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Protocols

Efficient delivery of signal-responsive gene carriers for disease-specific gene expression via bubble liposomes and sonoporation

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ABSTRACT

Sonoporation is a promising method to intracellularly deliver synthetic gene carriers that have lower endocytotic uptake than viral carriers. Here, we applied sonoporation to deliver genes via polyethylene glycol (PEG)-grafted polymeric carriers that specifically respond to hyperactivated protein kinase A (PKA). PEG-grafted polymeric carrier/DNA polyplexes were not efficiently delivered into cells via the endocytotic pathway because of the hydrophilic PEG layer surrounding the polyplexes. However, the delivery of polyplexes into cells was significantly increased by sonoporation. The delivered polyplexes exhibited PKA-responsive transgene expression in PKA-overexpressing cells, but not in cells with low PKA activation. These results show that the sonoporation-mediated delivery of PEG-modified PKA-responsive polyplexes is a promising approach for safely applying gene therapy to abnormal cells with hyperactivated PKA.

1. Introduction

The delivery system of suicide genes for cancer therapy requires exceptional cancer cell selectivity because genes encoding toxic proteins will destroy all transfected cells [1,2]. Thus, artificial gene carriers modified with specific ligand molecules for cancer cells have been developed to exclusively target tumor cells [3–5]. However, the non-specific distribution of gene carriers to normal healthy cells is inevitable.

With the aim of creating novel gene carriers with cancer cell specificity, we have developed polymeric carriers that will release therapeutic genes in response to dysregulated intracellular signaling molecules in malignant cells [6–9]. Dysregulated intracellular signaling is frequently caused by the hyperactivation of certain protein kinases (PKs). These carriers contain a main chain polymer grafted with PK-specific cationic peptide substrates as a side chains, which form polyplexes with plasmid DNA (pDNA) through electrostatic interactions. When the peptide substrates are phosphorylated by hyperactivated PKs in abnormal cells, the cationic charge of the peptides is reduced, causing the polyplexes to dissociate, which allows activation of transgene expression. These

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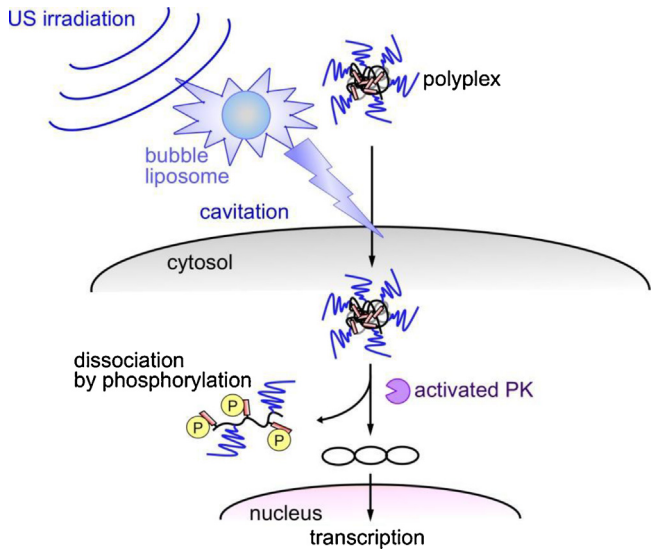

Fig. 1. The sonoporation-mediated delivery of PEG-modified PK-responsive polyplexes. PEG-modified carriers show significantly increased stability, but reduce endocytotic uptake because their low affinities to the cell surface. However, the delivery of PEG-modified PK-responsive polyplexes can be significantly enhanced by sonoporation. After the sonoporation-mediated transfection of polyplexes, the polyplexes are phosphorylated in abnormal cells with hyperactivated PKs. These reactions reduce the cationic charge of peptides, resulting in increased dissociation of polyplexes that allows activation of transgene expression.

polyplexes have been successfully applied for intracellular signal-responsive transgene expression in vivo and in vitro [6–9].

In a previous report, we improved the colloidal stability of such polyplexes by incorporating polyethylene glycol (PEG) onto the main chain of these polymeric carriers [10]. PEG-modified carriers showed significantly increased stability, but reduced affinity to the cell surface, resulting in less endocytotic uptake. To solve this problem, we used sonoporation to deliver PEG-modified PK-responsive gene carriers into cells (Fig. 1). Sonoporation utilizes microbubbles to sensitize ultrasound (US) irradiation [11–13], which induces microbubble explosion, also known as cavitation [14]. The high pressure produced by cavitation forms transient pores in the cell membrane, which increase membrane permeability for macromolecules, including pDNA [11], siRNA [15], decoy DNA [16], and quantum dots [17]. Sonoporation-mediated delivery is advantageous to endocytotic delivery because it reduces lysosomal pDNA degradation by directly transferring genes into the cytoplasm [11–16].

Furthermore, the cyclic AMP-dependent protein kinase A (PKA) is a serine/threonine protein kinase and exists in the cytosol as an inactivated form, in which the regulatory domain interacts with the catalytic domain. After extracellular stimulation, PKA is activated by the binding of cyclic AMP to the regulatory domain of PKA and the alternation of its conformation. The activated PKA phosphorylates target molecules (e.g., peptide substrate and proteins) in the cytosol and these phosphorylation reactions lead to the stimulation of downstream signaling pathways. The activated PKA participates in proliferation, differentiation, metastasis, survival of several cancers such as melanoma, breast cancer, and colon cancer [18–20]. The Kemptide (LRRA**S**LG), a peptide substrate derived from porcine liver pyruvate kinase, is commonly used as a specific substrate for PKA [21].

In this study, we investigated whether the sonoporation-mediated delivery of PEG-grafted polymeric carrier/pDNA polyplexes increased transgene expression in PKA-overexpressing cells via a new microbubble, bubble liposome (BL), which is a submicron-sized liposome encapsulating perfluoropropane [11,12].

2. Materials and methods

2.1. Synthesis of the PEG-modified PKA-responsive polymer

The PEG-modified PKA-responsive polymer was synthesized as described in a previous report [10]. Briefly, 2,2′-azobis (2-methylpropionitrile; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) as an initiator was added into a solution containing *N*-isopropylacrylamide (NIPAM; Wako) and *N*-acryloyloxysuccinimide (NAS; Kokusan Chemical Co., Ltd., Tokyo, Japan) in anhydrous *N,N*-dimethylformamide (DMF; Kanto Chemical Co., Ltd., Tokyo, Japan), and polymerization was carried out at 60 °C for 18 h under a nitrogen atmosphere. Poly(NIPAM-*co*-NAS) was collected by re-precipitation from diethyl ether and dried in vacuo. The molar ratio of each monomeric unit was determined by ^{1}H NMR.

To graft protected peptides onto the poly(NIPAM-*co*-NAS) main chain, poly(NIPAM-*co*-NAS) dissolved in DMF/chloroform (1:1, v/v) was mixed with side group-protected peptides, and the solution was stirred at room temperature for 3 d. The resulting polymers were collected by re-precipitation from diethyl ether and dried in vacuo. These polymers were dissolved in DMF/chloroform, and amino-terminated PEG (MW: 5000; NOF Co., Ltd., Tokyo, Japan) was added to the solution to modify PEG onto the polymer. This reaction was carried out at room temperature for 3 d with stirring. To cap the remaining activated ester groups, ten-equivalents of isopropylamine (Wako) were added to the solution, and the resulting mixture was stirred at room temperature for 3 d. The polymers were collected by re-precipitation from diethyl ether and dried in vacuo. To remove the protecting groups from the grafted peptide, the polymers were treated with 94% TFA (Kanto Chemical) containing 2.5% triisopropylsilane, 2.5% water and 1.0% 1,2-ethanedithiol (Sigma-Aldrich, St. Louis, MO, USA). After re-precipitation from diethyl ether, the resulting polymers were dialyzed for 3 d against water using a dialysis membrane bag with a M_w cut-off of 10,000 Da. The peptide and PEG contents of the polymers were determined by the UV-absorption of tryptophan (ε_{280} = 5500 M^{-1} cm^{-1}) and elemental analysis, respectively.

2.2. Preparation of BLs

BLs were prepared as described in a previous report [15]. Briefly, PEG liposomes comprising 1,2-dipalmitoyl-*sn*-glycero-phosphatidylcholine (NOF Co., Ltd.) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-polyethyleneglycol (DSPE-PEG$_{2000}$; NOF Co., Ltd.) at a molar ratio of 94:6 were prepared by reverse-phase evaporation. Liposome size was adjusted to 200 nm using extruding equipment and a sizing filter (pore size: 200 nm). The liposome concentration was calculated using a phospholipid C test (Wako). BLs were prepared from liposomes and perfluoropropane gas.

2.3. Cell culture

The HepG2 cell line was purchased from Health Science Research Resources Bank. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako) containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin-B (all from Invitrogen, Carlsbad, CA, USA). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air.

2.4. Sonoporation-mediated transfection

HepG2 cells were cultured in 48-well culture plates (1.5×10^4 cells/well) for 2 d in 0.2 mL DMEM containing 10% FBS. The medium was replaced with 500 μL Opti-MEM (Invitrogen) containing poly-