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Signal Processing – Imaging Systems – Assist Systems – Implants – Sensors and Instrumentation – Computers

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Development of a ferrocene-mediated needle-type glucose sensor covered with newly designed biocompatible membrane, 2-methacryloyloxyethyl phosphorylcholine -co-n-butyl methacrylate

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Key words: Artificial endocrine pancreas, diabetes mellitus, needle-type glucose sensor, biocompatibility, subcutaneous tissue glucose concentration, continuous glucose monitoring

Abstract

To prepare the long-life and stable glucose sensor, we developed the ferrocene-mediated needle-type glucose sensor covered with newly designed biocompatible membrane, 2-methacryloyloxyethyl phosphorylcholine -co-n-butyl methacrylate (MPC-co-BMA) membrane. In this membrane, the hydrophilic phosphorylcholine chains were grafted on the hydrophobic polymer surface. 1. The poly(MPC-co-BMA) membrane inhibited platelet activation and protein adhesion on the surface, showing excellent biocompatibility. These results suggested that the hydrophilic phospholipids chains might have the potential for suppressing activation and adsorption of biochemical molecules. 2. The ferrocene-mediated needle-type glucose sensor covered with poly(MPC-co-BMA) membrane achieved excellent results in vitro. Subcutaneous tissue glucose concentrations were measured in a wide range from 1.7 to more than 16.7 mmol/l. The correlation between subcutaneous tissue (Y) and blood (X) glucose concentrations was $Y = 1.04X + 0.12$ ($r = 0.98$). The subcutaneous tissue glucose concentrations could be monitored precisely for 7 days without any in vivo calibrations, and for 14 days by introducing in vivo calibrations. We therefore conclude that this sensor is stable and reliable, as compared to any other glucose sensors we developed.

Introduction

In 1982, Shichiri et al. [1] developed a highly sensitive needle-type glucose sensor covered with polyvinyl alcohol (PVA) membrane suitable for in vivo use. Then, we developed a wearable artificial endocrine pancreas, which consisted of a needle-type glucose sensor, a microcomputer system, insulin and glucagon infusion pump systems and a battery. With this system, physiological glycemic regulations were established in ambulatory diabetic patients [2]. However, the major obstacle in extending the term of glycemic control was failure to develop a stable and reliable implantable glucose sensor. At present, the output current of the glucose sensor decreases with time when in vivo subcutaneous tissue glucose monitoring is undertaken. We consider the major reason for sensor decay to possibly be protein fixation on the membrane surface. The protein fixation on the membrane surface causes a diffusion barrier for glucose and oxygen. The insufficient availability of oxygen in glucose sensor inserted in subcutaneous tissue causes a decrease in the maximal output current of glucose sensor, resulting in the lowering the measuring range of glucose concentrations.

To prolong the life-time of the sensor, the biocompatible membrane is crucial. We studied the biocompatibility of many other hydrophilic membranes available in Japan, such as segmented polyurethane, polyvinyl alcohol conjugating heparin, polyethylene oxide and alginate-polylysine-alginate membrane, but

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none of these hydrophilic membranes showed superiority to polyvinyl alcohol membrane [3].

If an artificial surface similar to the cell wall is constructed, the surface would show excellent biocompatibility. Thus, to extend the biocompatibility of the glucose sensor, we have developed a cell surface like membrane, butyl methacrylate (BMA) having phosphorylcholine moiety, 2-methacryloyloxyethyl phosphorylcholine (MPC) was synthesized, poly(MPC-co-BMA) [4].

In this report, the biocompatibility of the poly(MPC-co-BMA) membrane is studied in vitro and in vivo. Then, the performances of a glucose monitoring system with a ferrocene-mediated needle-type glucose sensor covered with this highly biocompatible poly(MPC-co-BMA) membrane were evaluated in healthy and diabetic volunteers.

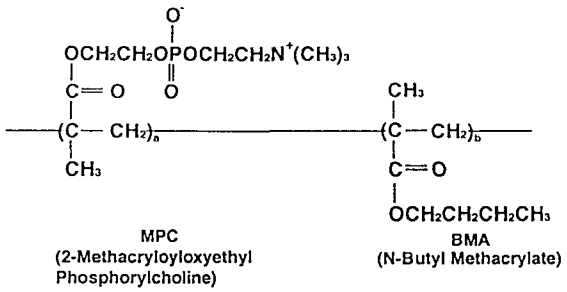

Fig. 1. Structural formula of poly(2-methacryloyloxyethyl phosphorylcholine(MPC)-co-n-butyl mehtacrylate(BMA)).

Methods

Preparation and biocompatible properties of poly(BMA-co-MPC) membrane

Preparation of poly(MPC-co-BMA) membrane
2-chloro-2-oxo-1,3,2-dioxaphospholane (COP) was synthesized according to the method of Edmundson [5] and purified by distillation under pressure, bp 98°C/1 mmHg (lit.: bp 79°C/0.4 mmHg). 2-hydroxyethyl methacrylate (HEMA) and n-butyl methacrylate (BMA) were distilled under reduced pressure of argon gas, and fractions of bp 65°C/3 mmHg and 60°C/30 mmHg were used, respectively. 2,2′-azoisobutyronitrile (AIBN) was recrystallized from methanol. Tetrahydrofuran (THF), acetonitrile, and triethylamine (TEA) were purified by conventional way.

The principle preparation of poly(MPC-co-BMA) membrane has been described in detail elsewhere [4]. Into a 500 ml three-necked flask equipped with a dropping funnel, thermometer, and drying tube, 0.154 mol of HEMA, 0.154 mol of TEA and 200 ml of dry THF were placed. After the solution was cooled at −20 °C, 0.154 mol of COP in 100 ml of dry THF were added dropwise to the stirred solution over a period of 1 hr. The temperature of the reaction mixture was maintained at −20 ~ −30 °C for 3 hr. Then, the precipitate in the reaction mixture which was triethylammonium chloride was filtered off. The filtrate was evaporated under reduced pressure. To the residue, 50 ml of dry ethyl ether were added to precipitate a small amount of triethylammonium chloride and precipitates were filtered. By evaporation of filtrate under reduced pressure, colorless liquid, 2-(2-oxo-1,3,2-dioxaphospholoyloxy)ethyl methacrylate (OPEMA) was obtained (yield: 35.5 g).

Into a 200 ml glass pressure bottle, 5.0 g of OPEMA and 30 ml of dry acetonitrile were placed. After the bottle was cooled at −20 °C, 2 ml of anhydrous triethylamine were rapidly added to the solution. The pressure bottle was closed and allowed to warm up to room temperature. After it was heated at 60 °C for 16 hr, the bottle was cooled at −20 °C. With this procedure, a white precipitate, MPC, began to form. The MPC was filtered off under argon gas atmosphere, washed with cold dry acetonitrile and dried under reduced pressure (yield: 3.2 g).

The desired amounts of MPC, BMA and AIBN were dissolved in methanol -THF mixture in polymerization tube. After oxygen in the tube was eliminated by bubbling of argon gas into the solution, the tube was sealed. Then the tube was shaken at 60 °C for 16 hr. The contents were cooled to stop the reaction and precipitated by pouring into hexane for copolymers with low MPC composition and into diethyl ether for those with high MPC composition. The precipitate was collected and dried in vacuo. The structure of poly(MPC-co-BMA) was characterized by IR and ^{1}H NMR spectra. The structural formula of poly(MPC-co-BMA) is shown in Fig. 1. In this experiment, we used copolymers with high MPC composition, since those with high MPC composition showed excellent biocompatibility in vitro [4].

Biocompatible properties
Polymer coating of acrylic beads was carried out by a solvent evaporation technique using 4% PVA (PVA coated beads) or 0.5% poly(MPC-co-BMA) solution (MPC coated beads).

Platelet activation. To test the thrombogenicity of the membrane, we have investigated the adenosine diphosphate (ADP)-induced maximum aggregation rate in platelet-rich plasma (PRP) against PVA membrane and poly(MPC-co-BMA) membrane. PRP was prepared from healthy subjects. Thirty six milliliters of blood were collected in a disposable syringe containing 4 ml of 3.8% sodium citrate solution. The citrated blood was immediately centrifuged at 750 rpm for 15 min to obtain citrated PRP. The number of platelets of PRP was adjusted to approximately 3×10^8 cells/ml by dilution with platelet-poor plasma, which was prepared from the citrated blood by centrifugation at 2600 rpm for 15 min. The number of platelets was counted with platelet counter (PL-100, Sysmex, Japan). After PRP (450 μl) was incubated with PVA or poly(MPC-co-BMA) coated beads (140 mg) in a glass cell for 3 hr at 37 °C, 50 μl of 10 μmol/l ADP solution were added to induce platelet aggregation. As control, PRP was incubated without membranes. The maximum aggregation rate was measured by aggregometer (LUMI AGGREGOMETER model 400, CHRONO-LOG, U.S.A.).

Protein adhesion. In in vitro experiments, after the MPC coated beads were soaked in 0.7 mmol/l phosphatidylcholine dipalmitoyl (DPPC; SIGMA Chemical Co, MO, U.S.A.) liposomal solution overnight, those acrylic beads (140 mg) were placed in test glass tubes filled with 70 g/l bovine serum albumin (BSA) solution for 1, 3, 5, 7, 10 and 14 days. After washing unbound protein with saline solution (NaCl 154 mEq/l), protein adsorbed on acrylic beads was eluted by Tris-SDS (Tris 125 mmol/l, SDS 80 mmol/l). Eluted solution was concentrated by precipitation using acetone at −20 °C. One volume of the eluted solution was mixed with four volumes of acetone previously stored at −20 °C. The mixture was kept at −20° C for 15 hr and centrifuged at 4000 rpm for 15 min. The pellet was dissolved in 500 μl of Tris (125 mmol/l, pH 7.4), transferred to an Eppendorf tube, achieving a degree of concentration of about 200 times and sonicated (10 s) to prevent aggregates [6]. The total protein content was determined with the micro BCA Protein Assay Reagent (PIERCE, Rockford, IL, U.S.A.). The amount of proteins adsorbed on the polymer beads was expressed as μg/cm^2 of the surface area of the beads.

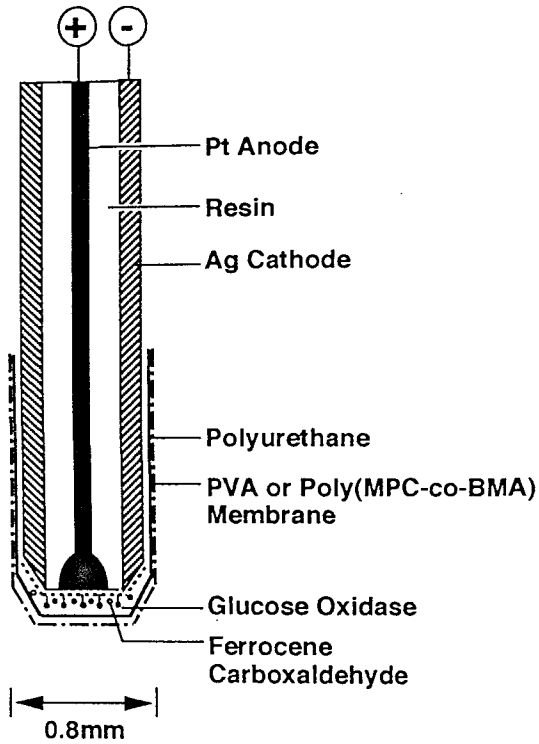

Fig. 2. Schematic diagram of a ferrocene-mediated needle-type glucose sensor covered with PVA or poly(MPC-co-BMA) membrane.

In in vivo experiments, those acrylic beads (140 mg) were implanted in subcutaneous tissue of normal Wister rats weighing about 300 g for 1, 3, 5, 7, 10 and 14 days (n=5, each). The amount of proteins adsorbed on the polymer beads were determined in the same manner as previously described.

Preparation and characteristics of ferrocene-mediated needle-type glucose sensor covered with poly(MPC-co-BMA) membrane

Preparation of ferrocene-mediated needle-type glucose sensor covered with poly(MPC-co-BMA) membrane
The preparation of a ferrocene-mediated needle-type glucose sensor has been described in detail elsewhere [7]. The principle structure of the ferrocene-mediated needle-type glucose sensor is shown in Fig. 2. The ferrocene-mediated needle-type glucose sensor was finally covered with 4% PVA membrane (Biomaterial Univers Co, Japan) (PVA sensor) or

0.5% poly(MPC-co-BMA) membrane (MPC sensor) using a solvent evaporation technique, 4% PVA or 0.5% poly(MPC-co-BMA) solution, respectively. The ferrocene-mediated needle-type glucose sensor was individually fabricated in our laboratory. Before using in in vitro and in vivo experiments, each sensor was sterilized by ethylene oxide gas at 40 °C for 5 hr, and was loaded with polarizing voltage of +0.4 V, using a potentiostat (HECS 318, Huso Co, Kawasaki, Japan). Current-time curves were recorded with a multi-pen recorder (R-63, Rikadenki Kogyo Inc, Tokyo, Japan).

Characteristics of ferrocene-mediated needle-type glucose sensor covered with poly(MPC-co-BMA) membrane

In vitro characteristics. After the MPC sensor was soaked in 0.7 mmol/l DPPC liposomal solution overnight, the in vitro characteristics of the sensor were assessed by placing the sensor in saline solution with varying glucose concentrations (0 – 27.8 mmol/l) in a temperature-controlled (37 °C) chamber. Response time to reach 90% of the final plateau value ($T_{90\%}$) was calculated by switching the saline-glucose solution from 5.5 to 11.1 mmol/l and vice versa. To test the decrease in sensor output due to protein fixation on the surface of the sensor, the sensor was placed in sodium azide (15 mmol/l) added saline-glucose solution (5.5 mmol/l) with or without BSA (30, 50 and 70 g/l) for 14 days. These results were also compared to the characteristics of the PVA sensor.

In vivo characteristics. After evaluating sensor characteristics in animal experiments, it was then assessed in 5 healthy subjects (age, 28.4 ± 2.3 yr; BMI, 22.6 ± 1.4 kg/m^2, mean ± SEM). After calibration was made by placing the PVA sensor or the MPC sensor in a saline solution with and without glucose (11.1 mmol/l) in vitro, the sensor was inserted into the subcutaneous tissue of forearm by the following procedure. The surface of the skin was punctured by an indwelling catheter (18 gauge, 18G 4HSSPP NP, MEDIKIT, Tokyo, Japan). The steel mandrin was removed, and the sensor was retrogradely inserted into the plastic cannula. The plastic cannula was then removed, thereby drawing the sensor under the surface of the skin. No local anesthesia was used. Subcutaneous tissue glucose monitoring by the sensor was initiated 2 hr after the implantation when output currents reached a steady state. Simultaneously, the whole blood glucose concentrations were also continuously monitored with the aid of a bedside-type glucose monitoring system (model STG-22, Nikkiso Co, Ltd, Tokyo, Japan) [8, 9]. In this system, a glucose oxidase membrane with a hydrogen-peroxide electrode was incorporated for continuous blood glucose monitoring. After monitoring the baseline for more than 30 min, each healthy subject was given a 0.42 mol glucose orally. After blood glucose returned to the fasting level (usually 1 to 2 hr after oral glucose tolerance test), insulin at a dose of 0.7 μmol/kg was injected intravenously. The 'relative' output current while kept in subcutaneous tissue was determined by comparing the sensor output with the simultaneously measured blood glucose concentration. The 'relative' response time was determined as the time lag between the times to reach the peak values of plasma and subcutaneous tissue glucose concentrations. These results were compared to the characteristics of the PVA sensor.

Subcutaneous tissue glucose monitoring with ferrocene-mediated needle-type glucose sensor covered with poly(MPC-co-BMA) membrane

Monitoring system

The glucose monitoring system (12.4 × 6.0 × 2.2 cm; weight, 330 g, SMP-X1, Nikkiso Co, Ltd, Tokyo, Japan) consists of a current-voltage converting amplifier, a 10 bit AD converter (voltage/frequency converter), an 8 bit CPU, an 8 KB PROM for storing measured glucose data, a liquid crystal display mechanism and a battery supply. In this monitoring system, the output current of glucose sensor is transformed into glucose concentration according to the following equation: $Y = G_{ST} \times I/(I_{ST}-I_0)$, where Y is glucose concentration in an unknown sample, G_{ST} is glucose concentration in a standard glucose solution (11.1 mmol/l), I_{ST} and I_0 are output currents when the glucose sensor is placed into a saline solution and a standard glucose solution, respectively, and I is output current against an unknown sample. The average glucose value is recorded every minute for a day, and the stored glucose data can be recalled using a data terminal (SDM-X1, Nikkiso Co, Ltd, Tokyo, Japan). Hypoglycemia beyond the prefixed threshold is notified by the sound of an alarm.

Continuous subcutaneous tissue glucose monitoring

This system of continuous subcutaneous tissue glucose monitoring was then assessed in 10 healthy subjects and 7 diabetic patients. All diabetic patients (7 patients

with NIDDM; age, 35.2 ± 10.9 yr; duration of diabetics, 5.7 ± 1.1 yr; BMI, 24.0 ± 3.4 kg/m^2) were hospitalized in our department, and treated with multiple insulin injection therapy. Their FBG and HbA1c values ranged between 3.8 and 10.4 mmol/l (7.8 ± 1.9 mmol/l) and between 7.2 and 8.9% (8.3 ± 0.2%), respectively. Their MBG and MAGE values ranged between 6.4 and 12.1 mmol/l (9.1 ± 0.3 mmol/l) and between 2.8 and 6.5 mmol/l (4.6 ± 0.2 mmol/l), respectively. None of diabetic patients had liver disease, kidney dysfunction nor chronic pancreatitis. Patients with severe diabetic microvascular complications were also excluded. Five healthy subjects were studied within a two-week period after the previous experiments on oral glucose load and intravenous insulin injection. Informed consent was obtained from each volunteer. This study was approved by the ethical committee of the Kumamoto University School of Medicine. During the experimental period, subjects were scheduled to take each meal at 8:00, 12:00 and 18:00 (1600–2200 kcal/day). The subjects were allowed to walk for 30 min starting at 1 hr after each meal.

After calibration with a standard glucose solution in vitro, the sterile PVA sensor or the sterile MPC sensor was inserted into the subcutaneous tissue of forearm in the same manner as previously described, and subcutaneous tissue glucose concentrations were continuously monitored. During continuous monitoring, the 'relative' output currents were calculated and compared. During continuous monitoring, when the 'relative' output current decreased with time due to protein fixation on the surface of the sensor, the in vivo calibration procedure was performed by dividing the sensor output by the 'relative' output current divided by one hundred, then multiplying a factor of 1.10. Since subcutaneous tissue glucose concentrations were identical to plasma glucose concentrations, but were 10% higher than whole blood glucose concentrations [10], a factor of 1.10 was multiplied to adjust apparent values estimated by whole blood glucose concentrations to true subcutaneous tissue glucose concentrations [11].

Blood samples were obtained from antecubital vein before and 1 and 2 hr after each meal and at bed time. Blood glucose concentrations in whole blood samples were measured by the glucose oxidase method.

Furthermore, scanning electron microscopic observation was done on the sensor surface. After removal of the glucose sensor, the sensor was washed with saline solution. The sensor was placed in a saline solution containing 0.2 mol/l glutaraldehyde to fix the adherent proteins. The sensor was rinsed with a water, dehydrated with ethanol, dried with critical point drying technique, and coated with gold. The sensor surface was observed with scanning electron microscope (Comtec CSM-501).

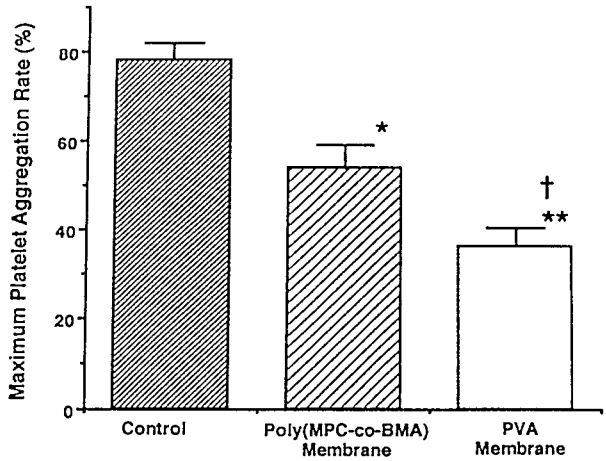

Fig. 3. Maximum platelet aggregation rates of platelet rich plasma (PRP; the number of platelets: 3 × 10^8 cells/ml) contacted with poly (MPC-co-BMA) and PVA coated acrylic beads for 3 hours at 37^{0}C. Platelet aggregation was induced by 0.1 μmol/l ADP. As control, PRP was incubated without membrane. Data are shown in mean±SEM. (*P<0.01 vs control, **P<0.005 vs control, †P<0.01 vs poly(MPC co-BMA) membrane).

Results are expressed as means ± SEM. Statistical analysis was performed with paired Student's t tests. Linear regression analysis was done by the method of least squares and the F-distribution test was used for statistical evaluation of the slopes of regression lines. The relationships between variables were estimated with Pearson's correlation coefficients.

Results

Biocompatible properties of poly(MPC-co-BMA)

The maximum rates of platelet aggregation were 78 ± 4% in control, 54 ± 5% in poly(MPC-co-BMA) membrane and 36 ± 4% in PVA membrane, respectively, reflecting greater consumption of platelets on PVA membrane (Fig. 3).

In vitro experiments, the amounts of protein adsorbed on the PVA or MPC coated beads were increased with time. The amounts of protein adsorbed on the PVA coated beads on 1st, 3rd, 5th, 7th, 10th and 14th day were 9.5 ± 0.6, 12.5 ± 0.9, 14.3 ± 1.1, 18.2

Table 1. Characteristics of ferrocene-mediated needle-type glucose sensor covered with poly(MPC-co-BMA) membrane

Tests	Performances (N=5)
1) *In Vitro Characteristics*	
Residual currents (nA)	0.3 ± 0.1
Output currents generated to 5.5 mmol/l glucose (nA)	7.4 ± 1.8
Baseline drift (%/24hrs)	1.0 ± 0.1
Linearity to glucose concentration (mmol/l)	0.6 - 22.2
$T_{90\%}$ response time to reach steady-state level	
From 5.5 to 11.1 mmol/l (min)	2.1 ± 0.3
From 11.1 to 5.5 mmol/l (min)	3.8 ± 0.4
2) *In Vivo Characteristics*	
'Relative' output current (%)	103.3 ± 1.8
'Relative' response time	
after oral glucose load (min)	4.6 ± 1.1
after intravenous insulin injections (min)	6.9 ± 1.6
Lower detection limit of glucose concentration (mmol/l)	1.8 ± 0.6
Correlation between subcutaneous tissue	
(Y;mmol/l) and blood glucose (X;mmol/l)	Y=1.04X+0.12
concentartions	(r=0.98)

Residual current denotes the output current of the sensor in the absence of glucose. Baseline drift is expressed as percentage change of output current in response to 5.5 mmol/l glucose solution during continuous operation. Linear relationships are studied in a regression analysis between output currents or glucose concentrations measured and glucose concentrations in the ranges indicated. $T_{90\%}$ denotes the response time to reach 90% of the final plateau value. The 'relative' output current while kept in subcutaneous tissue was determined by comparing the sensor output with the simultaneously measured blood glucose concentration. The 'relative' response time was determined as the time lag between the times to reach the peak values of blood and subcutaneous tissue glucose concentrations. Lower detection limit listed in in vivo characteristics was defined as the lowest level of subcutaneous tissue glucose concentration measured after intravenous insulin injection (0.7 μmol/kg). Blood glucose concentrations in whole blood samples were measured by glucose oxidase method.
Results are expressed as means±SEM.

± 1.3, 27.9 ± 1.5 and 35.6 ± 1.7 $\mu g/cm^2$, respectively. Whereas, those on the MPC coated beads were 2.2 ± 0.4, 4.4 ± 0.5, 5.7 ± 0.7, 6.2 ± 0.8, 12.1 ± 1.1 and 14.0 ± 1.4 $\mu g/cm^2$, respectively (Fig. 4 (A)). During 14 days experimental period, the amounts of protein adsorbed on MPC membrane were significantly smaller than those on PVA membrane ($P < 0.01$). In vivo experiments, the amounts of protein adsorbed on the PVA coated beads on 1st, 3rd, 5th, 7th, 10th and 14th day were 11.3 ± 1.3, 14.9 ± 1.4, 16.9 ± 1.4, 19.6 ± 2.0, 24.6 ± 2.1 and 31.2 ± 2.8 mg/cm^2, respectively. Those on the MPC coated beads were 3.8 ± 0.4, 6.0 ± 0.6, 6.3 ± 0.7, 7.3 ± 0.9, 12.4 ± 1.2 and 16.9 ± 1.4 $\mu g/cm^2$, respectively (Fig. 4 (B)). During 14 days of experimental periods, the amounts of protein adsorbed on MPC membrane was significantly smaller than those on PVA membrane ($P < 0.01$).

Characteristics of ferrocene-mediated needle-type glucose sensor covered with poly(MPC-co-BMA) membrane

In vitro characteristics

Table 1 shows the in vitro characteristics of the MPC sensor. The residual currents were measured 10 min after polarization when the output currents reached a steady state and were 0.3 ± 0.1 nA. Output currents generated to 5.5 mmol/l were 7.4 ± 1.8 nA. A linear relationship between glucose concentration and sensor output was observed in the range between 0.6 to 22.2 mmol/l. The response times to reach 90% of maximum or minimum values ($T_{90\%}$) were 2.1 ± 0.3 min or 3.8 ± 0.4 min, respectively. Similar sensor characteristics in vitro were obtained with the PVA sensor.

To test the decrease in sensor output due to protein fixation on the surface of the sensor, the sensor was placed in sodium azide added saline-glucose solution (5.5 mmol/l) with or without BSA (30, 50 and 70 g/l)

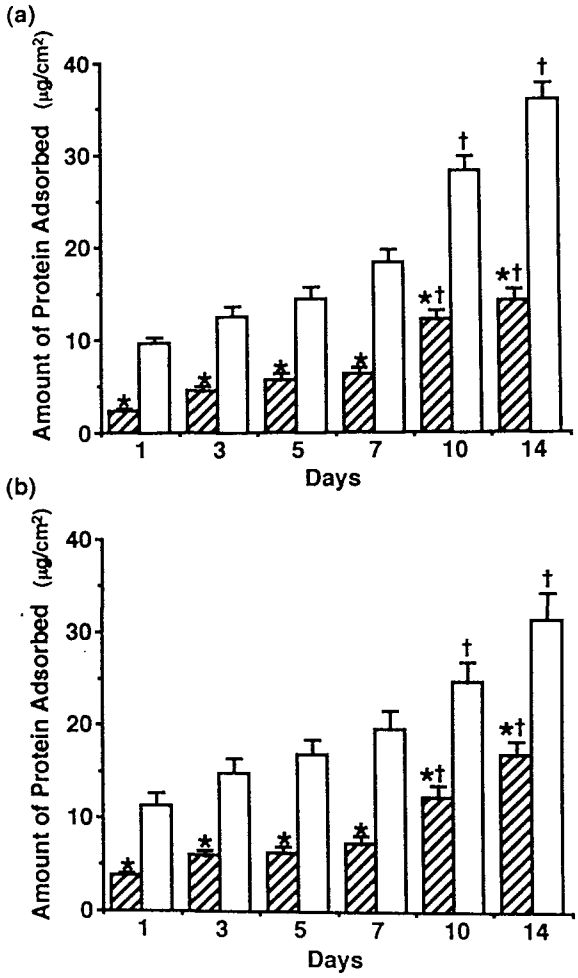

Fig. 4. The amounts of protein adsorbed on PVA or poly(MPC-co-BMA) coated beads in vitro and in vivo. In in vitro experiments (A), polymer coated acrylic beads were placed in test glass tubes filled with BSA solution (70g/1) for 1, 3, 5, 7, 10 and 14 days. After washing unbound protein with saline solution, protein adsorbed on acrylic beads was eluted and determined. The amount of protein adsorbed on the polymer beads are expressed as $\mu g/cm^2$ of the surface area of the beads. In in vivo experiments (B), polymer coated acrylic beads were implanted in subcutaneous tissue of normal Wister rats (n=5) for 1, 3, 5, 7, 10 and 14 days. The amount of protein adsorbed on the polymer beads were determined. Data are shown in mean±SEM. □: PVA, ■: poly(MPC-co-BMA). (*P<0.01 vs PVA, †P<0.01 vs amount of protein adsorbed on polymers at initial day).

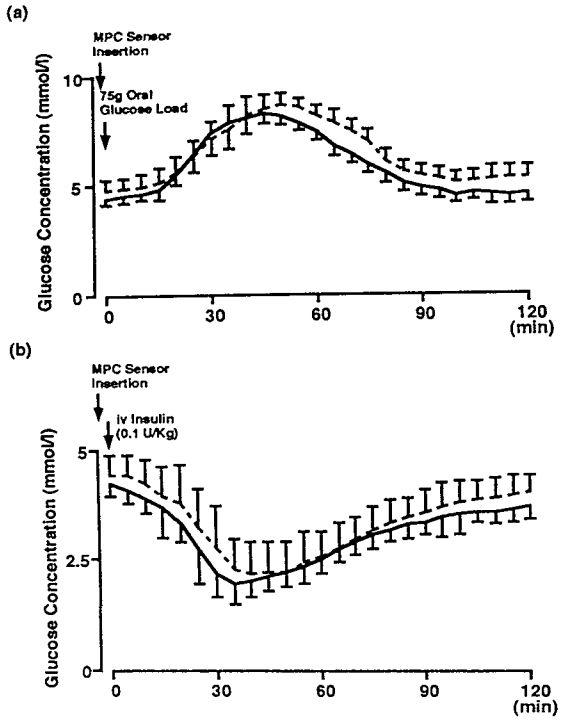

Fig. 5. Continuous monitoring of subcutaneous tissue glucose concentrations measured by the MPC sensor after 0.42 mol oral glucose load (A) and after intravenous insulin injection (0.7 μmol/kg) (B) in 5 healthy subjects. Subcutaneous tissue glucose concentrations measured by the MPC sensor (...) were compared with blood glucose concentrations (—) measured by the blood glucose monitoring system (STG-22, Nikkiso Co., Ltd., Tokyo, Japan). Results are analyzed at 5 min intervals of the continuous monitoring records, and expressed as means±SEM.

for 14 days. With either the PVA sensor or the MPC sensor, output currents were fairly constant during 14 days experimental periods in saline-glucose solution without BSA (100 ± 1.1%, expressed as percentage of initial value). With the PVA sensor, output currents were maintained at the same level as initial for up to 3rd day, and then decreased to the level of 78.5 ± 5.8, 76.1 ± 6.3, 74.3 ± 4.8% on 7th day and 67.1 ± 6.5, 62.1 ± 5.1, 49.2 ± 6.8% on 14th day, in the solutions with 30, 50, 70 g/l BSA, respectively. With the MPC sensor, output currents were not significantly changed during incubation period in the solutions with 30, 50 and 70 g/l BSA for 7 days, then gradually decreased to the level of 92.7 ± 3.2, 92.8 ± 5.3 and 89.8 ± 5.2% of the initial value on 14th day, respectively.

In vivo characteristics

As shown in Fig. 5, during oral glucose loads and intravenous insulin injections, there was a clear parallel relationship between the changes in blood glucose concentrations and subcutaneous tissue glucose concentrations measured. In vivo characteristics of the MPC sensor were also shown in Table 1. The mean 'relative' output current was 103.3 ± 1.8. Response

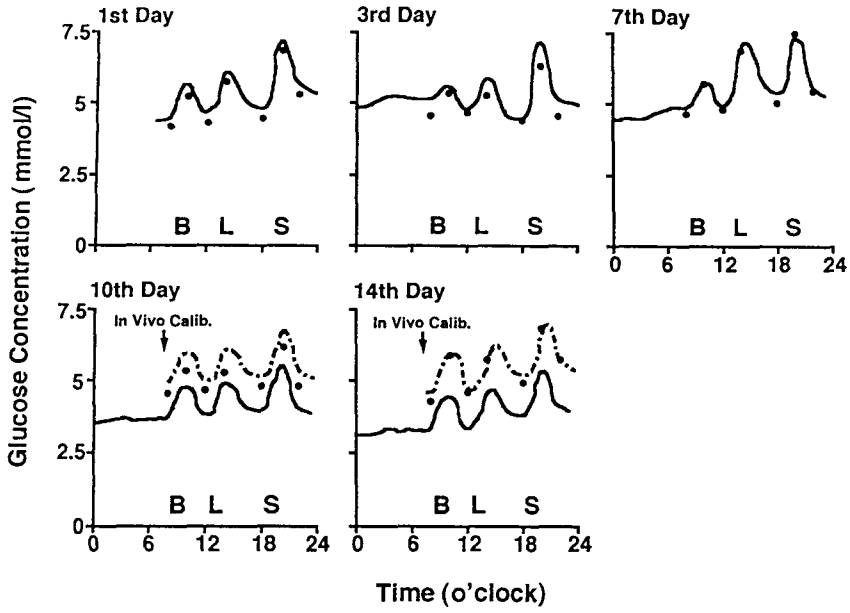

Fig. 6. Continuous subcutaneous glucose monitoring with the glucose monitoring system incorporated with the MPC sensor during 14 days in a representative case of healthy subject. After 7 days continuous monitoring, the in vivo calibration with fasting blood glucose every morning was introduced. B, L and S denote breakfast, lunch ans supper, respectively. The in vivo calibration procedure in this monitoring system was performed by dividing the sensor output by the 'relative' output currents dividing by one hundred, then multiplying a factor of 1.10. (—: apparent subcutaneous tissue glucose concentrations measured by the glucose monitoring system with the MPC sensor without in vivo calibration, ---: apparent subcutaneous tissue glucose concentrations measured by the MPC sensor with in vivo calibration, •: blood glucose concentrations determined by glucose oxidase method).

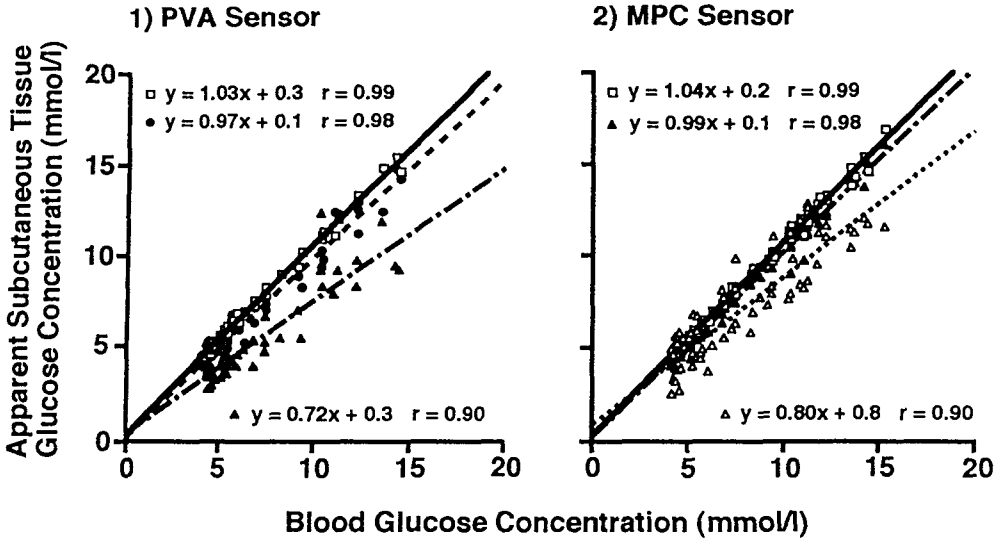

Fig. 7. Linear regression analyses between apparent subcutaneous tissue glucose concentrations measured by the ferrocene-mediated needle-type glucose sensor (Y: mmol/l) and blood glucose concentrations determined by glucose oxidase method (X: mmol/l) in healthy subjects and in diabetic patients: With the PVA sensor, subcutaneous tissue glucose concentrations were measured in 5 healthy subjects and 2 diabetic patients for 7 days. With the MPC sensor, subcutaneous tissue glucose concentrations were measured in 5 healthy subjects and 5 diabetic patients for 14 days. (□, —: data obtained at 1st day, •, ...: at 3rd day, ▲, ...: at 7th day, △, ...: at 14th day).

Table 2. Changes in 'relative' output current during continuous subcutaneous glucose monitoring with the MPC sensor for 14 days or the PVA sensor for 7 days in healthy subjects and diabetic patients.

Sensors	No. studied	'Relative' output current (%)					
		Days after the sensor implantation					
		1	3	5	7	10	14
PVA sensor	7	106.2 ± 2.3	97.2 ± 3.2	86.8 ± 3.8*	71.3 ± 4.2*	–	–
MPC sensor	10	104.0 ± 1.4	102.8 ± 1.8	100.6 ± 1.8	96.7 ± 3.9	88.6 ± 4.9*	80.4 ± 6.8*

The 'relative' sensor output while kept in subcutaneous tissue was determined by comparing the sensor output with the simultaneously measured blood glucose concentration. Blood samples were also obtained from anticubital vein before and 1 and 2 hr after each meal and at bed time. Blood glucose concentrations in whole blood samples were measured by the glucose oxidase method.
*: $P<0.01$, as compared to the 'relative' sensor output obtained at the first day.
Results are expressed as means±SEM.

times to reach the peak values after oral glucose loads and the nadir values after intravenous insulin injections were 4.6 ± 1.2 min and 6.9 ± 1.6 min, respectively. With this monitoring system, hypoglycemic ranges were detected (1.7–2.4, 1.8 ± 0.6 mmol/l) and notified by the sound of an alarm. Subcutaneous tissue glucose concentrations (Y) measured by the monitoring system were 3.3 ± 1.8% higher than blood glucose concentrations (X) ($Y = 1.04X + 0.12$, $r = 0.98$, $n = 240$ determinations in 5 healthy subjects). Similar in vivo sensor characteristics were obtained with the PVA sensor in short term experiments.

Subcutaneous tissue glucose monitoring with ferrocene-mediated needle-type glucose sensor covered with poly(MPC-co-BMA) membrane

Figure 6 shows a representative case of subcutaneous tissue glucose monitoring with the MPC sensor in a healthy subject for 14 days. The subcutaneous tissue glucose concentrations were measured precisely for 7 days without in vivo calibration. The apparent subcutaneous tissue glucose concentrations measured by the MPC sensor then decreased gradually in comparison to blood glucose concentrations. However, by introducing the in vivo calibration technique every morning after 7 days continuous monitoring, the apparent subcutaneous tissue glucose concentrations measured by the MPC sensor again correlated with the glycemic excursions for 14 days.

Changes in the 'relative' output currents during continuous monitoring with the PVA sensor and the MPC sensor in healthy subjects and diabetic patients are summarized in Table 2. As shown in Table 2, with the PVA sensor, the 'relative' output currents obtained at 3rd day of implantation were not significantly different from those obtained at 1st day of implantation. However, after 3 days, the 'relative' output currents decreased significantly to the levels of 86.8 ± 3.8% and 71.3 ± 4.2% of blood glucose concentrations at 5th and 7th day, respectively. On the other hand, with the MPC sensor, the 'relative' output currents were not significantly different until 7th day. After 7th day, the 'relative' output currents decreased gradually, but significantly to the levels of 88.6 ± 4.9% and 80.4 ± 6.8% at 10th and 14th day, respectively.

Figure 7 shows the linear regression analysis between apparent subcutaneous tissue glucose concentrations with the MPC and PVA sensors, and blood glucose concentrations during continuous monitoring. With the PVA sensors, the regression line obtained at 3rd day of implantation ($Y = 0.97X + 0.1$, $r = 0.979$) was not significantly different from that obtained at 1st day ($Y = 1.03X + 0.3$, $r = 0.985$). However, that obtained at 7th day ($Y = 0.72 + 0.3$, $r = 0.901$) was significantly different from that obtained at 1st day. On the other hand, with the MPC sensors, the regression line obtained at 7th day of implantation ($Y = 0.99 X + 0.1$, $r = 0.977$) was not significantly different from that obtained at 1st day of implantation ($Y = 1.04X + 0.2$, $r = 0.990$). The regression lines obtained at 10th and 14th day of implantation ($Y = 0.89X + 0.4$, $r = 0.953$ at 10th day and $Y = 0.80 X + 0.8$, $r = 0.902$ at 14th day) were significantly different from that obtained at 1st day. As shown in Table 3, however, by introducing the in vivo calibration technique every morning after 7 days continuous monitorings, the slopes of the regression lines obtained at 10th and 14th day (1.02 ± 0.03 and 0.99 ± 0.05) were not significantly different from

Table 3. Linear regression analyses between apparent subcutaneous tissue glucose concentrations measured by the MPC sensor and blood glucose concentrations determined by glucose oxidase method during continuous subcutaneous tissue glucose monitoring for 14 days.

Experimental design	Regression line	Days after implantation				
		1	3	7	10	14
(A) Subcutaneous tissue glucose monitoring without in vivo calibration	Slope	1.04 ± 0.01 (r=0.99)	1.04 ± 0.01 (r=0.98)	0.99 ± 0.02 (r=0.98)	0.89 ± 0.03* (r=0.95)	0.80 ± 0.05* (r=0.90*)
(B) Subcutaneous tissue glucose monitoring with in vivo calibration	Slope	—	—	—	1.02 ± 0.03 (r=0.95)	0.99 ± 0.05 (r=0.90*)

The overall regression lines between apparent subcutaneous glucose concentrations and blood glucose concentrations are calculated from the results obtained at 1st, 3rd, 7th, 10th and 14th day. Subcutaneous tissue glucose concentrations were monitored continuously with the MPC sensors for 14 days in 5 healthy subjects and 5 diabetic patients. After 7 days monitoring, the in vivo calibration procedure is introduced in each morning.
*: $p<0.01$, as compared to the results obtained at 1st day.
Results are expressed as means±SEM.

that obtained at 1st day (1.04 ± 0.01), but the results were significantly varied at 14th day.

When the results obtained during oral glucose loads, intravenous insulin injections and 7 days monitoring with the MPC sensors without in vivo calibration in all healthy subjects and diabetic patients studied were calculated together, a close relation ($Y = 1.02X - 0.20$, $r = 0.96$, $n = 740$ determinations in 5 healthy subjects and 5 diabetic patients) was found between subcutaneous tissue glucose concentrations and blood glucose concentrations.

Scanning electron microscopic examinations on the surface of MPC membrane revealed no protein fixation at all for up to 7 days and slight protein fixation at 14 days.

Discussion

The clinical application of wearable artificial endocrine pancreas remains an elusive goal that awaits the development of a reliable mean of blood glucose monitoring on a long-term basis. In 1982, Shichiri et al. reported the needle-type glucose sensor covered with PVA membrane, which worked for at most 4 days when the subcutaneous tissue glucose concentrations were monitored in diabetics [1]. During subcutaneous tissue glucose monitoring for 1 to 4 days, however, sensor output decreased with time. We have considered that major reasons for sensor decay with time when in vivo continuous monitoring is undertaken, might be due to the protein fixation on the membrane surface of the sensor, resulting in the insufficient availability of oxygen in glucose sensor inserted in subcutaneous tissue where oxygen tension is very low and easily changeable.

To solve the oxygen limitation problems, a ferrocene-mediated needle-type glucose sensor that does not require oxygen has been developed [7]. We demonstrated that the ferrocene-mediated glucose sensor possessed excellent sensor characteristics and showed minimal sensitivity to relevant oxygen concentrations. To prevent the protein adhesion on the membrane surface, then, we studied the biocompatibility of the needle-type glucose sensor covered with several types of membrane, such as segmented polyurethane, polyvinyl alcohol conjugating heparin, polyethylene oxide and alginate-polylysine-alginate membranes. However, in vitro and in vivo characteristics of these sensors covered with these membranes showed that none of these membranes were superior to PVA membrane [3, 12]. Recently, Reach's research group have reported the newly designed needle-type glucose sensor covered with polyurethane [13–15]. Moussy et al. have reported the needle-type glucose sensor covered with perfluorinated ionomer, Nafion [16, 17]. However, no investigators have succeeded the development of a stable, reliable and long-life glucose sensor for clinical applications.

We assumed that if an artificial surface similar to cell membrane was constructed by an arrangement of phospholipids from blood on the surface, the surface would show excellent biocompatibility. Phospholipids molecules have a self-organizing property with each

other and form a bilayer membrane structure. Therefore, polymers having phospholipid polar group are expected to have a strong affinity for phospholipid molecules. Ishihara et al. prepared a methacrylate with a phospholipid polar group in the side chain, 2-methacryloyloxyethyl phosphorylcholine (MPC) and its copolymers [18]. The poly(MPC-co-BMA) membrane could be used to construct a cell membrane-like structure by adsorbing phospholipids from blood or tissue fluids, and organizing them on the polymer surface. This phenomenon was indicated by X-ray photoelectron spectroscopic (XPS) and differential scanning calorimetry (DSC) analyses [18]. Thus, these hydrophilic phospholipids chains might have the potential for suppressing adsorption of biochemical molecules. Ishihara et al. evaluated its characteristics in vitro, such as permeability, thrombogenicity and protein adhesion on MPC copolymers [18, 19]. The poly(MPC-co-BMA) membrane was demonstrated to cut off the permeates by molecular weight of 5×10^4. They also found that the MPC moiety in poly(MPC-co-BMA) suppressed platelet adhesion and activation on the polymer surface, and prevented adsorption of serum proteins.

In this experiment, we also confirmed that comparing the results of PVA membrane, platelet aggregation on poly(MPC-co-BMA) membrane was significantly inhibited, and the amount of protein adsorbed on poly(MPC-co-BMA) membrane was also significantly lower than those on PVA membrane when polymer coated acrylic beads were incubated with BSA solution for 14 days. From these results, using MPC copolymers, Ishihara et al. are currently working to improve biocompatibility for such medical devices as a cellulose hemodialysis membrane [20, 21], and a polyolefin membrane for an oxygenator. The MPC copolymers were shown to inhibit thrombosis, resist protein adsorption, and allow solutes to permeate, making ideal for such devices. This kind of newly designed membrane might extend the longevity of the glucose sensor by inhibiting protein adsorption on the membrane surface and inactivating host-reaction of the implanted sensor. Therefore, we have developed the ferrocene-mediated needle-type glucose sensor covered with poly(MPC-co-BMA) membrane.

The ferrocene-mediated needle-type glucose sensor covered with poly(MPC-co-BMA) membrane had excellent sensor characteristics in vitro and in vivo. In vitro experiments, a linear relationship between glucose concentration and sensor output was observed in the range between 0.6 to 22.2 mmol/l. In vivo short-term experiments, the mean 'relative' output current was 103.3 ± 1.8. Response times to reach the peak values after oral glucose loads and the nadir values after intravenous insulin injections were 4.6 ± 1.2 min and 6.9 ± 1.6 min, respectively. Subcutaneous tissue glucose concentrations (Y) measured by the monitoring system were $3.3 \pm 1.8\%$ higher than blood glucose concentrations (X) ($Y = 1.04X + 0.12$, $r = 0.98$). With this sensor, hypoglycemia (1.7 – 2.4 mmol/l) observed during intravenous insulin injection test were precisely detected and notified by the sound of alarm.

With the MPC sensor, output currents were not significantly changed during incubation with 70 g/l BSA solutions for the period of 7 days, then gradually decreased to the level of $89.8 \pm 5.2\%$ of the initial value at 14th day. In vivo experiments, with the MPC sensor, the 'relative' output currents were not significantly different until 7th day. After 7th day, the 'relative' output currents decreased gradually, but significantly decreased to the levels of $80.4 \pm 6.8\%$ at 14th day. As compared to the PVA sensor, the MPC sensor showed a higher biocompatibility.

During continuous subcutaneous tissue glucose monitoring of blood glucose excursions, a highly significant correlation between subcutaneous tissue glucose concentration (Y), and blood glucose concentration (X) was observed (overall correlation during 7 days monitoring is $Y = 1.07X - 0.30$, $r = 0.96$). But the regression lines obtained at 10th and 14th day were significantly different from that obtained at 1st day, demonstrating that the apparent subcutaneous tissue glucose concentrations were significantly lower than blood glucose concentrations. However, by introducing the in vivo calibration technique every morning after 7 days continuous monitoring, subcutaneous tissue glucose concentrations measured by this sensor correlated again with the glycemic excursions for 14 days.

Scanning electron microscopic examinations on the surface of MPC membrane revealed no protein fixation at all for up to 7 days and slight protein fixation at 14 days. After removal of the glucose sensor, histological examination was done on the subcutaneous tissue surrounding the sensor in rats. In histological examinations at the sensor insertion site, after 7 days use, migration of leukocyte and slight fibrin deposition were recognized in both sensors covered with PVA and MPC membranes. With the PVA sensor, significant granulation tissues containing fibroblast, capillaries and chronic inflammatory cells were observed around sensor insertion site at 14th day. On the contrary, gran-

ulation tissue was slightly observed in the tissue around the MPC sensor. These histological changes of the subcutaneous tissue surrounding the sensor, probably due to the mechanical stimulation of the implanted sensor, might explain the finding that the sensor decay when the sensor was inserted into subcutaneous tissue was faster than that observed in in vitro experiments with BSA solutions.

Since GOD as heterogeneous protein produces allergic reaction to human being, the subcutaneous application of glucose sensor requires absolute prevention of enzyme leakage. We already confirmed no leakage of immobilized GOD through membrane covered on glucose sensor in in vitro incubation test and in vivo immunological test [22]. In dog experiments, it has been reported that 30 mg/kg of ferrocene for 6 months produced hemosiderosis, chronic iron deposition, but no other side effects were observed [23]. In the present experiments, very small amount of ferrocene carboxaldehyde (1 μg) were used, and we also confirmed no leakage of immobilized ferrocene carboxaldehyde in in vitro experiments. Acute and chronic toxicity experiments in vitro and in vivo on MPC polymers, including cytotoxicity, immune response and mutagenicity studies, showed no observable findings (unpublished data). Therefore, application of sensing system to human being has been considered to be safe.

In conclusion, poly(MPC-co-BMA) membrane had excellent biocompatibility. Then, using a ferrocene-mediated needle-type glucose sensor covered with poly(MPC-co-BMA) membrane, subcutaneous tissue glucose concentrations could be measured precisely and continuously for at least 7 days without in vivo calibration. Then, after day 7, with the introduction of in vivo calibration, subcutaneous tissue glucose concentration was able to be well monitored for 14 days. By replacing the sensor every 7th day, this sensor, therefore, can be applied for the self blood monitoring system and for the sensing system of wearable artificial endocrine pancreas in ambulatory diabetic patients for longer-periods.

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