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Immobilization of anticoagulant-loaded liposomes on cell surfaces by DNA hybridization

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ABSTRACT

An unresolved obstacle in transplantation of islets of Langerhans is the early graft loss caused by thrombotic reactions on the surface of islets after intraportal transplantation. We investigated a versatile method for modifying the surface of islets with liposomes carrying the anticoagulant argatroban using an amphiphilic poly(ethylene glycol)-phospholipid conjugate derivative (PEG-lipid) and DNA hybridization. Argatroban was gradually released from the liposomes on the islets, and antithrombic activity was detected in culture medium. Modified islets retained the ability to control insulin release in response to glucose concentration changes. Although we mainly examined surface modification of islets, this technique may be useful for immobilizing various types of small molecules on cells and tissues and thus may have many applications in cell therapy and regenerative medicine.

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1. Introduction

Type 1 diabetes is caused by the autoimmune destruction of insulin-producing pancreatic beta cells and inevitably leads to dependence upon exogenous insulin for control of blood glucose. Transplantation of islets of Langerhans (islets) has been proposed as a safe and effective treatment for type 1 diabetes patients and has been performed clinically for more than 15 y. Increased rates of insulin independence and glycemic stability after intraportal transplantation of islets have been achieved using the Edmonton protocol. However, some problems remain unresolved. For example, several pancreas donors are still necessary to achieve insulin independence in one recipient because more than 50% of islets are destroyed immediately after intraportal transplantation [1] [2], and [3]. Early graft loss is reported to be caused by the instant blood mediated inflammatory response (IBMIR) [4] and [5].

Systemic administration of anticoagulants and thrombin inhibitor have been administered to control IBMIR [6], [7], [8] and [9], and urokinase and soluble domains of thrombomodulin have been immobilized on islet surfaces [10], [6], [11], [12], [13], [14] and [15]. The efficacy of these techniques has been examined in animal islet transplantation models, but a standard protocol has not been yet established. In previous investigations, high molecular weight substances, such as heparin, thrombomodulin, and urokinase, were immobilized on islets. Low molecular weight drugs that act as inhibitors of blood coagulation enzyme, platelet, and complement are good candidates for immobilization and subsequent controlled release from the surface of islets. However, the methods that were used for immobilization of high molecular weight substances cannot be applied to immobilizing low molecular weight drugs on islet surfaces.

In this report, we investigated a new method to immobilize a small drug on the cell surface with subsequent sustained release from the cells. Liposomes, which can be used to encapsulate small drugs inside of a membrane, were used as the drug carrier. Poly (ethylene glycol)-phospholipid conjugates (PEG-lipids) carrying ssDNA (oligo$(dT)_{20}$ and oligo$(dA)_{20}$) were used to immobilize liposomes on the cell surface. When oligo$(dT)_{20}$- and oligo$(dA)_{20}$-PEG-lipids are applied to cells and liposomes, respectively, their lipid portions anchor into the lipid bilayers of cell membranes and liposomes; thus, the cells and the liposomes present oligo$(dT)_{20}$ and oligo$(dA)_{20}$ on their surface. We hypothesized that liposomes would be immobilized on the cell surface by hybridization between oligo$(dT)_{20}$ and oligo$(dA)_{20}$ when cells and liposomes were mixed. We applied this method to immobilize the thrombin inhibitor argatroban on the surface of islets and detected the release of argatroban by measuring antithrombin activity in the culture medium of the islets.

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2. Materials and methods

2.1. Materials

α-*N*-Hydroxysuccinimidyl-ω-maleimidyl poly(ethylene glycol) (NHS-PEG-Mal, MW 5000) and 1,2-dipalmitoyl-*sn*-glycerol-3-phosphatidylethanolamine (DPPE) were purchased from NOF Corporation (Tokyo, Japan). Chloroform, dichloromethane, triethyl amine, diethyl ether, heparin sodium salt, and penicillin–streptomycin mixed solution (PC/SM) were purchased from Nacalai Tesque (Kyoto, Japan). RPMI 1640 medium and Dulbecco's phosphate-buffered saline (PBS) were purchased from Invitrogen (Carlsbad, CA) and Nissui Pharmaceutical Company (Tokyo, Japan), respectively. Fetal bovine serum (FBS) was purchased from BioWest (Miami, FL, USA), and enzyme-linked immunosorbent assay (ELISA) kits for insulin were purchased from Shibayagi Company (Gunma, Japan). 1-Palmitoyl-2-[12-[(7-nitro-2-1, 3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphoethanolamine (NBD-lipid) was purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). L-α-phosphatidylcholine from egg yolk, Type XVI-E (EggPC), Oligo(adenine)$_{20}$ (oligo(dA)$_{20}$), and oligo(thymine)$_{20}$ (oligo(dT)$_{20}$) carrying a protected SH group at the 5′-end (oligo(dA)$_{20}$-SH, oligo(dT)$_{20}$-SH) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The protecting group was removed from the -SH group by reduction of the disulfide bond with dithiothreitol (DTT) according to the manufacturer's instructions. Argatroban [16], a thrombin inhibitor, was purchased from Yoshindo, Inc. (Toyama, Japan). The SensoLyte 520 Thrombin Activity Assay Kit was purchased from AnaSpec, Inc. (Fremont, CA), and the Phospholipids C-test Kit was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Nitrocellulose membranes (0.8 μm pore size), Millex-GP 0.22 μm filter units, and Isopore 0.1 μm membrane filters were purchased from Millipore Co. (Billerica, MA, USA). Sephadex G-25 M was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). CCRF–CEM cells (established from acute lymphoblastic leukemia cells) were obtained from the Health Science Research Resources Bank (Tokyo, Japan).

2.2. Synthesis of oligo(dA)$_{20}$– or oligo(dT)$_{20}$– conjugated PEG lipids

Oligo(dA)$_{20}$- or oligo(dT)$_{20}$-conjugated PEG-lipids (oligo(dA)$_{20}$-PEG-DPPE or oligo(dT)$_{20}$-PEG-DPPE) (Scheme 1(a)) were synthesized following the method reported previously [13,15]. They were used for the surface modification of liposomes and cells without any further purification.

2.3. Preparation of liposomes

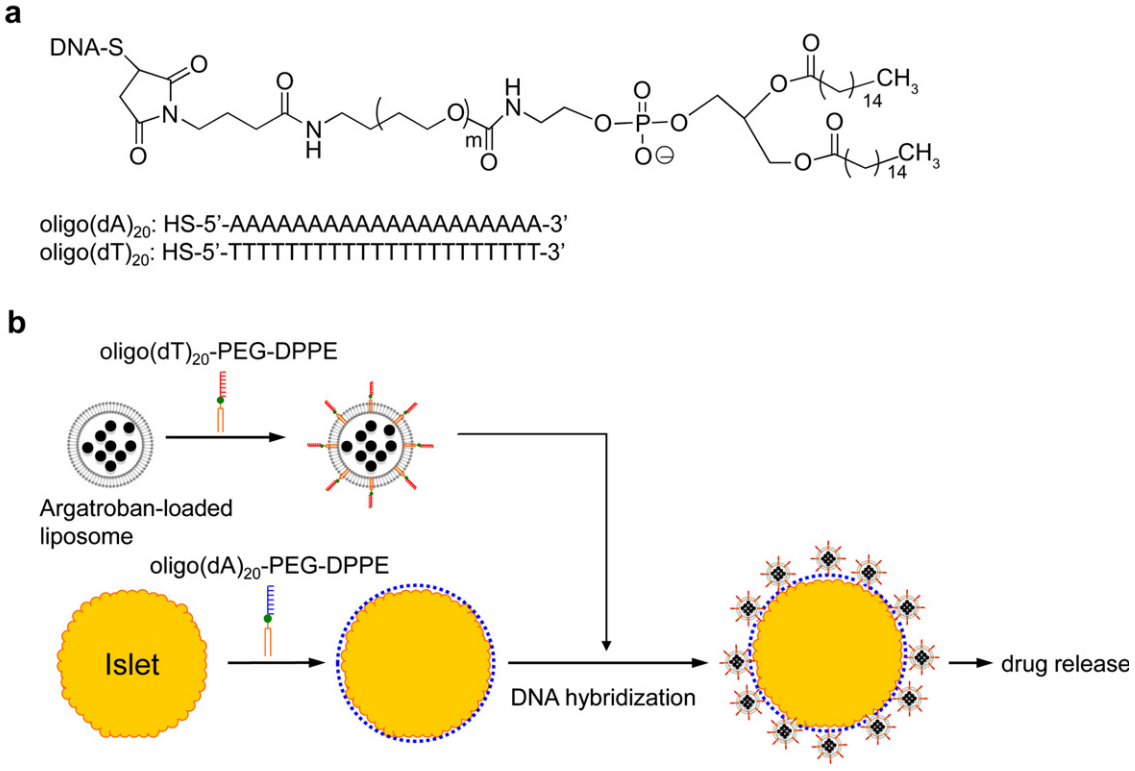
EggPC (20 mg) was dissolved in 3.0 mL of chloroform, and the solution was dried in a vacuum to prepare a dry thin lipid film on the inner surface of a round-bottom flask using a rotary evaporator. The lipid film was hydrated with 2 ml of 0.1 mM argatroban in PBS solution and then the suspension was vigorously stirred for 3 h at room temperature (RT) to prepare the lipid vesicles. The suspension was extruded through a series of membrane filters with different pore sizes—0.8 μm, 0.22 μm (two times), and 0.1 μm (ten times)—to form small unilamellar vesicles or liposomes. The diameter of the liposomes was determined by dynamic light scattering (DLS-7000; Otsuka Electronics Co., Ltd, Osaka, Japan). The resultant liposome suspension was applied to Sephadex G25 M to separate the liposomes carrying argatroban (liposome-argatroban) from free argatroban. The fraction was collected every 1.0 mL. The concentrations of argatroban and lipid in each fraction were determined by UV-VIS absorption at 331 nm (DU 640; Beckman Coulter Inc., Brea, California, USA) and with the Phospholipids C-test Kit, respectively. To prepare fluorescence-labeled liposomes, 20 mg of EggPC in 2 mL of chloroform was mixed with 0.4 mg of fluorescence-labeled lipid (NBD-lipid) yielding a final concentration of 1.8 mol%. The procedures to prepare small unilamellar liposomes carrying NBD (liposome-NBD) were same as those described above. Oligo(dT)$_{20}$ was introduced to the liposome surface by adding 20 μL of oligo(dT)$_{20}$-PEG-DPPE (0.5 mg/mL in PBS) to the liposome-argatroban suspension (10 mg/mL in PBS) and incubating for 2 h at RT.

2.4. Immobilization of oligo(dA)$_{20}$ on cell surfaces

CCRF–CEM cells were cultured 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. CCRF–CEM cells were collected by centrifugation. After 25 μL of oligo(dA)$_{20}$-PEG-lipid (0.5 mg/mL in PBS) solution was added to the cell pellet (1.0×10^6 cells), the suspension was incubated with gentle agitation for 1 h at RT. The cells were washed with PBS and collected by centrifugation. A suspension of liposome modified with oligo(dT)$_{20}$-PEG-lipid and NBD (oligo(dT)$_{20}$-liposome-NBD) (25 μL, 10 mg/mL in PBS) was added to the cells, and the cell suspension was left to incubate for 10 min at RT. The cells carrying liposome-NBD were washed with PBS and collected by centrifugation. The cells were resuspended in 2 mL of culture medium, observed over time under a confocal laser scanning microscope, and analyzed by a fluorescence-activated cell sorter (FACS) (Guava EasyCyte Mini; Millipore, Billerica, MA, USA) equipped with a 488 nm diode laser. Data collected from 50,000 cells were used to generate a histogram. Untreated cells were used as a negative control.

2.5. Immobilization of liposomes on islet surfaces by DNA hybridization

All animal experiments were approved and accepted by the animal care committee of the Institute for Frontier Medical Sciences, Kyoto University, Japan.



Scheme 1. Schematic illustration of immobilization of liposomes onto islet surfaces. (a) Chemical structure of ssDNA-PEG-lipid conjugate. (b) Schematic illustration of immobilization of liposomes onto islet surfaces via ssDNA-PEG-lipids. ssDNA-PEG-lipid anchored to the membrane through hydrophobic interactions between the alkyl chains of ssDNA-PEG-lipid and the lipid bilayer. Oligo(dT)$_{20}$ on liposomes hybridized with oligo(dA)$_{20}$ at the end of PEG chains on the islet surface.

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