Plasma-Assisted Immobilization of Heparin onto Low-Density Polyethylene Surface

Shin-ichi KONDO,*[,a] Yasuyo FUKUNAGA,[a] Michinori OIKAWA,[a] Yasushi SASAI,[a] and Masayuki KUZUYA[b]

[a] *Laboratory of Pharmaceutical Physical Chemistry, Gifu Pharmaceutical University; 5–6–1 Mitahora-Higashi, Gifu 502–8585, Japan: and* [b] *Department of Pharmaceutical Physical Chemistry, Faculty of Pharmaceutical Science, Matsuyama University; 4–2 Bunkyo-cho, Matsuyama, Ehime 790–8578, Japan.*

Received January 25, 2008; accepted April 14, 2008; published online April 15, 2008

In this study heparin was covalently immobilized onto LDPE-VEMAC sheet fabricated by the introduction of carboxyl groups to the surface of low-density polyethylene (LDPE) using a plasma technique. The plasma irradiation time influenced the density of carboxyl groups on the LDPE-VEMAC sheet. Heparin was immobilized on the LDPE-VEMAC sheet using a condensation reagent, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC). We confirmed the immobilization of heparin from the ATR-FT-IR spectrum of the sheet obtained. Where heparin was directly immobilized on the LDPE-VEMAC sheet, the density of the immobilized heparin depended on that of the carboxyl groups. Heparin was also immobilized with a spacer, hexamethylene diamine, and the density of such heparin was about 1.6 times that of the directly immobilized heparin. This result suggests that the introduction of a spacer may be an effective way to increase the density of immobilized heparin.

Key words immobilization; plasma technique; heparin; low-density polyethylene

Considerable interest has focused on the immobilization of biomolecules onto hydrophobic materials, due to their potential applications in biochips,[1—3] biosensors,[4,5] biocompatible materials[6—8] and so on.[9—11] Polymers are often used as hydrophobic materials to immobilize biomolecules, using covalent bonds, ionic bonds or physical adsorption. The polymer surface can be modified to introduce reactive groups for biomolecular immobilization by several methods, such as coating, plasma surface treatment and photo-irradiation.

We have recently reported a novel method to introduce durable surface hydrophilicity and minimize its decay with time on several hydrophobic polymers, such as polyethylene-naphthalate, polyethylene and nylon-12.[12—15] Figure 1 shows the outline of the fabrication of durable surface hydrophilicity. This method involves sorption of methylvinylether-maleic anhydride copolymer (VEMA), which is commercially known as a GANTREZ, into the surface layer and immobilization by a plasma-assisted cross-link reaction. Hydrolysis of VEMA follows, to generate hydrophilic carboxyl groups on the surface. Durable surface hydrophilicity introduced in this way has been confirmed both by the measurement of the water contact angle and by demonstration of the long-term stability of the surface lubricity on the urethane-made catheter. It is expected that reusable bio-chips may be fabricated by this polymeric material possessing durable hydrophilicity, since biomolecules can be strongly immobilized over a long period *via* surface carboxyl groups.

In a previous paper, we reported our preliminary work on the plasma-assisted immobilization of oligo-DNA on a low-density polyethylene (LDPE) surface.[16,17] LDPE was chosen because of its low cost, chemical and mechanical properties, and ready availability in various forms such as films, beads, nets, tubes, and sheets. To obtain a LDPE-VEMAC sheet, durable surface hydrophilicity was introduced onto the LDPE sheet according to the method shown in Fig. 1. 6-mer or 12-mer oligo-DNA, as model DNA, was immobilized on the LDPE-VEMAC sheet. The sheet thus obtained was able to hybridize with complementary oligo-DNA, but not with its single nucleotide polymorphism (SNP). Furthermore, it was shown that this LDPE-VEMAC sheet immobilizing the oligo-DNA was reusable.

We have studied the plasma-assisted immobilization of biomolecules onto hydrophobic polymers. Heparin was selected as a model biomolecule in the present study. Heparin is a complex polysaccharide composed of repeating disaccharides of uronic acid and glucosamine, and is a well-known biomolecule for many biological activities.[18—20] Some researchers have used ionic immobilization of heparin onto polymer surfaces possessing positive charges[21]; however, there has been a shift of focus to the covalent immobilization of heparin onto modified polymer surfaces due to more effective long-term retention.[22—27] In this paper, we report the covalent immobilization of heparin on a LDPE-VEMAC sheet, as confirmed by ATR-FT-IR spectrum measurement. We examine the effect of the plasma operational conditions for the preparation of the LDPE-VEMAC sheet on the density of immobilized heparin, and also study the ef-

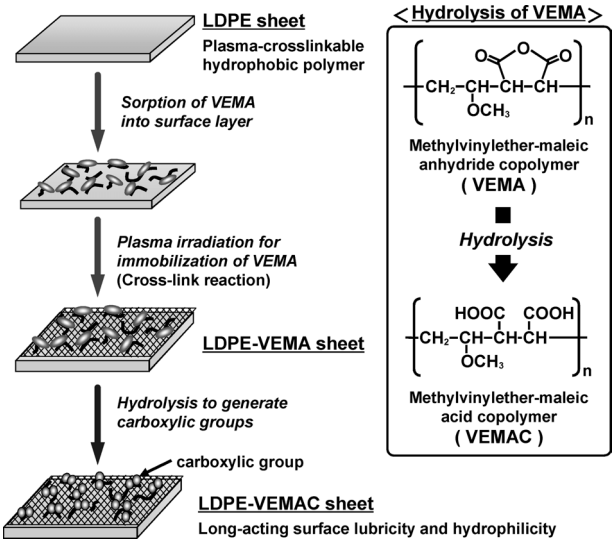

Fig. 1. Fabrication of Durable Surface Hydrophilicity onto a Hydrophobic Polymer Surface

* To whom correspondence should be addressed. e-mail: skondo@gifu-pu.ac.jp

fect of a spacer group inserted between heparin and the LDPE-VEMAC sheet.

Results and Discussion

Direct Immobilization of Heparin on the Surface of LDPE-VEMAC Sheet It is assumed that the density of heparin immobilized onto a LDPE-VEMAC sheet depends on that of the carboxyl groups on the sheet. Thus, it is important to gain insight on the density of the carboxyl groups on the LDPE-VEMAC sheet prepared by various plasma operational conditions. The density of the carboxyl groups on the LDPE-VEMAC sheet was evaluated from the uptake of Toluidine Blue O.[28] Figure 2 shows the progressive changes in the density of the carboxyl groups on the surface of the LDPE-VEMAC sheet against the plasma irradiation time. Although a small amount of Toluidine Blue O adsorbed on the surface of LDPE, it was significantly smaller than that on the LDPE-VEMAC sheet. The density of the carboxyl groups on the LDPE-VEMAC sheet continued to increase for *ca.* 30-s of plasma irradiation and then tended to decrease. This result is considered as follows. A shorter treatment time causes an insufficiency of crosslinking reactions, so that a small amount of VEMAC is immobilized on the surface of LDPE. On the other hand, a longer treatment time causes greater degradation of VEMAC (decarboxylation), resulting in a decrease in the surface density of carboxyl groups. The selection of operational conditions appears to be an important factor in the preparation of a LDPE-VEMAC sheet possessing suitable carboxyl group density.

It was shown that a LDPE-VEMAC sheet irradiated for 30 s possessed the maximum carboxyl group density within our experimental conditions. We directly immobilized heparin using a condensation reagent, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), onto the surface of a LDPE-VEMAC sheet irradiated for 30 s (Chart 1). The ATR-FT-IR spectrum of the resulting sheet, termed the LDPE-VEMAC-HE sheet, was measured to confirm the immobilization of heparin. Figure 3 shows the ATR-FT-IR spectrum of the LDPE-VEMAC-HE sheet together with spectra of the LDPE-VEMA sheet, the LDPE-VEMAC sheet and heparin.

The spectrum of the LDPE-VEMA sheet showed the peak at 1773 cm^{-1} with a shoulder peak (1730 cm^{-1}) in the 1600 to 1800 cm^{-1} region, whereas only the peak at 1730 cm^{-1} was observed in the LDPE-VEMAC sheet. The peaks at 1773 and 1730 cm^{-1} were ascribed to an acid anhydride and a carboxyl group, respectively.[16,17] Thus, it is suggested that acid anhydride groups are completely converted to carboxyl group in the LDPE-VEMAC sheet. On the other hand, the characteristic broad peak of heparin at 1600 cm^{-1} was observed in the spectrum of the LDPE-VEMAC-HE sheet, although there were no peaks at 1600 cm^{-1} in those of the LDPE-VEMA sheet and the LDPE-VEMAC sheet. It was also confirmed that the ATR-FT-IR spectrum of the LDPE-VEMAC-HE sheet remained almost the same after it was kept in water for one month. These results indicate that heparin can be covalently immobilized onto the surface of the LDPE-VEMAC sheet.

Effect of the Plasma Operational Conditions on the Density of Heparin Immobilized onto the Surface of the LDPE-VEMAC Sheet As described above, the density of the carboxyl groups on the LDPE-VEMAC sheet depended on the plasma operational conditions. It is assumed that the density of the carboxyl groups would influence the amount of

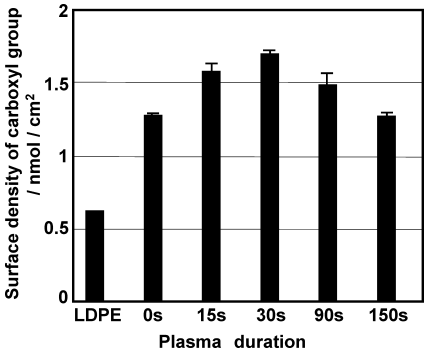

Fig. 2. Progressive Changes in the Density of a Carboxyl Group on a LDPE-VEMAC Sheet

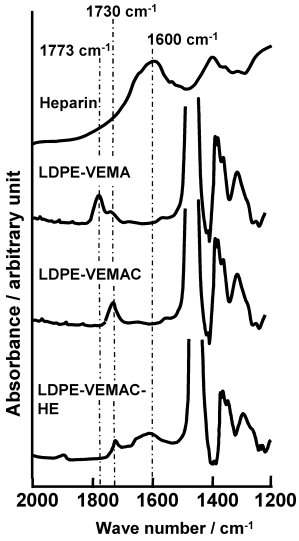

Fig. 3. ATR-FT-IR Spectra of LDPE-VEMAC-HE Sheet Together with a LDPE-VEMAC Sheet and Heparin

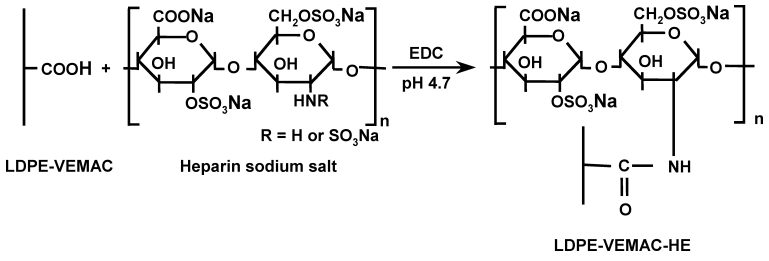

Chart 1

heparin immobilized on the LDPE-VEMAC sheet. Figure 4 shows the density of heparin immobilized onto the surface of LDPE-VEMAC sheets prepared by various plasma operational conditions, together with the carboxyl group density. It was shown that the density depended on that of the carboxyl group on the LDPE-VEMAC sheet and that the maximum value of the density was *ca.* 100 ng/cm^2 under our experimental conditions (Table 1). We noticed that the density of physically absorbed heparin—ionically immobilized heparin—was *ca.* 60 ng/cm^2 on a LDPE-VEMAC sheet plasma-irradiated for 30 s (Table 1). The amount of heparin physically adsorbed was significantly smaller than that of covalently immobilized heparin on the LDPE-VEMAC sheet plasma-irradiated for 30 s.

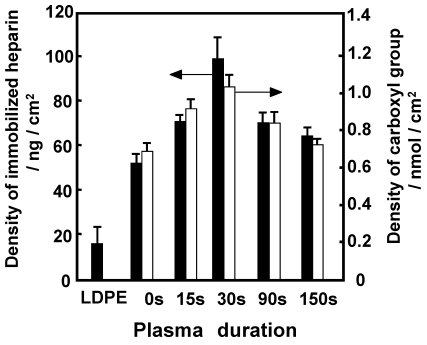

Fig. 4. Surface Density of Immobilized Heparin and a Carboxyl Group on LDPE-VEMAC Sheet

Immobilization of Heparin through a Spacer onto LDPE-VEMAC Sheet To increase the amount of immobilized heparin, we tried to immobilize heparin onto a LDPE-VEMAC sheet through a spacer. Hexamethylenediamine (HMDA) was used as a model spacer compound and was immobilized onto the LDPE-VEMAC sheet using EDC. The resulting sheet was termed the LDPE-VEMAC-HMDA sheet. Subsequently, heparin activated by EDC was reacted with the LDPE-VEMAC-HMDA sheet, resulting in the LDVE-VEMAC-HMDA- HE sheet (Chart 2).

The ATR-FT-IR spectrum of the sheet was measured to confirm the immobilization of heparin. Figure 5 shows the

Table 1. Density of Heparin Immobilized on Various LDPE Sheet (n=3)

	Absorbance at 633 nm	Density of immobilized heparin/ng/cm^2
Blank	0.418±0.001	
LDPE	0.415±0.002	14.6±9.7
LDPE-VEMAC[a)]	0.406±0.002	58.4±9.7
LDPE-VEMAC-He[a)]	0.397±0.002	102.2±9.9
LDPE-VEMAC-HMDA	0.407±0.001	53.5±4.9
LDPE-VEMAC-HMDA-HE	0.385±0.002	160.6±10.1

a) Plasma irradiation time; 30 s.

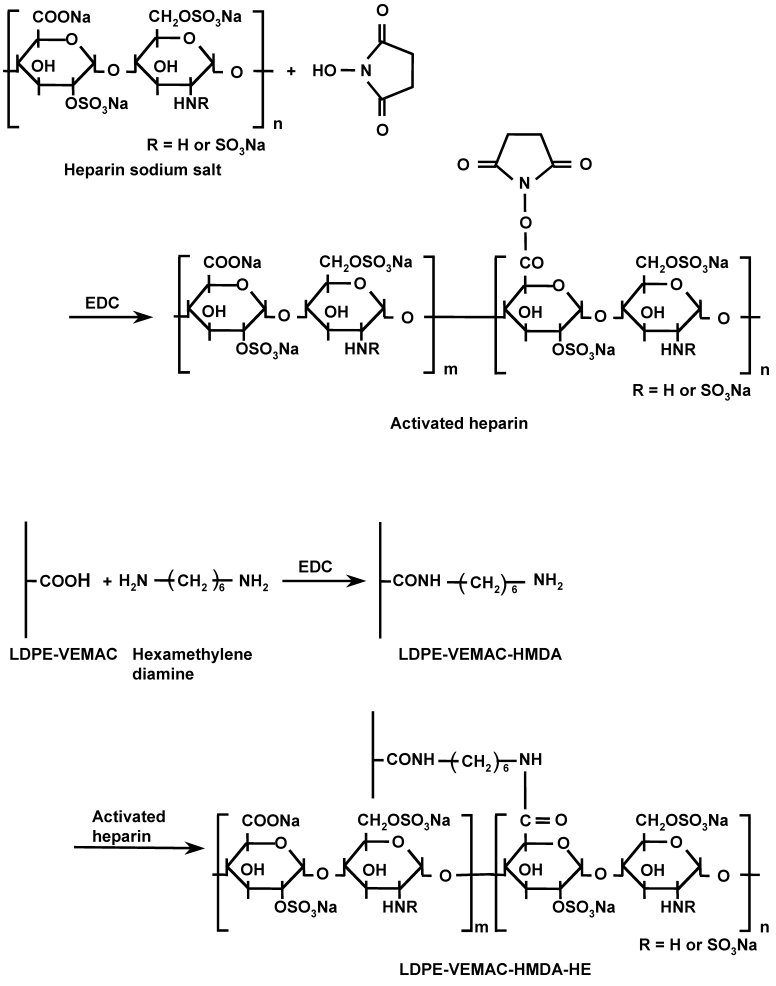

Chart 2

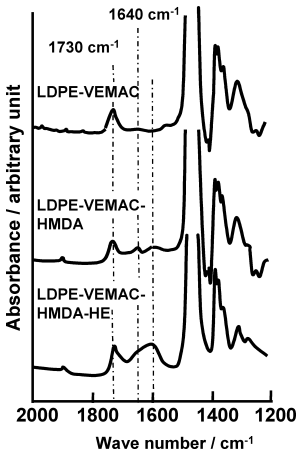

Fig. 5. ATR-FT-IR Spectra of a LDPE-VEMAC-HMDA-HE Sheet Together with LDPE-VEMAC and LDPE-VEMAC-HMDA Sheets

ATR-FT-IR spectrum of the LDPE-VEMAC-HMDA-HE sheet, together with spectra of the LDPE-VEMAC and LDPE-VEMAC-HMDA sheets. The characteristic peak of an amide group at 1640 cm^{-1} was observed in the spectrum of the LDPE-VEMAC-HMDA sheet, in contrast to that of the LDPE-VEMAC sheet. We estimated the density of immobilized HMDA at *ca.* 300 nmol/cm^2, to gain insight into the reactive ratio of carboxyl groups. It was shown that *ca.* 60% of carboxyl groups on the LDPE-VEMAC sheet reacted with HMDA. On the other hand, the characteristic broad peak of heparin at 1600 cm^{-1} was observed in the spectrum of the LDPE-VEMAC-HMDA-HE sheet. It was suggested that heparin was immobilized on the LDPE-VEMAC-HMDA sheet.

We also estimated the density of immobilized heparin on the LDPE-VEMAC-HMDA sheet. The density of immobilized heparin was *ca.* 160 ng/cm^2 on the LDPE-VEMAC-HMDA-HE sheet (Table 1). On the other hand, the density of heparin physically adsorbed on the LDPE-VEMAC-HMDA sheet was *ca.* 55 ng/cm^2 (Table 1). Thus, the density of covalently immobilized heparin was greater than that of physically adsorbed heparin, suggesting that a greater amount of heparin could be immobilized through a spacer onto a LDPE-VEMAC sheet.

Conclusion

The conclusions drawn from the present study can be summarized as follows. We have covalently immobilized heparin on a LDPE-VEMAC sheet. The density of heparin directly immobilized on the LDPE-VEMAC sheet depended on that of a carboxyl groups on the surface. It was also shown that the density of heparin immobilized with a spacer was about 1.6 times greater than that of heparin directly immobilized on the LDPE-VEMAC sheet. This result suggests that the introduction of a spacer may effectively increase the density of immobilized heparin. It is hoped that a more practical application for the immobilization of heparin will be developed from the work in progress.

Experimental

Low-density polyethylene (LDPE) sheets with an average thickness of 0.04 mm (Unipack E-4, Seinichi Co., Ltd.), maleic anhydride–methylvinylether copolymer (VEMA) (GANTREZ-AN 139, Mw=41000, GAF Co. Ltd.), and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) (Sigma) are commercially available and were used without purification. Heparin sodium salt was purchased from Nacalai Tesque. Sodium chloride–sodium citrate (SSC) buffer solution (2×) was prepared by diluting SSC buffer stock solution (20×) (Nacalai Tesque) with distilled water. Phosphate buffered saline (PBS) was prepared by diluting PBS (10×) (Nacalai Tesque) with water. Other reagents and solvents were of the best grade commercially available.

Preparation of a LDPE-VEMAC Sheet A LDPE-VEMAC sheet was prepared according to the method previously reported.[12—17] A LDPE sheet (10 mm×30 mm) was soaked in cyclohexanone solution containing 2% VEMA and 5% *p*-xylene for 6 h at 60 °C to deposit VEMA into the LDPE surface layer. This sheet was dried *in vacuo* overnight. The LDPE sheet was then soaked in tetrahydrofuran (THF) for 10 s to remove excess VEMA. After drying, the treated LDPE was subjected to Ar plasma-irradiation to immobilize VEMA onto the LDPE surface layer. The plasma state was generated by radio frequency discharge of inductive coupling with a five loop antenna at 13.56 MHz and a 20 W power supply. The flow volume (50 ml/min) and pressure (0.5 Torr) of argon gas were controlled by changing the evacuating speed. The sample sheet was placed on a glass-tripod in a reaction chamber (230 mm long, 45 mm ϕ in diameter) to ensure homogeneous exposure to plasma gas.

After plasma-irradiation, the hydrolysis of maleic anhydride linkage in VEMA was conducted by immersing the LDPE sheet in 1 mol/l NaOH aqueous solution. The hydrolyzed LDPE-VEMAC sheet was soaked in 1 mol/l HCl at room temperature, thoroughly washed with water, and then dried.

Determination of Surface Density of Carboxyl Groups on a LDPE-VEMAC Sheet Surface densities of carboxyl groups were evaluated from the uptake of basic dyes according to the literature.[28] Carboxyl groups on the LDPE-VEMAC sheet were complexed with 5×10^{-4} mol/l Toluidine Blue O of pH 10 at 30 °C for 5 h. Non-complexed dye was removed with 1×10^{-4} mol/l NaOH and then desorption of the dye molecules complexed to the carboxyl groups on the sheet was conducted with 50% acetic acid solution. Absorbance at 633 nm was used for colorimetry.

Immobilization of Heparin on the Surface of LDPE-VEMAC Sheet (LDPE-VEMAC-HE Sheet) LDPE-VEMAC sheet was soaked into 5 ml of 0.01 mol/l sodium citrate–HCl buffer (pH 4.7) containing 100 mg of EDC at room temperature for 2 h. After then 25% heparin solution (500 μl) was added to the reaction mixture, and this mixture was kept at room temperature for 20 h. This sheet was washed with 0.5 mol/l NaCl solution (pH 7.5) and 0.01 mol/l sodium citrate–HCl buffer (pH 4.7) to obtain LDPE-VEMAC-HE sheet.

Determination of Surface Density of Heparin Immobilized on LDPE-VEMAC Sheet[29] Surface density of immobilized heparin was estimated essentially according to the method reported by Smith *et al.*[29] A standard curve representing a concentration range of approximately 10—70 μg of heparin was obtained as follows: the standard heparin solution was prepared by dissolving heparin (16.6 mg) in 0.2% NaCl solution (100 ml). In several tubes, varying amount of the standard heparin solution and 2.5 ml of 0.005% Toluidine Blue O solution were added. Each tube was then diluted with 0.2% NaCl to a total volume of 5 ml and agitated by a Vortex mixer for 30 s. Hexane (5 ml) was added to each tube and the tubes were shaken vigorously for another 30 s to separate the heparin-dye complex formed. The aqueous layers of all the tubes were sampled and diluted 1 : 10 with absolute ethanol. The absorbance at 633 nm was then read within 30 min.

The 6 sheets of LDPE-VEMAC-HE was soaked into the mixture of 0.005% Toluidine Blue O solution (2.5 ml) and 0.2% NaCl solution (2.5 ml). The absorbance of the solution was measured at 633 nm and compared with standard heparin solutions. The amount of Toluidine Blue O adsorbed on the LDPE-VEMAC-HE sheet was estimated.

Immobilization of Hexamethylene Diamine on the Surface of a LDPE-VEMAC Sheet (LDPE-VEMAC-HMDA Sheet) Three sheets of LDPE-VEMAC were soaked in a mixture of water (5 ml) and 0.25 mol/l EDC solution (1 ml) at 30 °C for 2 h. After adding 0.25 mol/l hexamethylene diamine (HMDA) solution (1 ml), these sheets were immersed in the solution at 30 °C for 20 h. These sheets were then washed with water to obtain a LDPE-VEMAC-HMDA sheet.

Determination of Surface Density of HMDA Immobilized on a LDPE-VEMAC Sheet The surface density of HMDA immobilized on a LDPE-VEMAC sheet was estimated from the unreacted carboxyl groups on the sheet, which were determined from the uptake of Toluidine Blue O, as described above.

Immobilization of Heparin on the Surface of a LDPE-VEMAC-HMDA Sheet (LDPE-VEMAC-HMDA-HE Sheet) Various concentrations of EDC solution (25 μl) were added to a 25% heparin sodium salt solu-

tion (500 μl) at room temperature. After 1 h, 0.25 mol/l *N*-hydroxy succinimide solution (25 μl) was added to the reaction mixture. This solution was kept at 30 °C for 20 h to obtain activated heparin solution and was used without further purification. A LDPE-VEMAC-HMDA sheet was soaked in a mixture of activated heparin solution (50 μl) and water (5 ml) at 30 °C for 24 h. It was then washed with water to obtain a LDPE-VEMAC-HMDA-HE sheet. The amount of heparin immobilized on the LDPE-VEMAC-HMDA-HE sheet was estimated according to the method described for the LDPE-VEMAC-HE sheet.

Acknowledgements This work was supported in part by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (Grant No. 17590036), which is gratefully acknowledged.

References

1) Brown P. O., Botstein D., *Nat. Genet.*, **21**, 33—37 (1999).
2) Lipshutz R. J., Fodor S. P. A., Gingeras T. R., Lockhart D. J., *Nat. Genet.*, **21**, 20—24 (1999).
3) Muguruma H., Hiratsuka A., Karube I., *Anal. Chem.*, **72**, 2671—2675 (2000).
4) Schuck P., *Annu. Rev. Biophys. Biomol. Struct.*, **26**, 541—566 (1997).
5) Consnier S., *Biosens. Bioelectron.*, **14**, 443—456 (1999).
6) Ratner B. D., Horbett T. A., Hoffman A. S., Hauschka S. D., *J. Biomed. Mater. Res.*, **9**, 407—415 (1975).
7) Tsuruta T., *Adv. Polym. Sci.*, **126**, 1—51 (1996).
8) Tanzi M. C., *Expert Rev. Med. Devices*, **2**, 473—492 (2005).
9) Nandivada H., Chen H. Y., Bondarenko L., Lahann J., *Angew. Chem. Int. Ed.*, **45**, 3360—3363 (2006).
10) Hwang I., Baek K., Jung M., Kim Y., Park K. M., Lee D. W., Selvapalam N., Kim K., *J. Am. Chem. Soc.*, **129**, 4170—4171 (2007).
11) Narita M., Hoshino F., Mouri M., Tsuchimori M., Ikawa T., Watanabe O., *Macromolecules*, **40**, 623—629 (2007).
12) Kuzuya M., Yamashiro T., *J. Photopolym. Sci. Technol.*, **8**, 381—384 (1995).
13) Kuzuya M., Matsuno Y., Yamashiro T., Tuiki M., *Plasmas and Polymers*, **2**, 79—89 (1997).
14) Kuzuya M., Yamashiro T., Kondo S., Tuiki M., *Plasmas and Polymers*, **2**, 133—142 (1997).
15) Kuzuya M., Sawa T., Yamashiro T., Kondo S., Takai O., *J. Photopolym. Sci. Technol.*, **14**, 87—90 (2001).
16) Kondo S., Sawa T., Kuzuya M., *J. Photopolym. Sci. Technol.*, **16**, 71—74 (2003).
17) Kondo S., Sasai Y., Kuzuya M., *Thin Solid Films*, **515**, 4136—4140 (2007).
18) Akashi M., Sakamoto N., Suzuki K., Kishida A., *Bioconjug. Chem.*, **7**, 393—395 (1996).
19) Zhou D., Too C. O., Wallace G. G., *React. Funct. Polym.*, **39**, 19—26 (1999).
20) Xu G. C., Hibino Y., Suzuki Y., Tanihara M., Imanishi Y., Awazu K., *J. Biomater. Sci. Polym. Ed.*, **12**, 503—514 (2001).
21) Ito Y., Sisido M., Imanishi Y., *J. Biomed. Mater. Res.*, **20**, 1017—1033 (1986).
22) Liu L. S., Ito Y., Imanishi Y., *Biomaterials*, **12**, 390—396 (1991).
23) Byun Y., Jacobs H. A., Kim S. W., *J. Biomater. Sci. Polym. Ed.*, **6**, 1—13 (1994).
24) Wirsen A., Ohrlander M., Albertsson A. C., *Biomaterials*, **17**, 1881—1889 (1996).
25) Marconi W., Benvenuti F., Piozzi A., *Biomaterials*, **18**, 885—890 (1997).
26) Kim Y. J., Kang I. K., Huh M. W., Yoon S. C., *Biomaterials*, **21**, 121—130 (2000).
27) Li Y., Neoh K. G., Cen L., Kang E. T., *Biotechnol. Bioeng.*, **3**, 305—313 (2003).
28) Sano S., Kato K., Ikada Y., *Biomaterials*, **14**, 817—822 (1993).
29) Smith P. K., Mallia A. K., Hermanson G. T., *Anal. Biochem.*, **109**, 466—473 (1980).