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Stabilized sensing of heparin in whole blood using the 'gate effect' of heparin-imprinted polymer grafted onto an electrode

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Abstract: A real-time heparin monitor is required to optimize the dosage of heparin and its antidote, protamine sulfate, during extracorporeal circulation procedures. The gate effect of molecularly imprinted polymer (MIP) is a potential tool for the rapid and selective sensing of heparin. We here present a method to stabilize the measurement of heparin concentration in whole blood using an MIP-grafted electrode. An initiator of radical polymerization, the diethyldithiocarbamic-benzyl group, was introduced onto the surface of an indium-tin oxide (ITO) electrode. Heparin sodium, methacryloxethyltrimethoxysilane, and acrylamide were dissolved in water, and methylenebisacrylamide was dissolved in dimethylformamide. A mixture of the two solutions was introduced into the 50 μm gap between the surfaces of a quartz crystal plate and the treated ITO electrode. Ultraviolet light was irradiated onto the surface of the ITO to graft the copolymer of the monomers, then the ITO was washed with a 1 M sodium chloride aqueous solution to remove the heparin template and obtain the MIP-grafted electrode. Cyclic voltammetry was performed with the MIP-grafted electrode in physiological saline or bovine whole blood containing 0-8 units/mL heparin and 5 mM ferrocyanide as a redox marker, and the relationship between the current intensity and the heparin concentration was analyzed. The current intensity decreased as the heparin concentration in either saline or whole blood increased, and the sensitivity of the electrode to heparin in blood was approximately 52% of its sensitivity to heparin in saline. The grafted-electrode was washed with a protease-containing detergent (Sterizyme® S, Maruishi Pharmaceutical) between measurements in blood. The heparin-sensitivity of the washed electrode in blood was 77% of that in saline. No sensitivity to chondroitin sulfate C was observed but sensitivity to low molecular weight heparin was demonstrated. We thus conclude that selective and stable sensing of heparin can be achieved using an electrode grafted with heparin-imprinted polymer.

Keywords: heparin, gate effect, blood, sensor, redox marker, hysteresis

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1 Introduction

In extracorporeal therapy (*e.g.,* hemodialysis, cardiopulmonary bypass), blood comes into contact with artificial materials inserted into 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, a glycosaminoglycan, is the most widely used injectable anticoagulant, and is metabolized rapidly by the human body (half-life time is 0.5-1 h) (Lesserson and Gravlee, 2007). 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 sulfate, are usually determined by measuring the "activated clotting time", or ACT. However, ACT does not always depend on the heparin concentration. A heparin-monitoring device (Hepcon®, Medtronic Co., Ltd., Minneapolis, MN) based on measuring ACT concentration by titration with protamine sulfate has been commercialised. Ohata *et al.* reported that the device is effective for optimizing the post-operative neutralization of heparin by protamine sulfate,

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and for reducing bleeding and inflammatory reactions after cardiopulmonary bypass surgery (1999). 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, there has been significant effort devoted to developing a simple method for sensing heparin in blood. For example, Krebs *et al.* attempted to quantify heparin by elastometric measurements of blood clots, but the dynamic range of the measurements did not include physiologically-relevant levels of heparin in blood (Krebs *et al.* 2015). Li *et al.* (2013) proposed a new heparin sensor based on potentiometry using a carbon electrode covered with heparin-specific molecularly imprinted polymer (MIP). MIP is a synthetic molecular recognition element containing template-shaped cavities in the polymer matrices with memory of the template molecules, and is created by copolymerization of crosslinking monomer and a functional monomer that has affinity for the template. However, Li *et al.* did not evaluate the ability of their sensor to sense heparin in blood. That same year, we proposed a new sensor for the quantification of heparin based on the “gate effect” of a heparin-specific MIP (Yoshimi *et al.*, 2013).

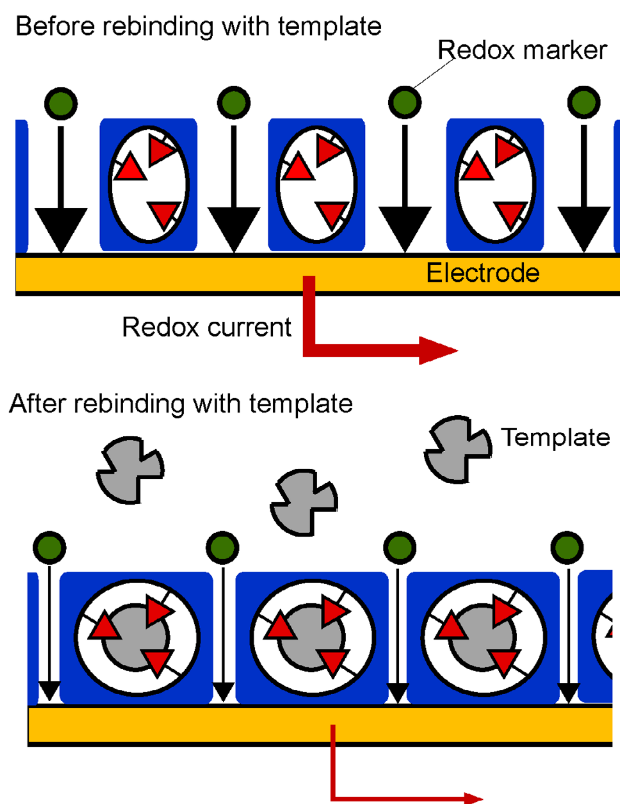

Fig. 1: Schematic diagram showing the mechanism by which the MIP layer on an electrode senses template binding using the gate effect.

The gate effect refers to the change in solute diffusive permeability in the MIP layer resulting from specific interactions with heparin, the molecule used to imprint the MIP, and can be used as a mechanism for signal transduction (Yoshimi *et al.*, 2001, 2009, 2013, 2015, Sekine *et al.*, 2007) as shown in **Fig. 1**. The redox current at the MIP-grafted electrode of the redox marker added to the sample solution is sensitive to the presence of the heparin template and this sensitivity is likely due to changes in the porosity of the template-specific MIP triggered by binding of the template. This change in porosity alters the accessibility of the electrode to the redox species. The MIP must be moderately flexible in order for the gate effect to be selective and sensitive (Yoshimi *et al*, 2015). Therefore, it should be possible to detect the template, heparin, using simple amperometric analysis by monitoring the change in the redox current at the MIP-grafted electrode.

In our previous study (Yoshimi *et al.*, 2013), cyclic voltammetry or chronoamperometry of ferrocyanide (hexacyanoferrate (II)), used as a redox marker, was performed using heparin-specific MIP grafted on an indium-tin oxide (ITO) electrode. The oxidative current was sensitive to heparin but was insensitive to other glycosaminoglycans. The response-time of the electrode to a change in the heparin concentration was less than 20 s, demonstrating that the MIP-grafted electrode holds promise for real-time monitoring of heparin concentration.

However, the practicality of this sensor is compromised due to the complicated relationship between the current and the heparin concentration. At the lower range of the detectable heparin concentration (< 0.04 unit/mL), current increases as the heparin concentration increases, but the current decreases as the concentration of heparin increases at the higher concentration detection range. These results suggest that there are two mechanisms governing the interaction of heparin with the heparin-specific MIP, depending on the heparin concentration. Limited reproducibility among the produced heparin-imprinted electrodes must be addressed because the relative effect of the two mechanisms could vary among electrodes. To be practical, the heparin-sensor should be disposable and one of the mechanisms should be removed. We here describe a method based on a single mechanism and demonstrate that the output of the redox current at the MIP-electrode accurately reflects the heparin-specific interaction with the MIP.

A practical sensor for monitoring blood heparin levels during extracorporeal therapy must be able to detect heparin in undiluted whole blood directly in order to provide the essentially real-time status of the anticoagulant effect. We therefore surface-treated the MIP-

grafted electrode in order to realize stable measurement of the heparin concentration in undiluted whole blood.

2 Experimental Section

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) was purchased from Dojindo Laboratories (Kumamoto, Japan). Sodium heparin (unfractionated; 130 units/mg; from porcine intestinal mucosa), (2-(methacryloxy)ethyl) trimethyl ammonium 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). Indium-tin oxide (ITO) positioned on a glass plate (IN-100, 10 ohm cm^{-2}) was purchased from Furuuchi Chemical Co. (Tokyo, Japan). Polytetrafluoroethylene (PTFE) film (thickness: 50 μm) was purchased from Chukoh Chemical Industries Ltd (Tokyo, Japan). Quartz plates were purchased from Monotech, Inc. (Saitama, Japan). Anticlotting coating material SEC-1™ was kindly donated by Toyobo Co., Ltd. (Osaka, Japan). The biocompatible polymer Lipidure®-CM 5206 was purchased from NOF Co., Ltd. (Tokyo, Japan). Low molecular weight heparin (LMWH) was purchased as Fragmin® (1,000 units/mL in physiological saline) from Pfizer Co., Ltd. (New York, NY, USA).

2.2 Conventional Grafting Procedure

The grafting of polymer molecularly imprinted with heparin onto an ITO electrode was performed as previously described (Yoshimi *et al.*, 2013) with minor modification of the composition of the monomer-template solution. The diethyldithiocarbamate benzyl group, a radical photopolymerization initiator, was introduced onto the surface of the ITO (1 cm × 5 cm pieces) using the previously described procedure (Yoshimi *et al.*, 2013). Sodium heparin (0.160 g; 20,800 units), METMAC (900 mg; 4.32 mmol) and acrylamide (1000 mg; 14.08 mmol) were dissolved in 6 mL distilled water. MBAA (1,000 mg) was dissolved in 18 mL DMF. The two solutions were mixed and deoxygenated by argon bubbling for 30 min. The initiator-coated electrode was soaked in the mixed solution in a quartz crystal tube and irradiated using a germicidal ultraviolet lamp (GL-6, 6W, Panasonic Co., Ltd., Osaka, Japan) for graft-polymerization. The grafted electrode was ultrasonicated in distilled water to remove the template (heparin) and provide the heparin MIP-grafted electrode. Bovine blood was purchased from Tokyo Shibaura Zoki Co. Ltd. (Tokyo, Japan). The blood was prevented from coagulating by the addition of 1.7 M sodium citrate (10 mL/1 L blood) soon after collection.

2.3 Modification of graft-polymerization.

The procedure for grafting the MIP onto the ITO was modified as follows. The ITO-deposited glass plate was cut into 3 cm x 5 cm pieces, then the diethyldithiocarbamate benzyl group was introduced as an initiator of photoradical polymerization, as previously described (Yoshimi *et al.*, 2013). A deoxygenated heparin solution and the monomers were sandwiched between the initiator-covered ITO-glass plate and a quartz plate separated with PTFE sheet spacers (50 μm thick) under an argon atmosphere as shown in **Fig. 2**. The sandwich was irradiated using a 200 W Xenon spotlight lamp (LC-5, Hamamatsu Photonics, Co. Ltd., Hamamatsu, Japan) for 1 h. The ITO plate was washed with ultrasonication in distilled water for 1 h, soaked in 1 M NaCl for 1 min, and flushed with distilled water again. The ITO-coated plate was dried under a nitrogen stream and cut into 1 cm × 3 cm pieces to obtain MIP-grafted electrodes.

2.4 Electrochemical measurements

Traditional cyclic voltammetry (CV) was performed using the MIP-grafted ITO as the working electrode in a typical

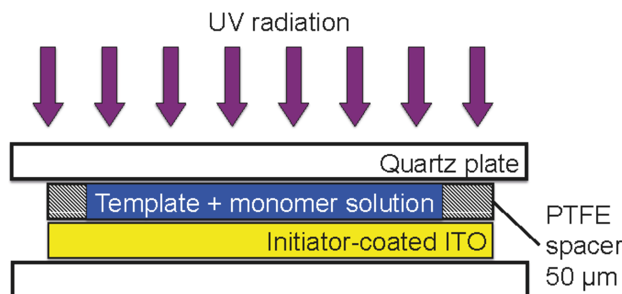

Fig. 2: Scheme showing the modified procedure using a thin layer of template-monomers solution for grafting MIP on the ITO.

miniature electrochemical cell (Plate Material Evaluating Cell, ALS Co., Ltd., Tokyo, Japan) with an internal volume of approximately 1.2 mL. A platinum and an Ag/AgCl electrode (66-EE008, Cypress Systems Inc., Lawrence, KS, USA) were used as the counter electrode and the reference electrode, respectively. The potential of the working electrode was scanned at a rate of 0.20 V/s using a potentiostat (HECS-326E, Fuso Co., Ltd., Tokyo, Japan). CV was conducted in a sample solution comprising 0.15 M aqueous sodium chloride (or physiological saline) as the supporting electrolyte, 5 mM potassium ferrocyanide as a marker, and 0–40 units/mL (0–300 mg/mL) of sodium heparin as the analyte. The sensitivity of the MIP–ITO sensor surface for heparin was evaluated using the dependence of the peak anodic current of ferrocyanide on the heparin concentration.

The sensitivity of the electrode for heparin in whole blood was evaluated by performing CV with bovine whole blood containing 5 mM ferrocyanide and 0-8 units/mL heparin. Heparin is usually dosed at 300 units/kg-patient weight for extracorporeal circulation therapy. Human blood comprises 8% of the body weight, and therefore the initial heparin concentration is approximately 4 units/mL blood. Since some patients require additional dosing, we examined a heparin concentration range of 0-8 units/mL in whole blood.

3 Results and Discussion

3.1 Resolution of hysteresis

Effective monitoring of the anticoagulant effect requires that the heparin-sensor have the same sensitivity whether the heparin concentration is increasing or decreasing, so hysteresis is an important factor in evaluating a sensor. **Figure 3** shows the relationship between the current density and the heparin concentration as the concentration increases and decreases. The first (circles) and second (triangles) cycles of heparin concentration change show a significant difference in current between the increasing (open) and the decreasing (closed) portions of the cycle in the absence of any treatment of the electrode between measurements during the cycle. In the third cycle, the MIP-grafted electrode was flushed with 1 M NaCl, rinsed with distilled water, and dried under a nitrogen stream between each measurement. This resulted in very small differences between the increasing and decreasing portions of the cycle and indicates that washing the electrode between measurements with a high concentration of saline is effective for decreasing hysteresis. The change in the current resulting from the interaction of MIP with heparin occurs in two stages: (1) specific binding of heparin and (2) a change in MIP porosity. The improved reproducibility obtained by simple washing of the electrode seems to indicate that one of these two stages is irreversible, *i.e.*, the MIP does not return to its original physical state when the heparin concentration is lowered, but requires a high concentration of saline to reset it to its original physical state. The view that the binding step is irreversible and that washing with high-concentration saline results in heparin being removed due to the ion shield effect appears simple at first glance, but if this hypothesis is followed, it would mean that the current density does not depend on the heparin concentration. Therefore, it is difficult to imagine that the binding step is irreversible because of the good relationship observed between the current density and the heparin concentration. On the other hand, if the MIP has a high plasticity, an immediate return to the original state is not possible even if the template is removed. Therefore, our results may suggest that the MIP layer is highly plastic and that the change in the permeability of the ferrocyanide anion across the MIP layer caused by a higher concentration of heparin is not

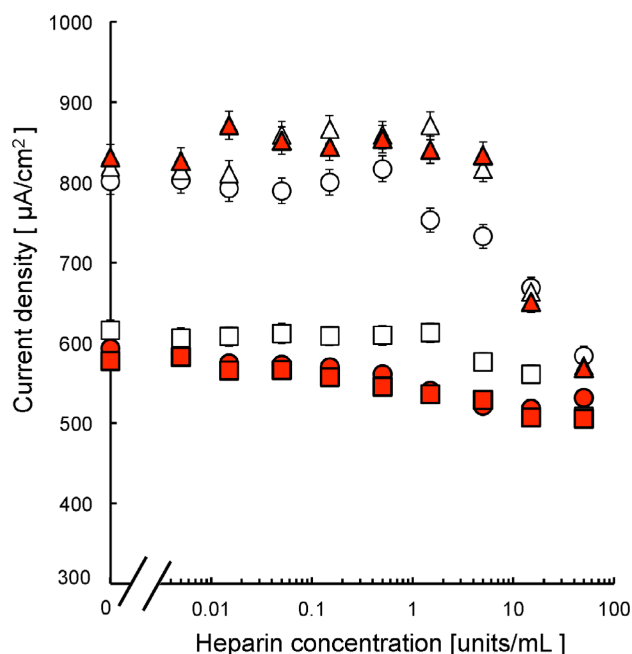

Fig. 3: The relationship between the anodic peak current density of 5 mM ferrocyanide at a MIP-electrode prepared by the conventional procedure and heparin concentration in physiological saline as the concentration increases (open symbols) and decreases (closed symbols). During the first (circles) and second (squares) cycles, the electrode was not washed between measurements, but it was washed with 1 M NaCl solution, rinsed with distilled water, and dried under a nitrogen stream for each measurement in the third cycle (triangles).

recovered following removal of the heparin as the heparin concentration decreases. However, the porosity of the MIP layer may recover immediately due to decreased repulsion between the trimethylammonium groups on the MIP by the ion shield effect of the concentrated saline.

The current density increased as the heparin concentration increased at the lower range (< 0.04 unit/mL) but decreased as the heparin concentration continued to increase to the higher end of the range in our previous work (Yoshimi *et al.*, 2013). These results indicated that there are two overlapping mechanisms, increasing the accessibility of ferrocyanide anion to the ITO electrode as the heparin concentration increases and then decreasing accessibility as the heparin concentration continues to increase. The balance between the two mechanisms would vary among grafted electrodes, thus decreasing the reproducibility of the sensitivity of the electrodes. However, the current decreased almost monotonically in this work. The difference might be due to the modification of the composition of the monomer-template solution. The modification would be effective to simplify the mechanism of change in permeability of the MIP layer by the template. The monotonically decrease of the current by the template may be due to electric repulsion between ferrocyanide anion and heparin anion adsorbed on the MIP layer. The simplified mechanism would be advantageous to improve the reproducibility of the sensitivity of the electrodes.

3.2 Stabilization by the modification of the grafting process.

An example of the relationship between the current intensity at the MIP-electrode, prepared by a conventional procedure using a quartz tube, and the heparin concentration is shown in **Fig. 4**. A remarkable difference in the current density was observed between the first and second cycles (time interval of approximately 1 h), indicating that the current drifted dramatically during the sequential measurements. An example of the relationship between the current intensity at the MIP-electrode prepared by the improved procedure described above and the heparin concentration is shown in **Fig. 5**. Although the measurements between each cycle were performed at 24 h intervals, the current densities during the three cycles were similar (maximum error was 5%),

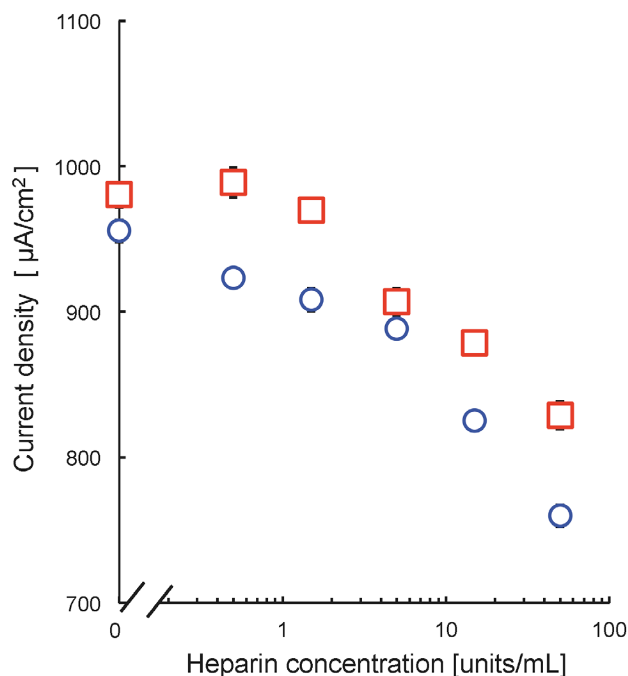

Fig. 4: The relationship between the current density of 5 mM ferrocyanide at the MIP-electrode generated by the conventional grafting process and the heparin concentration. Circles: first cycle, squares: second cycle. The interval between cycles was approximately one hour (n=3). In this grafting process, light from a 6W-gercimidal lamp was irradiated for 24 h on the initiator-coated electrode immersed in monomers-template solution contained in a quartz crystal tube.

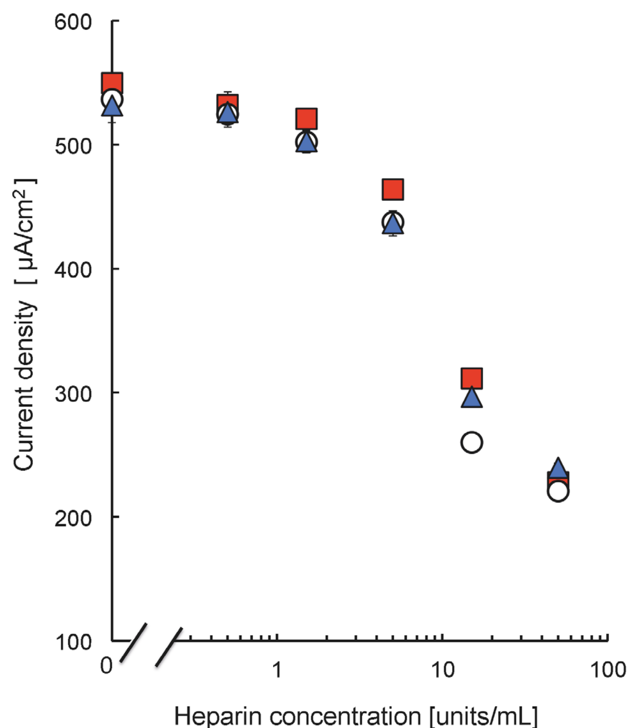

Fig. 5: Daily difference variation in the relationship between the current density of 5 mM ferrocyanide at the MIP-electrode generated by the modified grafting process and the heparin concentration. Triangles: first day, circles: the second day, squares: the third day (n=3). In this modified grafting process, light from a 200 W-xenon lamp was irradiated on the initiator-coated electrode through a 50-μm thick layer of the monomer-template solution, as shown in Fig. 2.

indicating that compared to the conventional method, the strong light source and small optical pathlength used in the monomers-template solution in the present method improve the efficiency of polymerization at the surface of the ITO. The MIP grafted on the electrode remained stable through multiple electrochemical measurements.

Additionally, the improved grafting procedure increased the success ratio for preparing heparin-sensitive electrodes. Only 10% of the electrodes treated by the conventional procedure were sensitive to heparin, while all of the electrodes treated by the improved procedure exhibited remarkable sensitivity to heparin.

3.3 Sensitivity in whole blood

An example of the change in peak current intensity of ferrocyanide oxidation at the MIP grafted electrode with changes in the heparin concentration is shown in **Fig. 6.** The current density decreased monotonically as the heparin concentration in whole blood increased. As shown in **Table 1**, the sensitivity of the current density to the heparin concentration is approximately half that of the heparin concentration in physiological saline, indicating that the sensitivity of the MIP-electrode is decreased by some component in blood even considering the confidence intervals of the data. The sensitivity in plasma is not higher than that in blood. (The average of the sensitivity in plasma is rather lower than that in blood, but the large confidence intervals of the data indicated that there is no significant difference), suggesting that this component may be plasma proteins rather than blood cells. We therefore tried coating the MIP-electrode with a "biocompatible material" to prevent protein adsorption onto the electrode. Two commercial materials for preventing protein-adsorption on the surface of clinical instruments were used: Lipidure®-CM 5206 (Mang *et al.*, 2005) (NOF Co. Ltd., Tokyo, Japan), a copolymer of 2-methacryloyloxyethyl phosphorylcholine and alkylmethacrylate), and SEC-1 ™ (Toyobo, Osaka, Japan) which contains polyethyleneglycol to increase surface hydrophilicity (http://www.toyobo-global.com/news/pdf/2011/12/press05122011.pdf).

A MIP electrode was soaked in a 4.1 mg/mL ethanolic solution of Lipidure®-CM 5206, for 1 min and dried under a nitrogen stream. Interestingly, this modification did not affect the sensitivity of the electrode in physiological saline, but essentially abolished the sensitivity in whole blood, indicating that Lipidure®-CM 5206 does not suppress protein adsorption on the MIP-electrode but rather promotes adsorption. In contrast, when SEC-1™ was coated on the MIP-electrode, the average sensitivity of the coated electrode to heparin in whole blood was approximately 74% of that in physiological

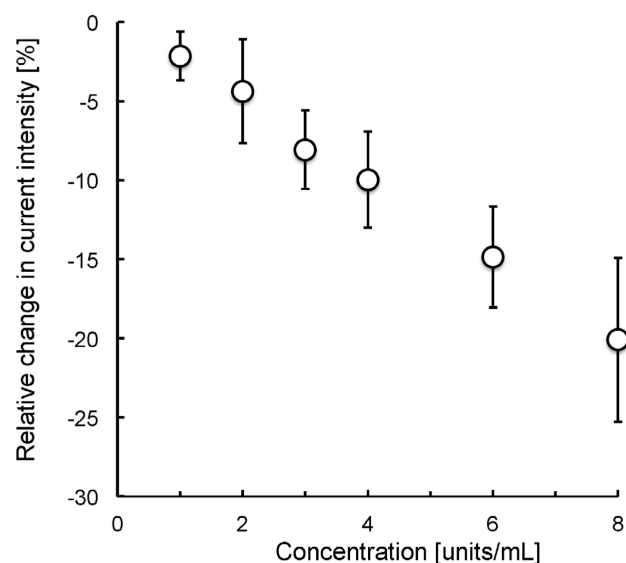

Fig. 6: Relationship between the relative change in the peak current density measured using cyclic voltammetry with a MIP-grafted electrode (generated using the modified method) and the unfractionated heparin concentration in bovine whole blood containing 5 mM ferrocyanide (n=3).

Table 1: Relative decrease in anodic peak oxidative current of 5 mM ferrocyanide at the MIP-grafted electrode by the addition of 8 units/mL heparin in whole blood, plasma or physiological saline, and the ratio of the decrease in anodic peak oxidative current when the heparin was present in whole blood compared to when heparin was in physiological saline. The plasma was obtained by the centrifugation of whole blood at 2,600 x *g* for 1 h (n=3).

Treatment of the MIP-electrode	In physiological saline	In whole blood	In plasma	*Ratio of sensitivity
None	38 ± 7 %	20 ± 5%	10 ± 5 %	0.52
Coated with Lipidure®-CM 5206	30 ± 5 %	< 5 %	-	<0.17
Coated with SEC-1™	35 ± 5 %	26 ± 12 %	-	0.74
Washed with Sterizime® S	31 ± 7 %	24 ± 3 %	-	0.77

*Ratio of sensitivity in whole blood to that in physiological saline

saline. However, the reproducibility of the sensitivity measurements was poor, as indicated by the large standard deviation. Then significant effect for retaining sensitivity in blood was not observed. We therefore cannot say that coating with SEC-1™ is a viable option for retaining the heparin sensitivity of the MIP-electrode in whole blood.

Another approach was also tried and involved washing the blood components from the MIP-electrode between measurements. Sterizime® S (Maruishi Pharmaceutical, Tokyo, Japan) is a protease-containing detergent commonly used for washing clinical tools contaminated with blood. The MIP electrode was washed in Sterizime® S 100-fold diluted with 1 M NaCl aqueous solution, rinsed with distilled water and dried with nitrogen between measurements. The average sensitivity of the coated electrode in whole blood was approximately 77% of that in physiological saline, and the standard deviation of the sensitivity was small, showing that washing with Sterizime® S is effective for retaining the heparin sensitivity of the MIP-electrode in whole blood. Furthermore, washing with a detergent is much more economical than coating with SEC-1™. The MIP electrode is essentially insensitive to chondroitin sulfate C, which, like heparin, is a glucosamine sulfate (**Fig. 7**) when present in whole blood, and the selectivity of the MIP is retained in blood by washing with the detergent. The current at the MIP electrode was affected by LMWH. The clinical use of LMWH is increasing due to its safety: unlike unfractionated heparin, it does not inhibit the function of thrombin or platelets (Korkmaz, 2005). However, the anticlotting effect of LMWH cannot be monitored by the measurement of ACT, currently the most common method for evaluating the anticoagulant effect of unfractionated heparin (Krishnaswamy and Kapadia, 2013). In contrast, the MIP electrode can measure LMWH in whole blood and thus will help expand the clinical utility of LMWH.

Automation of the electrode washing process between measurements is facile. Thus, a real-time heparin monitoring apparatus for use with extracorporeal circulation therapy can be achieved by using a MIP-grafted electrode in conjunction with a washing system.

4 Conclusion

We here described procedures for improving an electrode grafted with MIP to sense different heparin concentrations. The stability of the electrode can be improved by grafting MIP in a thin layer of a mixed solution of monomers and template. The hysteresis in the response of the electrode grafted with MIP towards heparin can be removed by washing with a highly concentrated sodium chloride solution between measurements. The sensitivity of the electrode to heparin is lower in whole blood than in physiological saline, but washing with a protein-removal detergent is effective for maintaining the high sensitivity of the electrode towards heparin in whole blood.

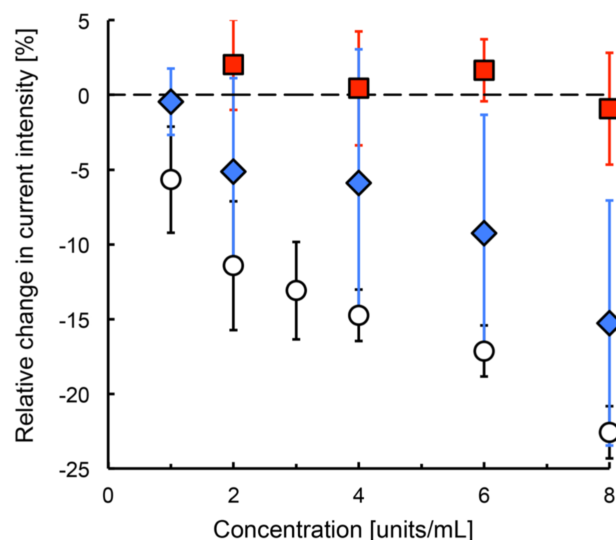

Fig. 7: Relationship between the peak current intensity of 5 mM ferrocyanide and unfractionated heparin (circles), LMWH (diamonds) or chondroitin sulfate C (squares) in whole blood. The MIP electrode was washed with Sterizime® S 100-fold diluted with 1 M NaCl aqueous solution, rinsed with distilled water, and dried with nitrogen between measurements. (n=3) Chondroitin sulfate C does not function as an anticoagulant. However, the concentration of chondroitin sulfate C was converted to units/mL using the relationship 130 units = 1 mg, in a manner similar to the conversion used for heparin and described in the text. This allowed convenient comparison of chondroitin sulfate C with heparin and LMWH.

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