is retained in diluted whole blood. These features of the electrode are very attractive for its use as a real-time blood heparin sensor during extracorporeal bypass procedures. The sensor would reduce dramatically the amount of blood required for the analysis of heparin.

However, some problems remain to be overcome, such as baseline-drift of the response current (as shown in Fig. 4) and differences in the current between different grafted electrodes prepared using the same procedure. These problems can be resolved by optimizing the grafting technique and/or the composition of the prepolymer solution (*i.e.*, the concentration of heparin or of the monomers) and thus enhancing the heparin-induced change in the current. Periodic calibration using standard samples of heparin may also be effective.

In future, heparin sensors using these improved MIP-grafted electrodes will improve the outcomes of extracorporeal circulation procedures.

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