Table 1
Sequence of DNA for cell surface modification.

	5′–3′
polyA20	HS – AAA AAA AAA AAA AAA AAA AA
polyT20	HS – TTT TTT TTT TTT TTT TTT TT
SeqA	HS – TGC GGA TAA CAA TTT CAC ACA
SeqA′	HS – TGT GTG AAA TTG TTA TCC GCA
SeqB	HS – TAG TAT TCA ACA TTT CCG TGT
SeqB′	HS – ACA CGG AAA TGT TGA ATA CTA

glycerol-3-phosphatidylethanolamine (DPPE) was from NOF Corporation (Tokyo, Japan). Dichloromethane, triethylamine, and diethyl ether was from Nacalai Tesque (Kyoto, Japan). Hanks' balanced salt solution (HBSS), minimum essential medium (MEM), and RPMI-1640 medium were from Invitrogen Co. (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Equitech-Bio, Inc. (TX, USA), and phosphate-buffered saline (PBS) was from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). PKH67 Green Fluorescent Cell Linker Kit (PKH green) and PKH26 Red Fluorescent Cell Linker Kit (PKH red) were from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). *n*-Hexadecyl mercaptan was from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Glass plates (22 mm × 26 mm; thickness: 0.12–0.17 mm) were from Matsunami Glass Ind., Ltd (Osaka, Japan). Dithiothreitol (DTT) was from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

2.2. Synthesis of DNA-conjugated PEG-phospholipid (polyDNA–PEG-lipid)

Mal-PEG-lipid was synthesized by combining NHS-PEG-Mal (180 mg), triethylamine (50 μL), and DPPE (20 mg) with dichloromethane and stirring for 36 h at room temperature (RT) [14]. After precipitation with diethyl ether, Mal-PEG-lipid was obtained as a white powder (190 mg, 80% yield). ^{1}H NMR analysis ($CDCl_3$, 400 MHz, δ ppm): 0.88 (t, 6H, $-CH_3$), 1.25 (br, 56H, $-CH_2-$) 3.64 (br, 480H, PEG), 6.71 (s, 2H, –HC=CH–, maleimide).

The DNA sequences used in this study are listed in Table 1. DNA was synthesized by Sigma–Aldrich Chemical Co. DNA-SH was prepared by reduction of the disulfide bond with DTT according to the manufacturer's instructions. A PBS solution of DNA-SH (1.0 mg) was mixed with Mal-PEG-lipid (5.0 mg) in PBS for 24 h at RT to prepare polyDNA–PEG-lipid. PolyDNA–PEG-lipid (500 μg/mL in PBS) was used for surface modification of cells without purification.

2.3. Cell cultures

Two cell lines, CCRF-CEM cells (a human T cell lymphoblast-like cell line) and HEK293 cells (a human embryonic kidney cell line) were obtained from the Health Science Research Resources Bank (Osaka, Japan). Suspension culture of CCRF-CEM cells was performed in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Invitrogen) at 37 °C under 5% CO_2. HEK293 cells that stably expressed enhanced green fluorescence protein (EGFP) (GFP-HEK) were the kind gift of Dr. K. Kato (Institute for Frontier Medical Sciences, Kyoto University). The GFP-HEK cells were maintained in MEM supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

2.4. Surface modification of cells with polyDNA–PEG-lipid and co-incubation of differentially modified cells

For visualization under a fluorescence microscope, CCRF-CEM cells were labeled with PKH red or PKH green according to the manufacturer's instructions. To exchange the culture medium, CCRF-CEM or GFP-HEK cells (4×10^6 cells) were washed twice with HBSS and collected by centrifugation (180g, 5 min, 25 °C). After the addition of polyDNA–PEG-lipid solution (50 μL, 500 μg/mL in PBS) to the cell suspension, cells were incubated for 30 min at RT with gentle agitation. The cells were then suspended in 10 mL HBSS, collected by centrifugation (180g, 5 min, 25 °C), washed with another 10 mL HBSS, and re-centrifuged to obtain polyDNA–PEG-lipid-modified cells.

After cells were treated with polyA–PEG-lipid or polyT–PEG-lipid, the polyDNA–PEG-lipid-modified cells were mixed together in culture medium with the following ratios of polyA-cells:polyT-cells: 10:1, 4:1, 2:1, and 1:1. The cells were incubated with rotation at 100 rpm for 1 h at RT, followed by incubation at 37 °C under 5% CO_2. The cells were observed over time using a confocal laser scanning microscope (FLUOVIEW FV500, Olympus, Tokyo, Japan) and a phase-contrast microscope (IX7, Olympus Optical Co. Ltd., Tokyo, Japan).

2.5. Immobilization of polyDNA–PEG-lipid modified cells to patterned substrates

SeqA-conjugated PEG-lipid and SeqB-conjugated PEG-lipid were used for cell surface modification. For testing immobilization of the modified cells, substrate surfaces were modified using SeqA′ and SeqB′, the sequences complementary to SeqA and SeqB.

Glass plates were cleaned with a piranha solution (7:3 mixture of concentrated sulfuric acid and 30% hydrogen peroxide solution), washed 3× with Milli-Q water, and stored in a 2-propanol solution. For experiments, glass plates were mounted on a rotation stage in a metal vapor deposition apparatus (V-KS200, Osaka Vacuum Instruments, Osaka, Japan). A 1.0-nm chromium layer was deposited on the glass, followed by deposition of a 19-nm gold layer. The resulting glass plates coated with a thin layer of gold were immersed in an ethanol solution of *n*-hexadecyl mercaptan (1 mM) to produce a surface with SAM-carrying methyl groups (CH_3-SAM). The CH_3-SAM surface was irradiated with an ultraviolet (UV) light at 180 mW/cm^2 using an Optical ModuleX (SX-UI 501HQ, Ushio, Inc., Tokyo) equipped with a super-high-pressure mercury lamp (Ushio, Inc.) through a photomask with an array of transparent 1- or 2-mm circular dots in ambient air for 4 h. The plates were washed with ethanol to remove photodegradation products. A PBS solution of DNA-SH (600 μg/mL, SeqA′ and SeqB′), was applied to the UV-irradiated spots by manual pipetting and allowed to incubate for 2 h at RT. The substrate-coated glass plate was washed with HBSS before use.

In the first series of experiments, SeqA-PEG-lipid modified CCRF-CEM cells (SeqA-PEG-cells) and SeqB-PEG-lipid modified CCRF-CEM cells (SeqB-PEG-cells) were mixed at the following ratios: 4:1, 2:1, 1:1, 2:1, and 4:1. The cell suspensions were applied to UV-irradiated spots that had been incubated with a 1:1 mixture of SeqA′ and SeqB′ (see above); cells were incubated on the immobilized-DNA surface for 10 min at RT. In a second series of experiments, the UV-irradiated spots were incubated with SeqA′:SeqB′ at the following molar ratios: 4:1, 2:1, 1:1, 2:1, and 4:1. A 1:1 mixture of SeqA-PEG-cells and SeqB-PEG-cells was then applied to the UV-irradiated spots containing immobilized DNA. After washing with HBSS, cells attached to the substrate were observed using an upright fluorescence microscope (BX51, Olympus, Tokyo, Japan) and a stereomicroscope (MZF LIII, Leica, Solms, Germany). The number of attached cells was analyzed using ImageJ software (NIH, Bethesda, MD, USA).

An inhibition assay was also performed using a solution of SeqA′ (200 μg/mL) that was added to the mixture of SeqA-PEG-cells and SeqB-PEG-cells. After incubation for 30 min, the mixture was applied to the SeqA′ and SeqB′-immobilized substrate and incubated for 10 min at RT. After washing with HBSS, the cells attached to the substrate were observed using an upright fluorescence microscope.

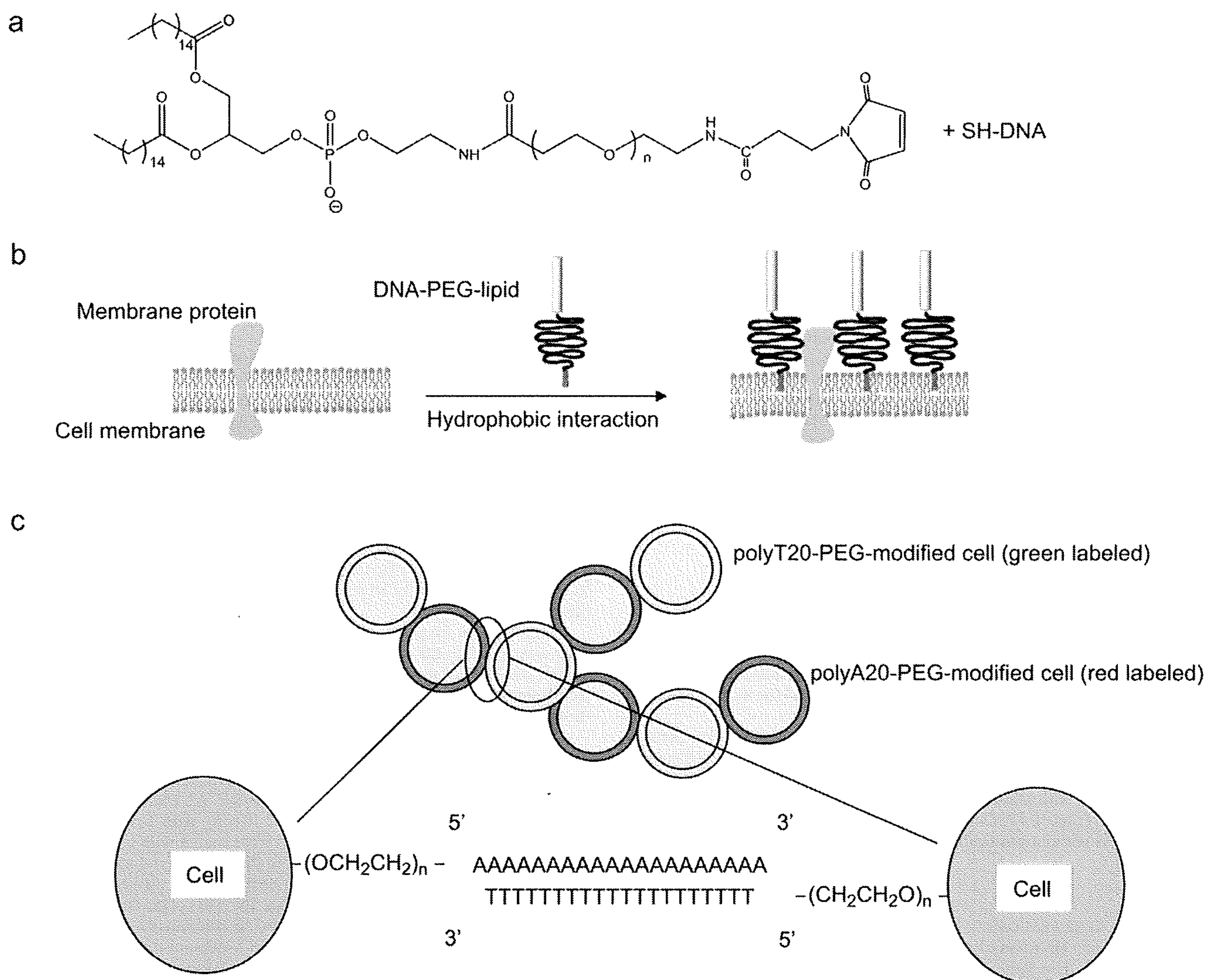
Substrates for cell attachment were also prepared using a contact printing technique. Poly(dimethylsiloxane) (PDMS) stamps were prepared as follows: A ledge pattern was fabricated on a PDMS surface using a laser beam machine (VLS2.30, Universal Laser Systems, Inc., Scottsdale, AZ, USA): The pattern consisted of unidirectional ledges (1 mm × 1 mm × 10 mm) with 1-mm intervals between ledges. The ledge surfaces on the stamps were coated with a solution of SeqA′ or SeqB′ DNA-SH (600 μg/mL) and applied to the gold-layered glass plates. A second stamp coated with a solution of SeqA′ or SeqB′ DNA-SH was applied to the surface perpendicular to the previous ledge design. The glass plate sat at RT for 2 h to dry. The glass plate was then immersed in an ethanol solution of *n*-hexadecyl mercaptan for blocking with CH_3-SAM and washed with ethanol and Milli-Q water. A 1:1 mixture of SeqA-PEG-cells and SeqB-PEG-cells were applied onto the patterned substrate and incubated for 10 min at RT with gentle agitation. After washing with HBSS, cells attached to the glass plate were observed using an upright fluorescence microscope.

3. Results

3.1. Intercellular attachment through hybridization of complementary polyDNA–PEG-lipid conjugates

Scheme 1 shows how cells carrying complementary polyDNA–PEG-lipid conjugates were tested for intracellular attachment. polyDNA–PEG-lipids were synthesized using a thiol/maleimide reaction between Mal-PEG-lipid and DNA-SH in which the SH group was introduced at the 5′-end of the DNA sequence. The DNA sequences used in this study are listed in Table 1. polyDNA–PEG-lipids carrying complementary sequences were prepared: polyA20 and polyT20, SeqA and SeqA′, SeqB and SeqB′. Our previous studies demonstrated that amphiphilic PEG-lipids are spontaneously incorporated into the cell membrane's lipid bilayer through hydrophobic interactions and that this incorporation has no cytotoxic effects [13–16,18,19]. We further showed that polyDNA could be introduced onto the cell surface using a PEG-lipid (Scheme 1b). The strategy in the present study was to mediate cell–cell interactions by hybridization between complementary DNA sequences that were incorporated into the cells' outer membranes (Scheme 1c).

Incorporation of polyA20–PEG-lipid into the cell membrane and its ability to hybridize with FITC-labeled polyT20 was examined



Scheme 1. (a) Synthesis of DNA-conjugated PEG-DPPE (polyDNA–PEG-lipid) from maleimide-PEG-lipid and DNA-SH. (b) Schematic illustration of the interaction between polyDNA–PEG-lipid and the lipid bilayer comprising the outer cell membrane. The polyDNA–PEG-lipid inserts into the cell membrane due to hydrophobic interactions between the acyl chain and the lipid bilayer. (c) Schematic illustration of cell–cell attachment through DNA hybridization between complementary polyDNA–PEG-lipids incorporated into the outer cell membranes.

first. A solution of polyA20–PEG-lipid was added to CCRF-CEM cells; after incubation, the cells were washed to remove unincorporated lipid, FITC-labeled polyT20 was added, and cells were observed using a confocal laser scanning microscope. As shown in Fig. 1a the FITC fluorescence was observed at the periphery of all cells, indicating that polyA20–PEG-lipids were incorporated into the outer cell membrane and that FITC-labeled polyT20 hybridized with the incorporated polyA20 DNA. When FITC-labeled polyA20 was added to polyA20–PEG-lipid modified cells, no fluorescence was observed on the cells. These results indicated that FITC-labeled polyT20 hybridized specifically with polyA20–PEG-lipids on the cell surface.

Intercellular attachments could also be mediated by hybridization between polyA20 and polyT20, as shown in Fig. 1c. CCRF-CEM cells labeled with PKH red were treated with polyA20–PEG-lipids (polyA20–PEG cells) and CCRF-CEM cells labeled with PKH green were treated with polyT20–PEG-lipids (polyT20–PEG-cells). Red polyA20–PEG-cells and green polyT20–PEG-cells were mixed at ratio of 1:1 and observed over time by a confocal laser scanning microscope (Fig. 1c). At 15 min after mixing, polyA20–PEG cells (red) and polyT20–PEG-cells (green) were attached to each other, with several cells attached in a linear fashion. At 60 min, even more cells had attached to each other. At 3 h, the linear cell aggregates had gathered to form clumps of cells. At 6 h, the cellular clumps were still present in the culture medium. As a control experiment, PKH red- and PKH green-labeled cells with no polyDNA–PEG-lipid treatment were mixed. These cells showed no attachment to each other (Fig. 1e). In addition, there was no self attachment between polyT20–PEG-cells. These results clearly showed that the attachment of different cells could be induced by hybridization between polyA20 DNA and polyT20 DNA on the cell surfaces. The ratio of the number of attachments between polyA20–PEG-cells and polyT20–PEG-cells to the total number of attachments for all cells was approximately 1 at 15 and 60 min of incubation, indicating the alternating attachment of polyA20–PEG-cells and polyT20–PEG-cells. At 3 h, the ratio had decreased to approximately 0.6, indicating that larger aggregates of cells had formed. Cell–cell attachments could also be induced between polyA20–PEG-lipid modified CCRF-CEM cells (red) and polyT20–PEG-lipid modified GFP-HEK cells (green), as seen in Fig. 1d). In contrast, no cell–cell attachments

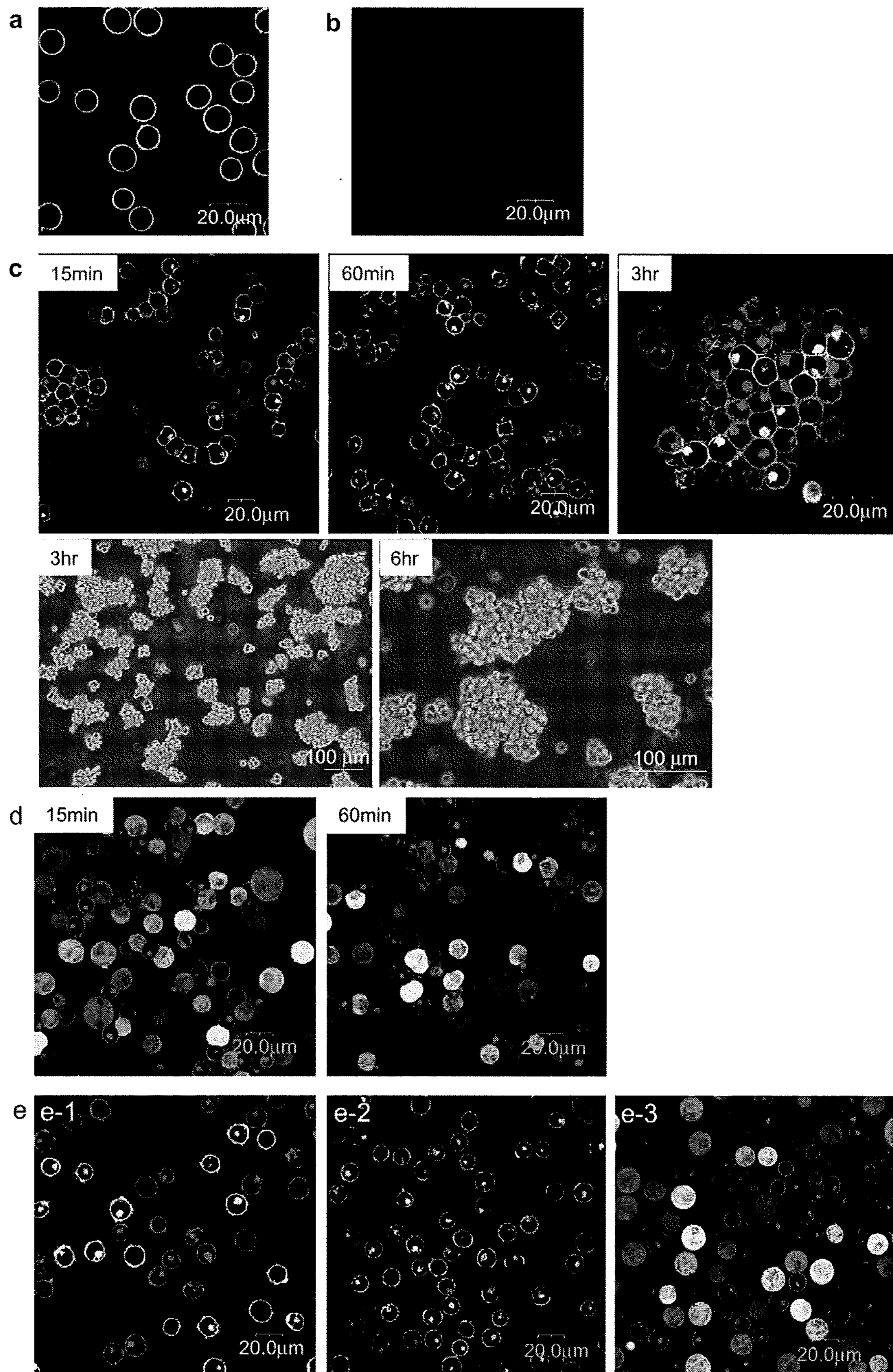


Fig. 1. Cell–cell attachment via DNA hybridization between complementary polyDNA–PEG-lipids on cell surfaces. CCRF-CEM cells incorporated polyA20–PEG-lipid into the outer cell membranes. Cells were observed by a confocal laser scanning microscope after polyA20–PEG-lipid modified CCRF-CEM cells were further treated with (a): FITC-labeled polyT20 and (b): FITC-labeled polyA20. (c): Cell–cell attachment between polyA20–PEG-lipid modified CCRF-CEM cells labeled with PKH red and polyT20–PEG-lipid modified CCRF-CEM cells labeled with PKH green in culture medium (cells were mixed in a 1:1 ratio). Cells were observed over time using a confocal laser scanning microscope and a phase-contrast microscope. (d): Cell–cell attachment between polyA20–PEG-lipid modified CCRF-CEM cells and polyT20–PEG-lipid modified GFP-HEK293 cells (cells were mixed in a 1:1 ratio). (e): Control experiments for cell–cell attachment by surface modification with polyDNA–PEG-lipid. (e–1): A mixture of CCRF-CEM cells labeled with PKH red and CCRF-CEM cells labeled with PKH green (no polyDNA–PEG-lipid modification). (e–2): PolyT20–PEG-lipid modified cells. (e–3): A mixture of CCRF-CEM cells labeled with PKH green and GFP-HEK293 cells after rotation culture at 100 rpm (no polyDNA–PEG-lipid modification).

were observed between CCRF-CEM cells and GFP-HEK cells without polyDNA–PEG-lipid modification (Fig. 1e). Thus, this method can be used to promote attachments between different kinds of cells.

3.2. Attachment of polyDNA–PEG-cells to complementary DNA immobilized on a solid substrate

Glass plates with a thin layer of gold were modified with CH_3-SAM and irradiated with UV light through a photomask with an array of 1- or 2-mm transparent circular dots. After washing the plates to remove photodegradation products, a solution containing DNA-SH was spotted on the dots in order to immobilize DNA via the Au/thiol reaction (Fig. 2a). PolyT20–PEG-cells labeled with PKH green were placed on the 2-mm spots where polyA20 molecules were immobilized and incubated for 10 min. After removal of unattached cells by washing with HBSS, the surface was observed using an upright fluorescence microscope. As shown in Fig. 2b, polyT20–PEG-cells attached to the polyA20-immobilized spot. Fig. 2c shows attachment of polyT20–PEG-cells onto a substrate with polyA20-SH and polyT20-SH spots. After polyT20–PEG-cells labeled with PKH green were applied and incubated for 10 min, and unattached cells were washed off with HBSS, the substrate was observed using a stereomicroscope (Fig. 2c). PolyT20–PEG-cells selectively attached to the polyA20-immobilized spots, with practically no attachment of cells to the polyT20-immobilized spots (dotted lines). These results showed that cells attached to the substrate through hybridization of DNA on the cell surface and on the substrate.

Next, a similar array of spots with immobilized SeqA′, SeqB′, and a 1:1 mixture of SeqA′:SeqB′ were prepared. A 1:1 suspension of SeqA-PEG-cells labeled with PKH red and SeqB-PEG-cells labeled with PKH green was incubated on the spots for 10 min. After removal of unattached cells with HBSS, the surface was observed using an upright fluorescence microscope. Fig. 2d shows SeqA-PEG-cells and SeqB-PEG-cells attached to SeqA′ and SeqB′-immobilized spots, respectively, and both SeqA-PEG-cells and SeqB-PEG-cells attached to spots where a mixture of SeqA′ and SeqB′ was immobilized. To test whether this interaction could be inhibited, SeqA′ was added to the mixture of SeqA-PEG-cells and SeqB-PEG-cells and the attachment of the cells to the substrate was examined. With the addition of SeqA′, there was no attachment of SeqA-PEG-cells to the SeqA′ spots, although SeqB-PEG-cells still attached to SeqB′ spots (Fig. 2e). This inhibition assay indicated that cells were specifically attaching to the immobilized DNA via complementary DNA hybridization.

The effects on cell binding to different ratios of immobilized SeqA′ and SeqB′ on the substrate spots were examined. Five spots of immobilized DNA were prepared using the following molar ratios of SeqA′:SeqB′: 4:1, 2:1, 1:1, 1:2, 1:4. A 1:1 mixture of SeqA-PEG-cells labeled with PKH red and SeqB-PEG-cells labeled with PKH green was incubated on the spots, and unattached cells were removed by washing with HBSS. The substrate was observed using

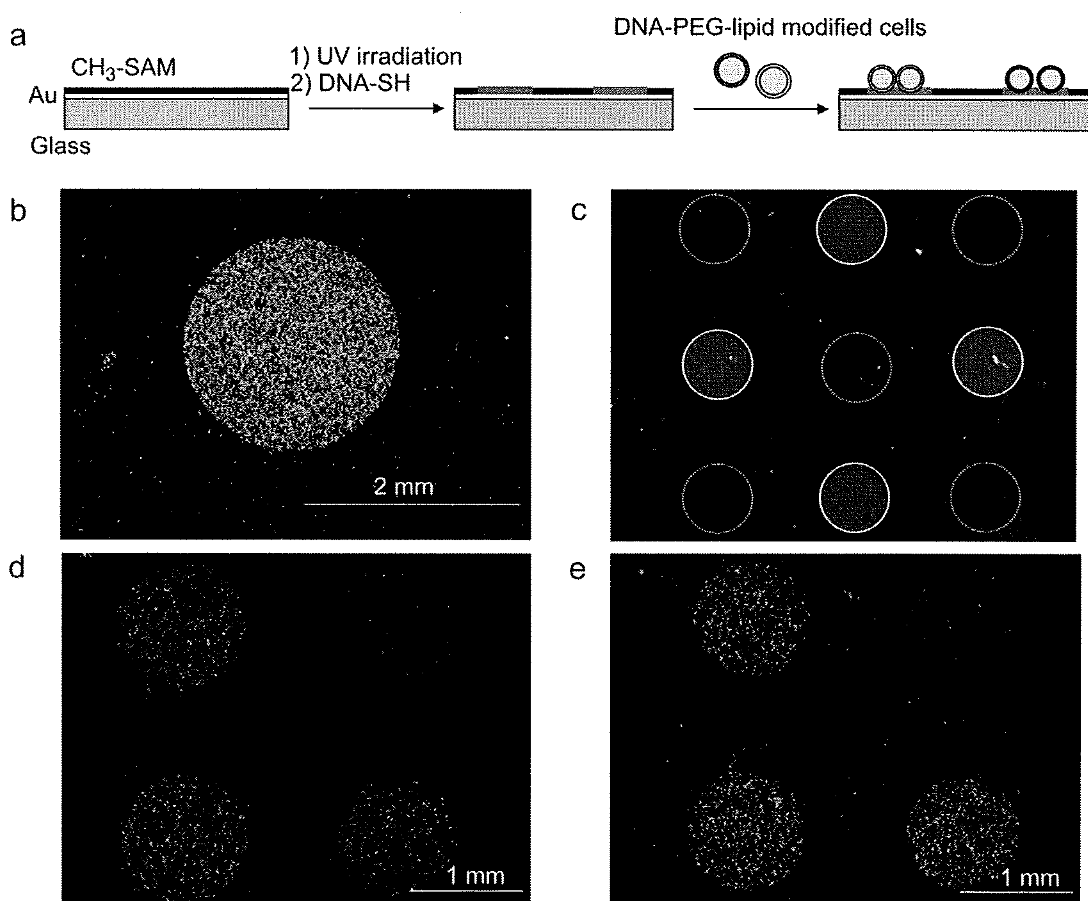


Fig. 2. Immobilization of polyDNA–PEG-lipid modified cells to a complementary polyDNA′ modified surface. (a): Scheme for preparation of DNA′-patterned substrate and immobilization of polyDNA–PEG-lipid modified cells. (b): Immobilization of polyT20–PEG-lipid modified CCRF-CEM cells labeled with PKH green to a single spot with immobilized polyA20-SH. The spot on the substrate surface was observed using an upright fluorescence microscope. (c): Attachment of polyT20–PEG-lipid modified CCRF-CEM cells to spots with immobilized polyA20-SH (solid lines) and polyT20-SH (dotted lines). The spots were observed using a stereomicroscope. (d): A mixture of SeqA-PEG-lipid modified CCRF-CEM cells and SeqB-PEG-lipid modified CCRF-CEM cells was incubated on DNA-immobilized spots where SeqA′ (top right), SeqB′ (bottom left), or a 1:1 mixture of SeqA′ and SeqB′ (top left and bottom right) were immobilized. (e): Inhibition assay for (d). A solution of SeqA′-SH was added to the mixture of SeqA-PEG-cells and SeqB-PEG-cells in advance and then the cells were incubated on the spots.

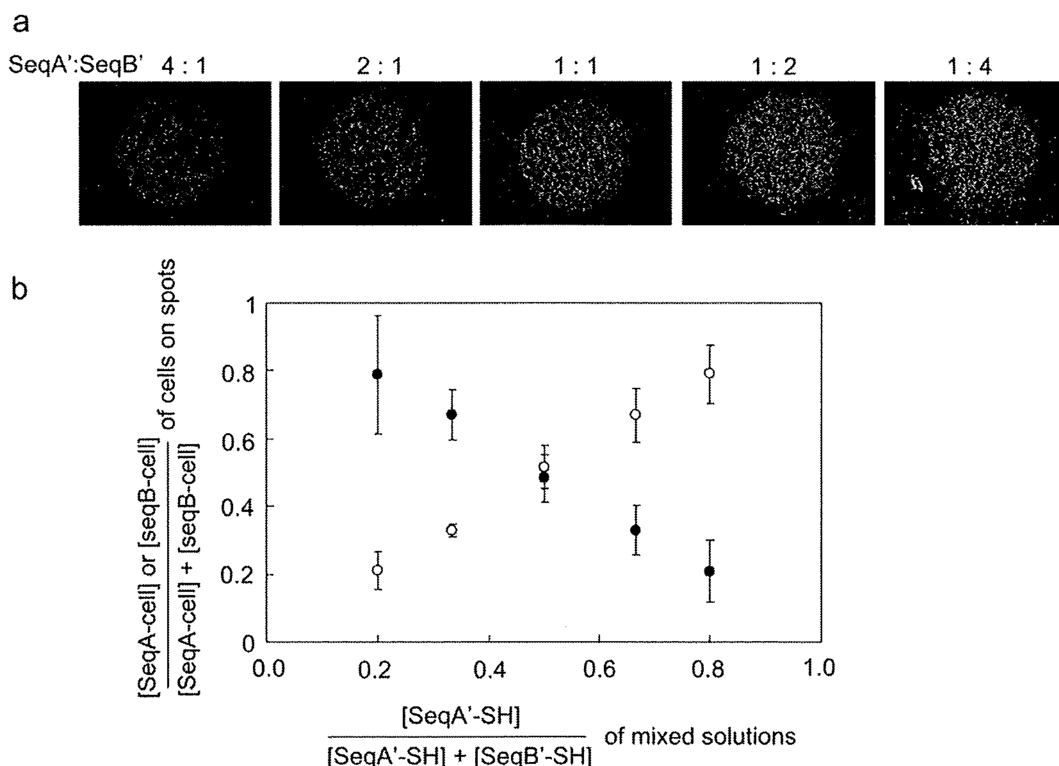


Fig. 3. Varying the ratios of immobilized SeqA′ and SeqB′ DNA in spots on the substrate surface and the effect on cell attachment. A 1:1 mixture of SeqA-PEG-cells labeled with PKH red and SeqB-PEG-cells labeled with PKH green were applied to the spots. (a): The surface was observed using an upright fluorescence microscope. (b): The ratios of SeqA-PEG-cells (open circles) and SeqB-PEG-cells (closed circles) attached to each spot were determined from fluorescence images using ImageJ software. The composition of cells are plotted against the SeqA′:SeqB′ ratios in the spots.

an upright fluorescence microscope (Fig. 3a). The number of cells that attached depended on the ratio of the complementary DNAs that were immobilized on the spots. The ratios of SeqA-PEG-cells to SeqB-PEG-cells attached to each spot were determined from fluorescence images using ImageJ software (open circles and closed circles in Fig. 3b, respectively). The cell ratios correlated well with the mixture ratios of SeqA′ and SeqB′.

We next examined the attachment of polyDNA–PEG-cells to a pattern on the substrate; the pattern was prepared by a contact printing method using a PDMS stamp. As shown in Fig. 4a, ledge surfaces on a PDMS stamp were coated with a solution of SeqA′ or SeqB′ DNA and pressed onto the gold surface. The same stamp was rotated 90° and again pressed to the surface, forming a cross pattern. A 1:1 mix of SeqA-PEG-cells and SeqB-PEG-cells was applied to the immobilized DNA, incubated, and washed with HBSS. Attached cells were observed using an upright fluorescence microscope. As shown in Fig. 4b, SeqA-PEG-cells and SeqB-PEG-cells selectively attached to the stripes containing immobilized SeqA′ or SeqB′ DNA, respectively, demonstrating that cells could attach via DNA hybridization to a DNA pattern prepared using a contact printing technique.

4. Discussion

Cell surface modification is generally achieved three ways: by covalent conjugation to the amino groups of membrane proteins; by

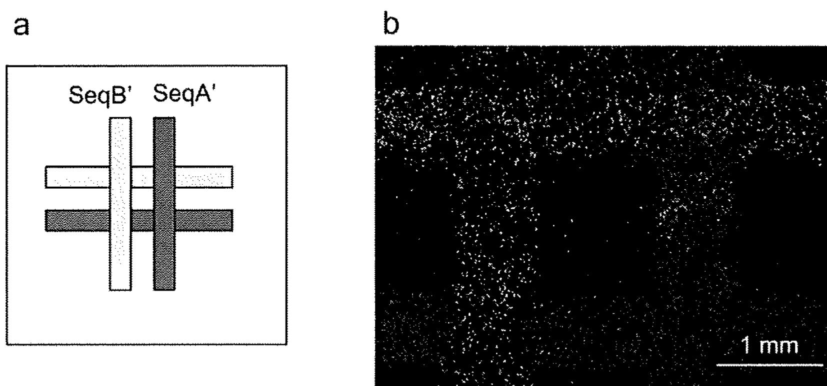


Fig. 4. Immobilization of cells on a patterned substrate prepared by a contact printing method using a PDMS stamp. (a): SeqA′–SH and SeqB′–SH were immobilized (red and green lines, respectively) in the pattern shown here. (b): A 1:1 mixture of SeqA-PEG-lipid cells labeled with PKH red and SeqB-PEG-lipid cells labeled with PKH green was applied to the patterned substrate containing immobilized DNA. The substrate was observed using an upright fluorescence microscope.

electrostatic interaction between cationic polymers and a negatively charged surface; and by incorporation of amphiphilic polymers into the lipid bilayer of the cell membrane by hydrophobic interactions [16]. We have studied cell surface modification using amphiphilic polymers such as PEG-lipid derivatives that incorporate spontaneously into lipid bilayers [16,18]. Notably, this surface modification technique does not cause protein denaturation or have cytotoxic effects. Further, functional groups such as amino groups, maleimide, and biotin can be incorporated into the cell membrane using PEG-lipid derivatives bearing these groups [13–15].

In the present study, polyDNA was introduced into the outer cell membrane using PEG-lipid. Cell–cell attachments between either the same types of cells or different types of cells were induced by incorporating complementary DNA sequences into two cell populations (Fig. 1); when mixed, the hybridization of the complementary sequences mediated cell–cell attachment. This DNA-hybridization technique was also used to attach DNA-modified cells to immobilized DNA on a substrate (Figs. 2 and 3). Antibody–antigen reactions, cell-extracellular matrix interactions, and hydrophobic interactions with amphiphilic polymers have all been used to immobilize cells on surfaces [20–22]. Using these techniques, cell suspensions must be applied to each spot to prepare arrays of cells. Not only is this a tedious and time-consuming process, cell viability is lost during the preparation of the array. In contrast, the technique described here is quite simple, since a suspension of cells with different DNA sequences can be applied to surfaces that have spots of immobilized complementary DNA sequences. Thus, this technique can be used for preparation of cell-based arrays for many types of studies.

To our knowledge, there are few previous studies that have achieved cell–cell attachment between different kinds of cells. We previously reported the immobilization of living cells to the surface of islets of Langerhans for microencapsulation using PEG-lipids and the biotin/streptavidin reaction [19]. It is also possible to attach feeder cells to embryoid bodies for the analysis of differentiation of ES cells into neurons [Iwata et al., unpublished report]. The simple and versatile methods described here have many applications in both regenerative medicine and in tissue engineering.

5. Conclusions

By incorporating complementary DNA sequences attached to amphiphilic PEG-lipids into the membranes of two cell populations, we induced cell–cell attachments that were mediated by DNA hybridization. This technique was also used to successfully induce cell attachment to a substrate containing immobilized DNA. This method shows promise for use in analyzing homogeneous and heterogeneous cell–cell interactions.

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Appendix

All figures with essential color discrimination in this article are difficult to interpret in black and white. The full color images can be found in the online version, at doi:10.1016/j.biomaterials.2009.11.098.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi: 10.1016/j.biomaterials.2009.11.098.

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Cell surface modification with polymers for biomedical studies

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Surface modification of living cells with natural or synthetic polymers is a powerful and useful tool in biomedical science and engineering. Various functional groups and bioactive substances can be immobilized to the cell surface through covalent conjugation, hydrophobic interaction, or electrostatic interaction. In this review, we provide an overview of the methods and polymers employed in cell surface modification, including: (1) covalent conjugation utilizing amino groups of cell surface proteins, (2) hydrophobic interaction of amphiphilic polymers with a lipid bilayer membrane, and (3) electrostatic interactions between cationic polymers and a negatively charged cell surface. We also discuss their applications in studies on cell therapy, cell–cell interaction analysis, cell arrangement, and lineage determination of stem cells.

1. Introduction

Surface modification of living cells with natural and synthetic polymers allows for new opportunities in biomedical engineering and science. A variety of functional groups and bioactive substances have been introduced onto the cell surface. The three methods generally employed in cell surface modification are covalent conjugation, hydrophobic interaction, and electrostatic interaction.

In this review, we provide an overview of the methods and polymers employed in cell surface modification in conjunction with their applications in biomedical engineering and science; applications discussed include: (1) adding biological functions, such as blood compatibility, to the cell surface, (2) controlling graft rejection in cell transplantation by masking surface antigens of cells with natural or synthetic polymers, and (3) aligning different kinds of cells through interactions between complementary units, such as oligoT and oligoA, introduced on different cell surfaces. Cell surface modification can be used to study cell–cell interactions and to control stem cell lineages in regenerative medicine. It is anticipated that cell surface modification technology, still an emerging research area, will be applied to the treatment of patients in the areas of regenerative medicine, tissue engineering, stem cell research, and embryology.

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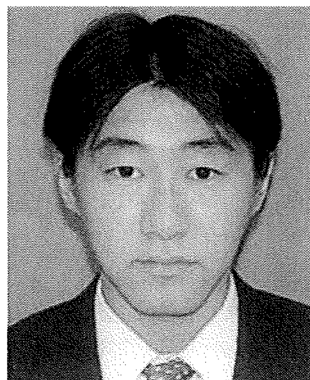
2. Methods for cell surface modification

Surface modification of cells has been generally achieved through three methods: (1) covalent conjugation to amino groups of cell surface proteins, (2) incorporation of amphiphilic polymers into the lipid bilayer membrane of cells by hydrophobic interaction and (3) electrostatic interaction between cationic polymers and a negatively charged surface. Attempts have been made with various kinds of synthetic and natural polymers for surface modification of cells and biomaterials.[1,2] The methods are summarized in Fig. 1 and Table 1.



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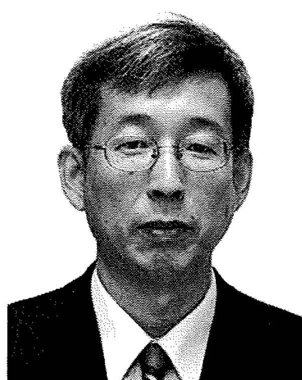

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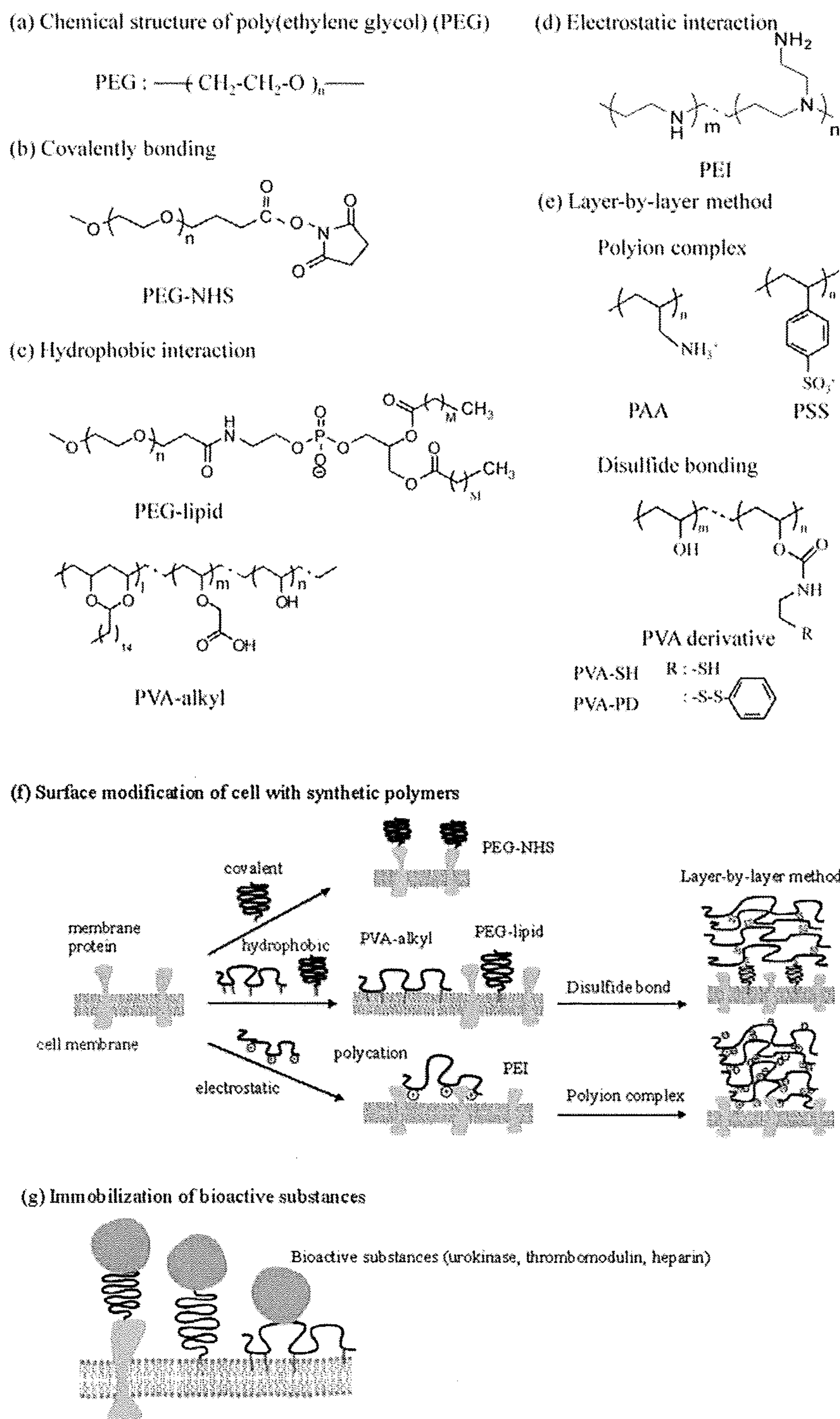

Fig. 1 Cell surface modification with synthetic polymers. Chemical structures of (a) poly(ethylene glycol) (PEG), (b) PEG carrying *N*-hydroxyl-succinimidyl ester (PEG-NHS), (c) PEG-conjugated phospholipid (PEG-lipid) and poly(vinyl alcohol) carrying side alkyl chains (PVA-alkyl), (d) poly(ethyleneimine) (PEI), and (e) poly(allylamine) (PAA), poly(styrene) sulfate (PSS), PVA derivative (PVA-SH, PVA-PD). (f) Schematic illustration of surface modification of a cell with synthetic polymers by covalent bonding, hydrophobic interaction, electrostatic interaction, and the layer-by-layer method. (g) Schematic illustration of the immobilization of bioactive substances to the cell surface *via* polymers.

2.1. Covalent bond method

For covalent conjugation of polymers, surface modification of living cells has been achieved through chemical or enzymatic treatment or by metabolic introduction.[3–11] As shown in Fig. 1b, the *N*-hydroxyl-succinimidyl ester (NHS) group and cyanuric chloride are frequently used to chemically form covalent bonds to amino groups of membrane proteins.[7–11] There is a potential that toxic effects are exerted on membrane proteins.[3,4] Enzymatic treatment and metabolic introduction have been also employed to add various functional groups such as biotin, azide, and ketone groups to living cell surfaces.[3–6] The technologies are

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Table 1 Methods for cell surface modification

	Modification sites	Treatment	Cell	Introduced functional groups or polymers	Reference
Covalent bond	Amino groups of membrane proteins	Chemical reaction	Red blood cell, islets, peripheral blood mononuclear cells, murine splenocytes.	PEG-NHS	7,8,11
				Biotin-NHS	9
				PEG-cyanuric chloride,	35–39
				Phosphine by Staudinger ligation, *etc.*	10
	Membrane protein	Metabolic reaction	Jurkat cells, HeLa cells, CHO cells, COS-7, HEK293	Azide, biotin and ketone groups	3,4,6
Electrostatic interaction	Negatively charged cell surface	Non-covalent reaction	Islets, human fibroblast cells, porcine brain capillary endothelial cells, MCF-7 breast cancer cells, Mouse mesenchymal stem cells	Poly(diallyldimethyl ammonium chloride)	21,24
				Poly(allylamine hydrochloride) (PAH)	21,23,24
				Poly(styrene) sulfate (PSS)	21,23
				Poly-L-lysine (PLL)	22,25
				Poly(ethyleneimine) (PEI)	24
				Alginate	22
				Hyaluronic acid, *etc.*	25
Hydrophobic interaction	Lipid bilalyer membrane	Non-covalent reaction	Islets, HEK293, CCRF-CEM	PEG-lipid	12–15,17,18
				PVA-alkyl	14,16

limited to the introduction of specified functional small molecules to cells and might perturb cell physiology.[5,6] Although covalent immobilization was expected to be stable for chemical degradation and present for a long period because of covalent bonding to membrane proteins, introduced polymers and functional groups disappeared from the cell surface with time.[7,9,14]

2.2. Hydrophobic interactions for cell surface modification using amphiphilic polymers

Amphiphilic polymers such as PEG-conjugated phospholipids (PEG-lipid) and poly(vinyl alcohol) bearing hydrophobic alkyl side chains (PVA-alkyl), have been used for cell surface modification (Fig. 1c).[5,6,12–18] In this method, hydrophobic alkyl chains of amphiphilic polymers are spontaneously anchored into the lipid bilayer membranes through the hydrophobic interaction. This interaction is shown in some detail in Fig. 2 for the case of PEG-lipid. This spontaneous incorporation was monitored by a surface plasmon resonance (SPR) instrument when a PEG-lipid solution was applied onto the supported lipid membrane formed on a SPR sensor surface. The spontaneous incorporation of PEG-lipid was observed and it is greatly affected by the length of alkyl chains of the phospholipids, that is, their hydrophobicity. Fluorescein isothiocyanate (FITC) was conjugated to the end of the PEG chain for fluorescence labeling. The FITC-PEG-lipid solution was added to CCRF-CEM (human cell line derived from T cell leukemia) cells and the mixture was incubated for 30 min. The images observed by a confocal laser scanning microscope are shown in Fig. 2c. The fluorescence of FITC was observed at the periphery of all cells, indicating PEG-lipids existed on the cell surface through anchoring of the lipid bilayer to the cell membrane by the hydrophobic interaction. PEG-lipids gradually disappeared from the cell surface without uptake into the inside of cells. They were dissociated from the cell surface into the medium.[14]

2.3. Electrostatic interactions

Several groups have explored the possibility of constructing thin polymer membranes on the surface of cells through electrostatic interactions between negatively charged cell surfaces and cationic polymers, and then further modified using a layer-by-layer technique of anionic and cationic polymers as shown in Fig. 1d and e.[21–25] Ionic polymers which have been employed are poly(allylamine hydrochloride), poly(styrene) sulfate, poly-L-lysine, and poly(ethyleneimine). A layer-by-layer method is a simple and attractive method to modify the cell surface.[26] The surface property can be controlled by the outermost layer of polymer. The thickness of the membrane is also controllable by the number of applications of polymer solution. Unfortunately, most polycations, such as PLL and PEI were found to be extremely cytotoxic and severely damaged treated cells. PEI which interacted with cells was not degraded and not excluded from the cell because PEI immediately destroyed the cell membrane after interaction with the cell surface.

2.4. Bioactive substances immobilized through an intermediary molecule

Bioactive substances have been immobilized on cell surface (Fig. 1g). There are two classifications of this method. One is immobilization of substances through an intermediary molecule which is covalently bound to membrane proteins on the surface *via* amide bonding with hetero- or homo-bifunctional cross-linkers. In one example, biotin molecules are covalently bound to the amino groups of membrane proteins through cross-linkers. The surface was then sequentially treated with avidin and heparin solutions. Heparin was immobilized to the surface through an electrostatic interaction with avidin (isoelectric point of avidin; pI = 10).[9] Phosphine molecules have also been covalently conjugated to amino groups of membrane proteins and then thrombomodulin was immobilized by Staudinger ligation.[10] The immobilization of serum albumins has been carried out using PEG carrying NHS groups at both ends; one NHS is used for anchoring to the amino group of membrane proteins and the other for the immobilization of serum albumin.[11] It has been reported these covalent modification methods exert minor toxic effects on cell viability and cell functions.[9,10,14]

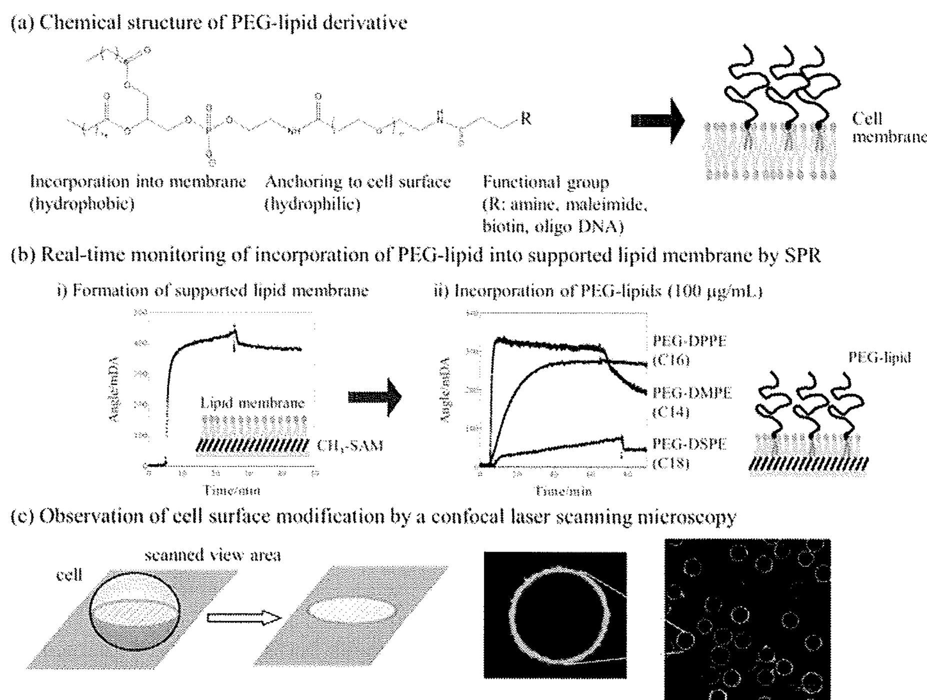

Fig. 2 Cell surface modification with amphiphilic PEG-lipid. (a) Chemical structure of PEG-lipid derivatives, R is a functional group such as an amino group, biotin, maleimide group, or oligo DNA. Schematic illustration shows the interaction between PEG-lipid and the lipid bilayer of a cell membrane. (b) Real-time monitoring of incorporation of PEG-lipid into a supported lipid membrane by a surface plasmon resonance (SPR) instrument. Suspension of a small unilamellar vesicle of egg yoke lecithin (70 μg mL^{-1}) was applied to a methyl-terminated self-assembly monolayer (CH_3-SAM) surface. A PEG-lipid solution (100 μg mL^{-1}) was then applied. Here we used three kinds of PEG-lipids with different length acyl chains; PEG-DMPE, PEG-DPPE, and PEG-DSPE have 14, 16, and 18 carbons, respectively, in an acyl chain. (c) Observation of FITC-labeled PEG-lipid modified CCRF-CEM cells by a confocal laser scanning microscope. The fluorescence of FITC was observed at the periphery of all cells, indicating PEG-lipids are anchoring to the lipid bilayer membrane of the cell surface by hydrophobic interactions.

The other method is formation of an intermediary polymer layer through hydrophobic interactions using amphiphilic polymers such as PEG-lipid and PVA-alkyl carrying various functional groups.[15,16] The functional groups introduced on cell surfaces through the amphiphilic polymers can be used for immobilization of bioactive substances. In one example, PVA-alkyl carrying SH groups is used for the immobilization of bioactive substances carrying maleimide groups. By the same means, PEG-lipid carrying maleimide at the end of the PEG chain is also useful for immobilization of bioactive substances carrying SH groups without cytotoxicity.

3. Biomedical application of cell surface modification

Biomedical application of cell surface modification can be found in the area of cell and organ transplantation. When cells or tissues (graft) from a donor are tranfused or transplanted to a patient, the immune system of the patient recognizes them as foreign materials and attacks and destroys them. When the patient carries pre-existing antibodies to the donor, hyperacute rejection is induced through a complement-mediated cytotoxicity in recipients. And T cells are activated by recognition of antigens displayed on the cell surface of the tissue or organ. The process initiates the adaptive immune response. Before the immune reaction is triggered, the blood coagulation and complement systems are activated by the graft when it was exposed to blood, and chemotactic factors, tissue factor, chemokines, and other inflammatory mediators are released during the early post-transplantation phase. An immediate blood mediated inflammatory reaction is activated, resulting in destruction of the transplanted graft.[27–32] Antigens on the cell surface should be masked or modified by surface treatment of the graft to inhibit or reduce these reactions.

Cell–cell interactions have been studied because they play important roles in natural embryo development and even in the bodies of mature animals. In the last ten years, differentiation of embryonic stem (ES) cells and somatic stem cells to functional cells has attracted attention in connection with regenerative medicine. The control of the attachment between heterogeneous or homogeneous cells by cell surface modification is expected as a useful tool in these studies.

In the following, we will present several biomedical applications of the cell surface modification.

3.1. Cell therapy of diabetes

Patients of type I diabetes have been successfully treated by transplantation of islets of Langerhans (islets), endocrine tissue releasing insulin, isolated from human donors, under administration of immunosuppressive drugs. Side effects of their long-term dose on recipients, however, are not fully understood. To resolve this issue, transplantation of islets after masking of cell

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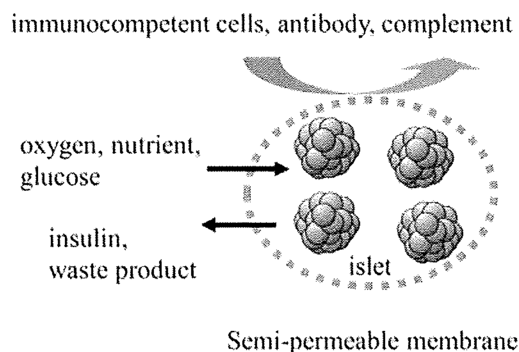
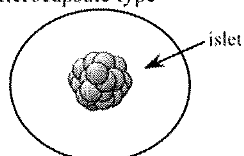
(a) Concept of immuno-isolation membrane

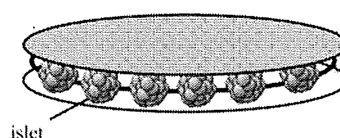


(b) Bioartficial pancreas

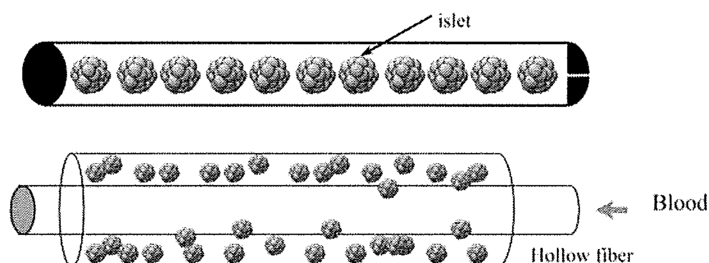
(i) Microcapsule type



(ii) Diffusion chamber type



(iii) Hollow fiber unit type



(c)

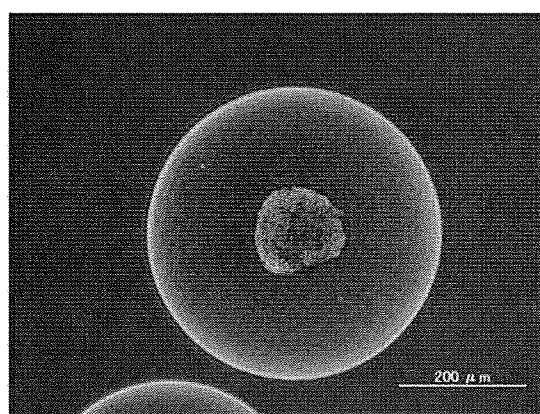


Fig. 3 Bioartificial pancreas. (a) The concept of an immuno-isolation membrane. (b) The types of bioartificial pancreas. (i) Microcapsule, (ii) diffusion chamber, (iii) hollow fiber-based diffusion chamber. (c) Enclosure of islets in agarose microcapsules.

surface antigens, that is a bioartificial pancreas, has been proposed and attempted (Fig. 3a). Islets are enclosed within a semi-permeable membrane. Oxygen and nutrients permeate through the membrane and are supplied to cells of islets. Insulin, secreted from islets in response to blood glucose increase, can diffuse through the membrane into the recipient blood circulation. It is possible that transplanted islets are protected in the recipient immune system without immuno-suppressive therapy

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and insulin release from the islets can control glucose metabolism for a long period. The bioartificial pancreas has been studied for over 30 years.[40] Firstly, its studies are briefly overviewed.

3.1.1. Brief overview of bioartificial pancreas. There have been three kinds of bioartificial pancreases including: a diffusion chamber type, hollow fiber unit, and microcapsule type (Fig. 3b).[41–48] In the first two types, islets form large aggregates in the chambers and the space between the housing and hollow fibers. Many cells located in the central part of aggregated islets are lost due to an insufficient oxygen supply. To prevent this, islets are kept within an alginate hydrogel to inhibit contact with each other. Another problem is that the size of the device became too large to be implanted. Efforts over the past 10 years have focused on the development of a third type bioartifical pancreas, that is, a microcapsule type.

Many groups have been studying a microcapsule type bioartificial pancreas (Fig. 3c).[49–55] Our group demonstrated that hyperglycemia in diabetic mice could be normalized for 200 days after allotransplantation of agarose-encapsulated islets. Yet, the volume of the encapsulated islets still restricts its clinical applications. The average diameter of islets is around 150 μm. The diameter of capsules is about three times as large as the original islets. According to an estimate, the total volume of the microcapsules would be 27 times as large as original volume of islets by a 3rd power increase of the radius. In the human clinical setting, 10 mL of islet suspension would be infused into the portal vein. The volume of the capsule suspension would be greater than 270 mL. It is too large to be infused into liver through the portal vein or implanted in other sites. Several trials have been done to reduce the size of capsules. Calafiore *et al.*[56] reported much smaller microcapsules, *i.e.* 300 μm diameter, which would provide a manageable total volume for clinical applications. However, the capsules with larger diameters than the islet itself are expected to plug larger blood vessels, imposing harmful effects on the patient's liver. The diameter of encapsulated islets must be much smaller to allow transplantation of the islets through portal veins. Thus, new methods for the microencapsulation of islets, without increasing the diameter of the implant are necessary.

3.1.2. Masking the surface antigens of islets with synthetic polymers. The concept of the bioartificial pancreas was to cover the cell surface with polymer chains or thin membranes to mask cell surface antigens. Several groups originally proposed methods for thin coverage of the surface of islets.[12,13,15,21] Through proper control of chain length of polymers and surface grafting density, antigenic sites on islets could be camouflaged with the polymers.[35–39]

Cell surface modification methods shown in Fig. 1 and listed in Table 1 have been employed in masking surface antigens of islets. PEG carrying activated ester group, *N*-hydroxyl-succinimidyl ester (NHS) group, at its end has been employed to cover the surface antigen. It was reacted with the membrane proteins or collagen layer on the islets surface.[38] Byun and coworkers[7,8] reported covering the surface of islets from a Sprague-Dawley rat with PEG and the modified 1200 islets were transplanted under the kidney capsule of streptozotocin (STZ)-induced diabetic Fisher 344 rats. Normoglycemia could be maintained for 1 year by combination of surface modification of islets with PEG-NHS and low doses of cyclosporine A, an immunosuppressive drug. On the other hand, normoglycemia could be prolonged for up to 12 days with low doses of cyclosporine A without PEG treatment. Contreras *et al.*[11] applied the PEG-NHS modification method to xenogeneic transplantation. No change was observed in the morphology of porcine islets and viability after PEG modification. NHS-PEG-modified porcine islets were transplanted into the liver through the portal vein of NOD-SCID mice which were previously transplanted with human lymph cells to establish a human immune-system prior to islet transplantation. In this study, the porcine islets were previously transfected with adenovirus expressing *Bcl-2* to suppress apoptosis. When naïve islets and naked *Bcl-2* expressing islets were transplanted, the blood glucose level was not normalized, but it was reduced to approximately 150 mg/dL and normalized for 15 days, respectively. Normoglycemia was maintained for 15 days by transplanting islets that expressed *Bcl-2* and were modified with PEG and albumin. It seems that PEG on the islet surface is supplemental against the immune rejection reaction. In fact, when PEG modified islets that did not express *Bcl-2* were transplanted, the blood glucose level was transiently normalized but increased again.

Krol *et al.*[21] attempted to encapsulate human islets by the layer-by-layer method and found there was minimal loss of islet function and viability when poly(allylamine hydrochloride) and poly(styrene) sulfate were employed. Although a layer-by-layer membrane could be formed on the islet surface using cationic and anionic polyelectrolytes, it was found that direct interaction between the cationic polymer and the cell surface should be avoided otherwise the cell membrane would gradually be destroyed.

Amphiphilic polymers, such as PEG-conjugated phospholipid (PEG-lipid)[19,20] and poly(vinyl alcohol) carrying side alkyl chains, have also been used to mask the surface antigens of islets.[12–18] Although a conformal PEG layer formed on the cells or islet surface at nanometre levels of thickness (Fig. 4), the PEG layer disappeared from the cell surface after approximately 3 days.[14] A more stable membrane should be employed for immuno-isolation. To enclosure the whole surface with a stable membrane, an additional polymer layer or a protein layer was further formed on the PEG-lipid layer on the cell surface. Various functional groups, such as maleimide and biotin, are introduced to the end of the PEG chain to be used as reaction points for formation of a layer-by-layer membrane on the cell surface. For example, it is possible to form a layer-by-layer membrane of poly(vinyl alcohol) (PVA) on the islets surface by the reaction between the thiol and maleimide groups.[12] The islets carrying PEG-lipid-maleimide are exposed to a solution of PVA carrying thiol groups (PVA-SH) (Fig. 1e). A maleimide group reacts with a thiol group to form a stable covalent bond under physiological conditions. A thiol/disulfide exchange reaction between a pyridyldithio group and thiol group can be also used to form a third layer on the islets as shown in Fig. 4c.

A multi-layer membrane can be formed using a biotin-streptavidin reaction. In our experiments, PEG-lipids carrying biotin at one end of PEG were applied to islets and anchored to the cell membranes. The PEG-lipid layer on the islets was further covered by streptavidin and sequentially treated with bovine

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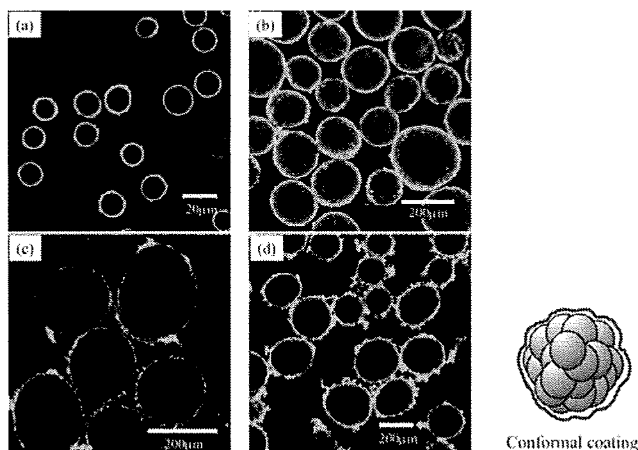

Fig. 4 Photos of surface modification of cells (CCRF-CEM) and enclosure of islets with thin polymer membranes *via* PEG-lipid derivatives, taken by a confocal laser scanning microscope. (a) FITC-PEG-lipid modified CCRF-CEM cells. (b) FITC-PEG-lipid modified islets. (c) Layer-by-layer membrane of PVA-coated islets. PVA was labeled with FITC. (d) Layer-by-layer membrane of biotin-BSA and FITC-streptavidin-coated islets.

serum albumin carrying biotin. This procedure was repeated 20 times (Fig. 4d).[15] The thickness of the membrane was about 30 nm, based on the calculation from surface plasmon resonance (SPR) analysis. A glucose stimulation test was performed to examine the ability of the modified islets to control insulin release in response to glucose level changes. No significant differences in insulin release were observed between groups of islets with/without surface modifications. We also examined the effect of PEG modification on graft survival when islets were transplanted into the liver through the portal vein of STZ-induced diabetic mice.[18] Although we found graft survival was improved by surface modification with PEG-lipid and cell damage to islets could be suppressed, its effect was limited. Cause of the failure is not clear yet. Antigenicity of streptavidin might induce immune reactions or dynamic behavior of the cell membrane, such as endocytosis, which might deteriorate the polymer layer, because PEG-lipid anchored to the cell membrane simultaneously internalized into the cell.

Our group also examined coating the islet surface with an alginate/poly-L-lysine/alginate multilayer, where NH_2-PEG-lipid was introduced into islet cell membranes to form a positively charged islet surface to facilitate electrostatic binding of negatively charged alginate.[13] The negatively charged surface was sequentially covered with poly-L-lysine and alginate. Islets coated with this membrane responded normally in a static glucose stimulation assay.

3.1.3. Control of immediate blood mediated inflammatory reaction. In clinical islet transplantation, islets are transfused into the liver through the portal vein.[57,58] It was reported that 50% of islets are lost in the early phase following transplantation, before the immune-rejection reaction.[33] The blood coagulation and complement systems are activated by islets, and chemotactic factors, tissue factor, chemokines, and other inflammatory mediators are released during the early post-transplantation phase. Immediate blood mediated inflammatory reactions are activated, resulting in destruction of the transplanted islets.[27–32] In animal studies, thrombin inhibitor melagatran,[34] activated protein C,[29] low molecular weight dextran sulfate,[30] and the water-soluble domain of complement receptor I (sCR1)[31,32] have been systematically administered to regulate early coagulation and blood-mediated inflammatory reactions. It is difficult, however, to apply these methods in the clinical setting because systemic administration is associated with an increased risk of bleeding. The effects of bioactive substances can be localized by the immobilization of such substances on the surface of islets.[9,10,15,16]

Nilsson and coworkers[9] proposed coating porcine islets with heparin to inhibit surface thrombosis formation. Biotin was covalently immobilized on the surface of porcine islets using activated ester chemistry and the surfaces were further treated with avidin. The islet surface was coated with macromolecular conjugates of heparin: ~70 heparin molecules covalently linked to an inert carrier chain through electrostatic interaction between positively charged avidin and negatively charged heparin. In allotransplantation in pigs, increases in plasma concentrations of thrombin–antithrombin (TAT) complexes and complement activation parameter (C3a) were suppressed for heparin-coated islets compared with naïve islets. The transient increase of insulin leaked from destroyed porcine islets was also suppressed. These results suggest heparinization of the islet surface helps prevent

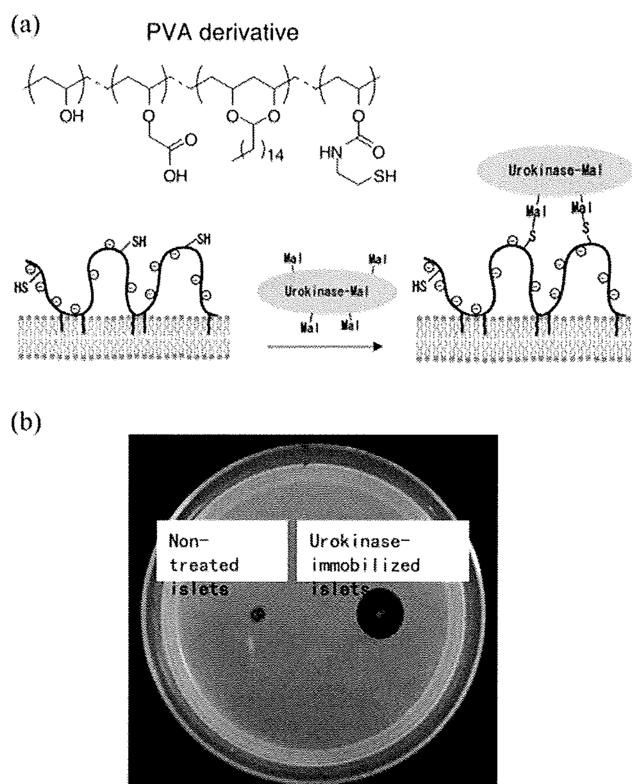

Fig. 5 Immobilization of urokinase on the surface of islets. (a) Immobilization of urokinase to the islet surface using amphiphilic PVA-alkyl. (b) Fibrin plate assay of urokinase-immobilized islets. Urokinase-immobilized islets and non-treated islets (100 islets each) were placed on a fibrin gel plate and incubated at 37 °C for 13 h. A large transparent area formed around the urokinase-immobilized islets due to dissolution of the fibrin gel by plasmin, which is produced from plasminogen by urokinase.

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the immediate blood mediated inflammatory reactions. Chaikof and coworkers[10] reported covalently immobilizing recombinant thrombomodulin to the surface of islets. Phosphine molecules were covalently conjugated to amino groups of membrane proteins to anchor recombinant thrombomodulin by Staudinger ligation. The presence of thrombomodulin on the islet surface resulted in a significant increase in the production of activated protein C with a reduction in islet-mediated thrombogenicity. Covalent immobilization of bioactive substances through membrane proteins is thought to deteriorate membrane protein functions and to perturb cell physiology.[3,4]

Our group developed non-covalent methods for immobilization of bioactive substances on islets using various amphiphilic polymers.[12–18] Fibrinolytic enzyme, urokinase, was non-covalently immobilized on the islet surface as schematically shown in Fig. 5a.[16] An amphiphilic PVA derivative carrying long alkyl side chains, thiol, and carboxylic groups was used for immobilization of urokinase on the islet surface. Thiol groups were introduced to the islets surface using the amphiphilic PVA derivatives through the hydrophobic interaction between the long alkyl side chains and lipid bilayer of the cell membrane (Fig. 5a). Urokinase was modified with a hetero-bifunctional cross-linker (sulfo-EMCS, *N*-(6-maleimidocaproyloxy) sulfosuccinimide) to introduce maleimide groups. The urokinase carrying maleimide groups was immobilized to the islet surface through the maleimide/thiol reaction. Fig. 5b shows results from the fibrin plate-based assay. To assess the fibrinolytic activity of the urokinase, urokinase-islets and naïve islets (100 islets each) were spotted on a fibrin gel plate and left for 24 h. The transparent area formed in the fibrin plate, representing dissolved fibrin, was measured. A large transparent area (diameter; 1.8 cm) was observed around the islets carrying urokinase, indicating urokinase activity. The transparent area was small around naïve islet spots.

These results suggest immobilization of bioactive substances to the surface of islets is promising and a possible means for inhibition of immediate blood mediated inflammatory reactions resulting in improving graft survival following intra-portal transplantation.

3.1.4. Enclosure of islets with cells. We recently attempted to enclose islets with living cells.[17] If the surface of islets can be covered with living cells such as vascular endothelial cells or fibroblasts derived from a recipient, compatibilities of islets with the recipient are expected to be significantly improved and evade the immune-rejection reaction. Therefore, we attempted to enclose islets with a living cell layer membrane.[17] To our knowledge, there have been no previous reports of enclosing islets with living cells. We used amphiphilic PEG-lipid and the biotin/streptavidin reaction to immobilize HEK293 cells on the surface of islets, as shown in Fig. 6a. After biotin molecules were introduced on the surface of HEK293 cells by biotin-PEG-lipid, streptavidin was immobilized to the surface. Then the streptavidin-immobilized HEK293 cells were added to biotin-PEG-lipid modified islets. HEK293 cells were immobilized on the islet surface (Fig. 6b). The surface of the islets was completely covered with a cell layer after 3 to 5 days in culture without central

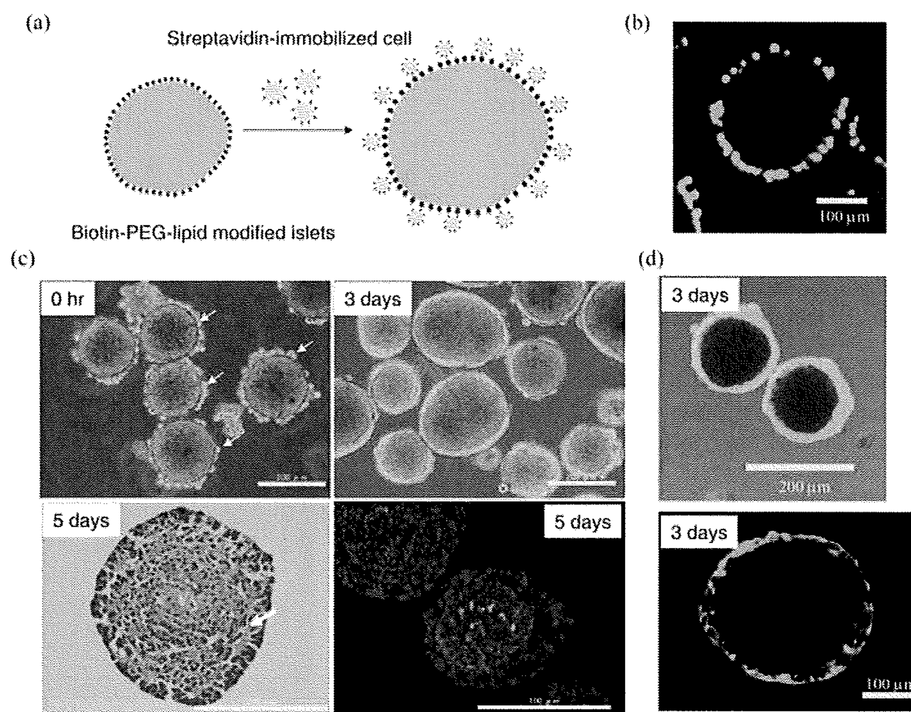

Fig. 6 Encapsulation of islets with living cells. (a) Schematic illustration depicting how to enclose an islet with living cells utilizing the avidin and biotin interaction. (b) Hamster islets modified with biotin-PEG-lipid were immobilized with streptavidin-immobilized HEK293 cells. HEK293 cells were labeled with CellTracker®. (c) Phase contrast microscopic images of islets carrying HEK293 cells and histochemical analyses (HE staining and immunostaining) of HEK293 cells-immobilized islets in culture. (c)-lower right; Alexa 488-labeled *anti*-insulin antibody and Hoechst 33342 dye for nuclear staining. The pictures are merged images from insulin and Hoechst 33342 staining. (d) GFP-expressing HEK293 encapsulated islets observed at 3 days culture and their sliced section at 3 days.

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necrosis of the islet cells (Fig. 6c and d). Insulin secretion was well maintained upon glucose stimulation of HEK293 cell-enclosed islets. We were successful in enclosing islets with a cell layer without damaging islet function, although HEK293 cells are a cell line.

We are trying to enclose islets with human vascular endothelial cells using the same technique. In a clinical setting of islet transplantation, islets are transfused into the liver through the portal vein. They are exposed to blood flow. As mentioned above, the immediate blood mediated inflammatory reactions are induced. Therefore, possibility of the new idea, enclosure of islets with patient's vascular endothelial cells, will be examined to overcome this issue.

3.2. Blood transfusion

Camouflaging surface antigens is also used to make universal RBC.[35–39] Methoxy(polyethylene glycol) (mPEG) was covalently bound to the surface of RBC through cyanuric chloride coupling. The reaction site of mPEG was accessible to the amino groups of lysine residues on membrane proteins. The surface modification of RBC with mPEG was shown to prevent host antibodies from recognizing blood group surface ABO antigens.[35–37] It was also possible to prevent receptor–ligand interactions by conjugation of mPEG to the surface of human peripheral blood mononuclear cells[38] and murine splenocytes.[39] These receptor–ligand interactions involved allorecognition, including weakening CD28-B7 co-stimulation which resulted in T cell apoptosis.

3.3. Cell-cell attachment

PEG-lipids can be used to introduce biotin or oligoDNA sequences onto a cell surface. Different kinds of cells can be attached or aligned through biotin/avidin interactions or hybridization of oligoDNA. Fig. 7 shows an example of cell–cell attachment through hybridization of oligoDNA at the end of PEG-lipid. The sequences of oligoDNA were;

polyA20: 5′-AAA AAA AAA AAA AAA AAA AA-3′
polyT20: 5′-TTT TTT TTT TTT TTT TTT TT-3′

CCRF-CEM cells which are derived from T cell leukemia and exist as floating state without attaching a substrate or each other were modified PolyA20-PEG-lipid or polyT20-PEG-lipid modified-cells. They were further labeled with fluorescent dye, PKH red and green, respectively (Fig. 7a). The two kinds of cells were mixed and incubated by rotation culture. Attachment between the two types of cells was examined by a confocal laser scanning microscope. They were alternatively aligned as shown in Fig. 7b. In contrast, no attachment was observed between cells not treated with oligoDNA-PEG-lipid, even after 3 h of rotation culture. These results suggest alternative cell alignment can be induced by hybridization of oligoDNA at the end of PEG-lipid. A biotin-PEG-lipid and streptavidin interaction can also be used to align cells.

The control over the attachment between heterogeneous or homogeneous cells is a useful tool in biomedical research. The technique can be applied to the analysis of cell–cell interaction, the induction of cell fusion, and so on. Cell–cell interactions should be expanded because they play important roles in embryo development. In addition, cell therapy is a promising method to treat various diseases such as type I diabetes, Parkinson's, Alzheimer's, ALS, and Huntington's disease. Its clinical applications, however, are mainly restricted by the shortage of cells from human donors. In this recent ten years, various stem cells, *i.e.* embryonic stem (ES) cells, various somatic stem cells and induced pluripotent stem (iPS) cells were found or developed and these are expected as promising cell sources.[67] Methods to differentiate these stem cells to functional cells have been extensively studied. In these, cell–cell interaction is accepted as an effective method to differentiate stem cells to functional cells.[68,69] It is expected to be much more powerful than other methods. Thus the control of cell–cell attachment will be also an important technique to obtain functional cells for cell therapy.

The attachment of different kinds of cells was applied to immobilize feeder cells, HEK293 cells (human endoderm kidney cell line) and PA6 cells (PA6 cells derived from skull bone

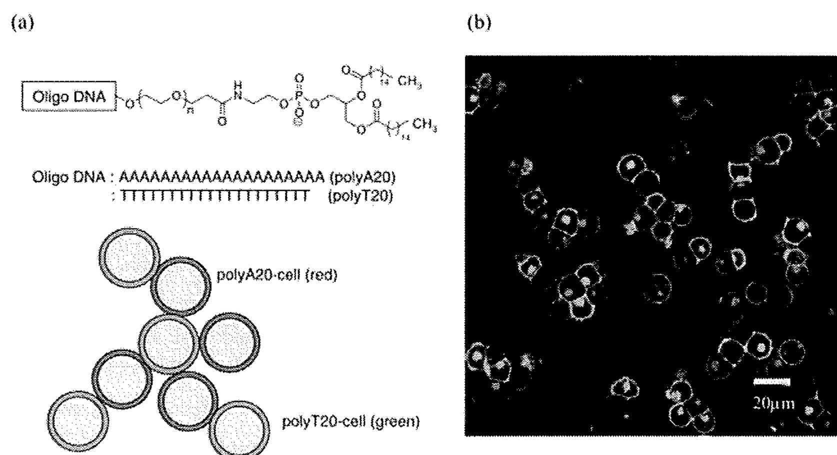

Fig. 7 Alignment of single cells by interactions between complementary oligoT and oligoA, introduced on different cell surfaces. (a) Chemical structure of oligoDNA-PEG-lipid and oligoDNA sequences (polyA20 and polyT20). (b) Alignment of single cells. PolyA20 and polyT20 cells are labeled with PKH red and PKH green, respectively. The cell attachment was induced by hybridization between polyA20 and polyT20 attached to PEG-lipid on the cell surface.

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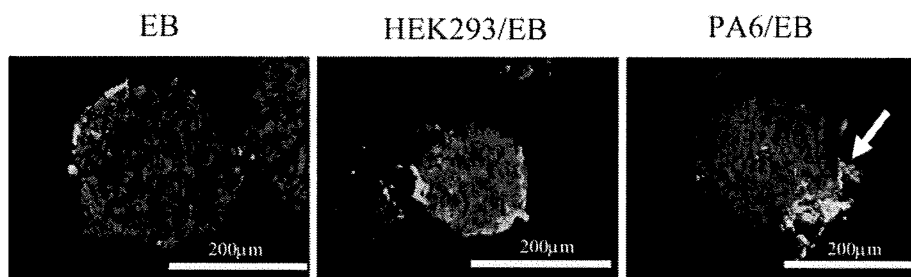

Fig. 8 Immunocytochemical analyses of EBs carrying GFP-HEK293 and PA6 cells after 6 days of hanging drop culture. Untreated EBs were used as controls. Frozen sample sections (were stained with antibodies for Nestin (red) and TuJ1 (green), which are markers of neural progenitor cells and mature neurons, respectively. Hoechst 33342 dye (blue) was used for nuclear staining. The pictures are merged images from Nestin, TuJ1, and Hoechst 33342 staining. The arrows indicate the local expression of Nestin and TuJ1.

marrow) onto the embryoid bodies (EBs), proliferating to cover the whole surface of the EBs (Fig. 8). The HEK293 cells uniformly attached and spread. The PA6 cells initially attached uniformly to the EBs; yet, they did not cover the EB surface, but penetrated into the EB. The frozen sections of EBs after 6 days coculture were analyzed by immuno-staining against neuron markers, Nestin and TuJ1. Nestin stained red, TuJ1 stained green, and the nuclei were stained with Hoechst blue (Fig. 8). The lineage fate of ES cells is thought to be determined by cell-cell interactions. Strong expression of Nestin and TuJ1 was found locally at the parts of the EBs where PA6 cells were attached. The ES cells were promoted to differentiate into neural cells when PA6 cells were immobilized on the EBs. In contrast, HEK293 cells completely suppressed the neuronal differentiation of ES cells. These results suggest our method is promising for studying the effects of intercellular contact interactions on the differentiation of ES cells. Although the results presented here are preliminary, we expect the application of this technology to stem cell research and embryology will be of much interest to the area of cell biology.

4. Conclusion and perspective

The cell surface modification methods are classified into three categories: (1) covalent conjugation to amino groups of cell surface proteins, (2) incorporation of amphiphilic polymers into the lipid bilayer membrane of cells by hydrophobic interaction, and (3) electrostatic interaction between cationic polymers and a negatively charged surface as summarized in Fig. 1 and Table 1. The last one is difficult to rationalize because cationic polymers are generally cytotoxic to most cells. The methods (1) and (2) are appropriate for cell surface modification due to minor or no cytotoxicity. Some problems still remain to be overcome. For example, PEG-lipids were dissociated from the cell surface into the medium and gradually disappeared from the cell surface. Another type of amphiphilic PVA derivative carrying many long alkyl side chains was expected to be more stable due to interactions with the cell membrane at multiple points. PVA-alkyls, however, gathered at an area on the cell membrane, similar to the capping phenomena observed on lymphocytes treated with polyvalent antibodies.[14] The surface modification by covalent conjugation is useful, however, there is a potential to impair functions of membrane proteins when the surfaces are densely modified. Although covalent immobilization was expected to be stable for chemical degradation, introduced polymers and functional groups disappeared from the cell surface with time.[7,9,14] From these facts, cell surface modification can be applied to only short term usages, such as scavengers of immediate blood mediated inflammatory reactions and cell alignment for *in vitro* study, due to their short life time. Its cause is not clear yet. Dynamic behavior of the cell membrane, such as endocytosis, might deteriorate the polymer layer on the cell membrane, because the polymer anchored to the cell membrane simultaneously internalized into the cell. This should be overcome to realize the clinical application of cell surface modification to cell therapy. If so, it will have a strong impact on the therapies of various diseases, such as type I diabetes and disorders of the central nervous system such as Parkinson's, Alzheimer's, ALS, and Huntington's disease because it is necessary to prevent the graft loss from the attack by the recipient defence system.[59–66]

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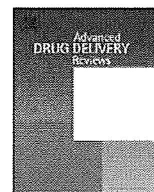

Bioartificial pancreas☆
Microencapsulation and conformal coating of islet of Langerhans

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ABSTRACT

Type 1 diabetes has been successfully treated by transplanting islets of Langerhans (islets), endocrine tissue releasing insulin. Serious issues, however, still remain. The administration of immunosuppressive drugs is required to prolong graft functioning; however, side effects of their long-term use on recipients are not fully understood, and cell transplantation therapy without the use of immunosuppressive drugs is desired. To resolve these issues, the encapsulation of isles with a semi-permeable membrane, or bioartificial pancreas, has been attempted. Many groups have reported that it functions very well in small animal models. Few of the bioartificial pancreases, however, were applied to human patients and their clinical outcome was not clear. In this review, we address obstacles and overview new techniques to overcome these issues, such as conformal coating and islet enclosure with cells.

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1. Introduction

Type 1 diabetes has been successfully treated by transplanting islets of Langerhans (islets), endocrine tissue releasing insulin [1,2]. The transplanted islets controlled insulin release in response to blood glucose concentration changes and can control glucose metabolism almost ideally. These promising results suggest efficacy of islet transplantation in clinical settings. Serious issues, however, still

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remain in the clinical application of islet transplantation, such as side effects of immunosuppressive drugs and a shortage of human donors. We expect that insulin-releasing cells will be derived from embryonic stem (ES) cells or induced pluripotent stem (iPS) cells in the near future [3]. A shortage of donors will not be a hurdle to the clinical application of cell therapy for diabetes. The administration of immunosuppressive drugs is required to inhibit immune reactions against allogeneic islets and control autoimmune reactions against insulin-releasing cells derived from iPS cells. The effects of their long-term use on recipients are not fully understood. Methods for cell transplantation therapy without the use of immunosuppressive drugs are desired. To resolve this issue, the encapsulation of islets with a semi-permeable membrane, or bioartificial pancreases, has been attempted, [4–6]. The membrane allows the permeation of oxygen, glucose, nutrients, waste products, and insulin, but inhibits the penetration of immunocompetent cells. Inhibition of contact between immunocompetent cells and the islets can compromise cell-mediated immunity. Various kinds of bioartificial pancreases have been developed. Few of the bioartificial pancreases, however, were applied to human patients and their clinical outcome was not clear. In this review, we address obstacles and overview new techniques to overcome these issues, such as conformal coating and islet enclosure with cells.

We review the recent progress of our works on the bioartificial pancreas.

2. Principle of the bioartificial pancreas

The idea of a bioartificial pancreas is derived from the success of cell transplantation at no vascularized sites, such as the brain ventricle or anterior chamber of the eye, which is called an immunoprivileged site. The principle of the bioartificial pancreas is shown in Fig. 1(a). Islets are encapsulated within a semi-permeable membrane and transplanted into a patient. The islets are expected to be isolated from the recipient's immune system, whereas oxygen, glucose, and nutrients are supplied through the membrane. Insulin secreted by the islets in response to changes in blood glucose levels can diffuse from the bioartificial pancreas to the recipient's body to maintain blood glucose metabolism. If the membrane suppresses the contact between immunocompetent cells and transplanted cells, a graft, especially an allogeneic graft, is expected to survive for a long period of time. Moreover, if the membrane suppresses the permeation of antibodies and complement proteins, a xenogeneic graft can survive in the host [7].

The graft survival after islet transplantation can be determined by the non-fasting blood glucose level and glucose tolerance test. When the blood glucose level does not reach the normoglycemia or it is returned to the hyperglycemia again after islet transplantation, islet transplantation has to be repeated. The same can be applied to the transplantation of microencapsulated islets.

3. Conventional bioartificial pancreas

3.1. Types of bioartificial pancreas and obstacles of clinical application

The history of the bioartificial pancreas extends for over 30 years [8]. Three major types of bioartificial pancreas have been developed: diffusion chamber, hollow fiber unit and microcapsule (Fig. 2). Some studies on these bioartificial pancreases will be briefly reviewed. The diffusion chamber and hollow fiber types of bioartificial pancreas are classified as a macrocapsule type. In diffusion chamber type pancreases, Nuclepore™ membranes with a pore size of 0.05–1 μm have been used as an immunoisolation membrane [9,10]. Hirotani et al. reported that normoglycemia was maintained for 30 weeks in streptozotocin (STZ)-induced diabetic rats by implanting a device that encapsulated mice pancreatic beta cell lines (MIN6) (xenotransplantation) [9]. Recently, Lee et al. reported human fetal pancreatic islet-like cell clusters (18–24 weeks) enclosed in devices made of poly (tetrafluorethylene) (PTFE) with a pore size of 0.4 μm that were transplanted at a subcutaneous site [10]. Ten weeks after transplantation into NOD mice, differentiated beta cell progenitors were found in the device. Although the progenitor cells were few, the blood glucose level was normalized, indicating efficacy. The hurdle for diffusion chamber types is the aggregation of islets in the space. When islets congregate and form larger aggregates, cells in the center are necrotic due to oxygen shortage. To prevent this issue, islets are suspended within alginate hydrogel prior to transfer into the device, which inhibits contact between islets [11]. The size of the device is an obstacle when applying the technology to human patients. The average islet diameter is roughly 150 μm. To suppress islet contact in the device (diameter should be less than 300 μm), the islets must be aligned in, at most, two layer sheets in the chamber. In clinical settings, more than 5×10^5 islet equivalents (IEQ) should be packaged into a chamber. The diameter of the device would be at least 30 cm when the islets are ideally deposited in a layer. A transplantation site in the human body that is large enough for such large devices is difficult to find.

Maki et al. developed a hollow fiber type bioartificial pancreas that is suitable for implantation into large animals [12,13]. The device consists of a chamber passing a semi-permeable tubular membrane that is connected to vascular grafts. Islets are placed between the housing chamber and the tubular membrane. The molecular weight cut-off for the tubular membrane is 80 kDa. Diabetes was controlled by implanting the device in an allotransplantation model, severely

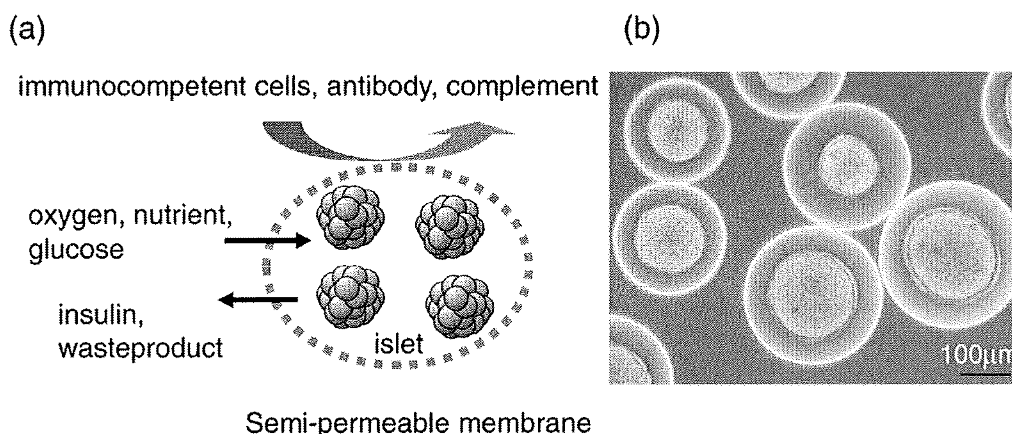

Fig. 1. Bioartificial pancreas. (a) The concept of the immunoisolation membrane. (b) Islets enclosed in agarose microcapsules.

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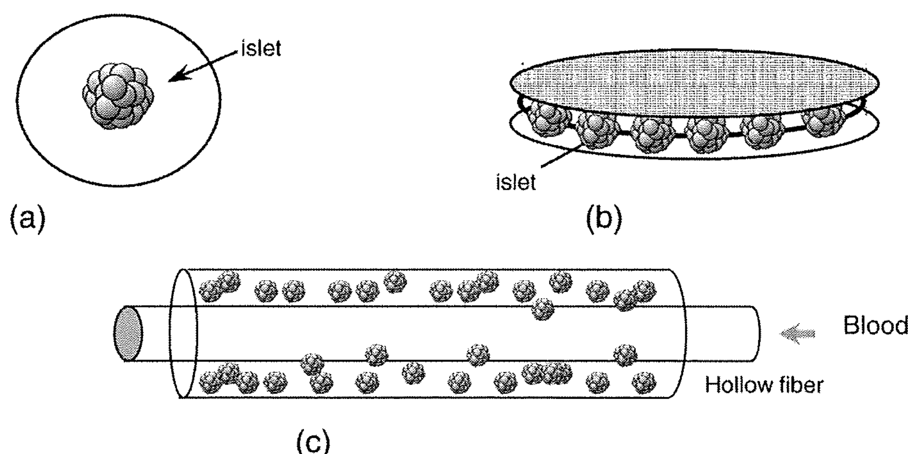

Fig. 2. The conventional bioartificial pancreas types. (a) Microcapsule, (b) diffusion chamber, and (c) hollow fiber-based diffusion chamber.

diabetic dogs that had undergone total pancreatectomies [12]. The efficacy of the device was also shown in xenotransplantation [13]. Blood coagulation should be controlled to inhibit thrombus formation in a hollow fiber unit. Aspirin was systematically administered in Maki et al.'s animal study. However, it is difficult to apply these methods in a clinical environment because systemic administration of anticoagulants is associated with an increased risk of bleeding. We meet serious difficulties in the diffusion chamber and the hollow fiber unit as mentioned above, therefore, recent studies have focused on the microcapsule type pancreases.

The microencapsulation of islets with alginate hydrogel was first reported in 1980 [14]. Islet-containing alginate solution is dropped through a nozzle into isotonic calcium solution to prepare an alginate capsule entrapping one or two islets. The surface is further coated with poly-L-ornithin and alginate for stabilization. There are many reports of successful *in vivo* experiments [15–17]. For example, porcine islets encapsulated within alginate capsules (30,000–70,000 capsules) have been transplanted into the intraperitoneal cavity of spontaneously diabetic monkeys [17]. Normoglycemia was then maintained in seven monkeys for 120–804 days without immunosuppressive therapy. The appropriate size of microcapsule is different for transplantation site. For the large space such as peritoneal cavity and subcutaneous site, the microcapsule size can be increased up to approximately 500 μm although the total implant volume would be expanded accordingly. For the transplantation into liver through portal vein, the size should be made as small as possible. Therefore, it is necessary to apply to islets with conformal coating.

Although many groups have been working on alginate microcapsules, there are still uncertainties. Some results, especially using large model animals, are hardly reproduced. Alginate microcapsules could not be transplanted into liver through the portal vein due to their large diameter. They have been transplanted into a peritoneal cavity in most of animal models. After transplantation, they form a large cluster, like a rice ball, and thus islets except those existing periphery of the cluster might be lost due to insufficient oxygen supply.

3.2. Islet encapsulation with agarose beads

We have been working on an agarose hydrogel to microencapsulate islets [18–22]. In test tubes, the islets are suspended in an agarose solution, warmed to 40 °C, and agitated to suspend the agarose solution in liquid paraffin. The agarose solution droplets were induced to gel by placing the tubes on ice, encapsulating the islets within agarose beads. Average diameters of islets and microcapsules which are shown in Fig. 1 are 140 μm and 260 μm, respectively. The transplantation of the agarose-encapsulated islets (Mic islets) was examined to evaluate their immunoisolation in allotransplantation. The effect of microencapsulation on islet allograft survival was determined using STZ-induced diabetic mice and NOD mice as recipients. All five STZ-induced diabetic BALB/c mice receiving Mic islets (from C57BL/6) maintained normoglycemia more than 100 days. When NOD mice were used as recipients, the blood glucose level in four of five grafts (islets from C3H/He mice) could be normalized for 80–200 days. The agarose hydrogel can effectively prolong allograft functioning in the STZ-diabetic mouse, and even in the NOD mouse, without the use of immunosuppressive drugs. These results suggest possibility that the agarose hydrogel membrane can be applied to transplantation of allogeneic islets for treating type I diabetic patients without immunosuppressive therapy.

The feasibility of the immunoisolation of the agarose microcapsule was also examined for the prevention of autoimmune recurrence after islet transplantation [22]. Islets were isolated from pre-diabetic NOD mice (6–8 weeks old) and microencapsulated in a 5% agarose hydrogel. The Mic islets were syngeneically transplanted into spontaneously diabetic NOD mice. The naked islet grafts were destroyed, and diabetes recurred within 2 weeks of transplantation in all 12 mice. On the other hand, 13 of the 16 mice implanted with Mic islets maintained normoglycemia for more than 100 days after transplantation of Mic islets. The microcapsulated islets exhibited well-granulated β cells with no mononuclear cell infiltration around the microcapsules or in the blood capillaries between the microcapsules. Agarose microcapsules were able to effectively protect NOD islets from autoimmune destruction.

In the near future, diabetic patients will be treated by transplantation of insulin-releasing cells which would be from iPS cells produced from patients' own cells. In this case, the cells should be protected from autoimmune destruction. Autoimmune reaction is hardly controlled even by administration of immunosuppressive drugs. The results obtained using NOD mice indicate that the agarose microcapsule which effectively protect cells from autoimmune destruction will be useful in transplantation of insulin-releasing cells derived patients own iPS cells.

3.3. Cryopreservation of bioartificial pancreas

Despite a number of reports of successful islet transplantation, several issues need to be resolved before the bioartificial pancreas becomes an accepted medical device. One of the requirements is a banking system [23–27] that allows for the long-term storage of bioartificial pancreases containing large quantities of living islets. There have been some reports on cryopreservation of microencapsulated

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