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Opsonized erythrocyte ghosts for liver-targeted delivery of antisense oligodeoxynucleotides

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ABSTRACT

The use of antisense oligodeoxynucleotides (AS-ODNs) in therapeutic applications requires the development of appropriate analysis and delivery systems. Here, we report a quantitation method and a carrier-mediated AS-ODN delivery system. AS-ODN levels were quantitated using an enzyme-linked immunosorbent assay (ELISA) in which biotinylated AS-ODNs bound to streptavidin-coated plates were detected by binding of a complementary, dinitrophenol-labeled detector ODN. The ELISA-based assay could detect AS-ODNs at the femtomole level. AS-ODN delivery systems based on opsinized erythrocyte ghosts (EGs) were developed using various combinations of hypotonic solution and resealing buffer to optimize AS-ODN encapsulation efficiencies. AS-ODN and polyethyleneimine (PEI) complex formation did not affect encapsulation into EGs. The ELISA-based assay showed that the pharmacokinetics of AS-ODNs differed significantly among the various delivery methods. Opsonized EG-encapsulated AS-ODNs exhibited a mean residence time (MRT) significantly shorter than AS-ODN encapsulated in EGs. The biodistribution of EG-loaded AS-ODNs depended on opsonization, with opsonized EG carriers producing 4.5-fold higher levels of AS-ODN in the liver compared with unopsonized EGs. These results indicate that opsonized EGs can be used for liver-targeted delivery of AS-ODN and suggest that an ELISA-based method may be useful for studying the *in vivo* fate of AS-ODNs.

1. Introduction

Antisense oligonucleotides (AS-ODNs) are single-stranded DNA molecules complementary to regions of a target gene that specifically inhibit gene expression by hybridizing to the gene's mRNA [1]. Owing to their potential to selectively downregulate single genes, AS-ODNs have been studied as one of a new generation of nucleic acid therapeutics. One such antisense drug, fomivirsen, has received approval for ocular therapy of human cytomegalovirus retinitis [2], and several other AS-ODNs are undergoing clinical trials.

Despite this progress, the issue of *in vivo* AS-ODN delivery remains a significant hurdle in the development of AS-ODNs as therapeutics. Owing to their negative charges and molecular size, AS-ODNs cannot effectively cross cell membranes on their own. They also suffer from short half-lives *in vivo* because they are rapidly secreted through the kidneys or degraded by enzymes in the serum. To overcome these problems, a number of delivery systems have been designed to protect nucleotide molecules from enzymatic degradation in the blood stream and deliver AS-ODNs to target tissues studied [3,4]. Crucial attributes of an effective AS-ODN carrier system are biocompatibility and biodegradability. Erythrocyte ghosts (EGs) satisfy both of these criteria and have been studied as potential plasmid DNA delivery systems [5]. In a previous study from our laboratory, plasmid DNA-loaded EGs effectively delivered encapsulated DNA into the blood, where they were maintained in circulation for an extended period. However, altering the distribution pattern of EGs to achieve liver targeting of AS-ODN may require modifications to the delivery system.

In parallel with the development of AS-ODN delivery systems, it is necessary to develop effective methods for quantifying AS-ODNs. Several such methods have been tested. These include capillary gel electrophoresis, which has been studied to quantify oligonucleotides in human plasma [6]; liquid chromatography and mass spectrometry, which have been developed to analyze oligonucleotides from rat plasma [7]; and surface plasmon resonance spectrometry,

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which has been used to detect oligonucleotides by an enzyme-catalyzed precipitation reaction [8]. However, owing to complexity and sensitivity problems associated with these methods, a need for a more convenient method for analyzing AS-ODNs remains.

Thus, the goals of this study were 2-fold: to develop an EG-based delivery system capable of targeting AS-ODNs to the liver, and to develop a simple and sensitive assay for quantifying AS-ODNs. Here, we report that AS-ODNs can be targeted to the liver using opsonized EGs as carriers and can be quantitated using an enzyme-linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. Marker-labeled ODNs

Various markers were used to label 18-mer ODNs. The marker-labeled ODNs were all custom-synthesized by Bioneer Co. (Daejeon, South Korea). The 5′-end of the AS-ODN was covalently linked to biotin (Fig. 1A). The detector ODN, with a sequence complementary to the AS-ODN, was 5′-labeled with dinitrophenol (DNP) (Fig. 1B). Fluorescein isothiocyanate (FITC)-labeled AS-ODNs, also purchased from the Bioneer Co., were used for fluorescence microscopy.

2.2. Quantitation of AS-ODNs by ELISA

AS-ODNs were quantitatively detected using modified standard ELISA techniques. Biotin-labeled AS-ODNs were serially diluted with phosphate-buffered saline (PBS) and added to streptavidin-coated 96-microwell plates (Nunc, Rochester, NY, USA). Following a 2-h incubation at 37 °C, each well was washed three times with PBS containing 0.1% Tween-20 and diethylpyrocarbonate (PBST-DEPC), after which 100 μL of the DNP-labeled detector ODN was added to each well and plates were incubated for 1 h at 18 °C. After washing each well three times with PBST-DEPC, a murine anti-DNP antibody (Sigma-Aldrich, St. Louis, MO, USA; 1/2500 dilution) was added and plates were incubated for 1 h at 18 °C. The plates were then washed three times with PBST-DEPC, after which 100 μL of 1/1000 diluted goat anti-mouse IgE-HRP antibody (ABD-Serotech, Oxford, UK) was added and plates were incubated for 1 h at 18 °C and then washed five times. One-hundred microliters of 1-step Turbo TMB-ELISA™ (Pierce, Rockford, IL, USA) were added to each plate and the plates were incubated for 2 min at room temperature. Finally, 100 μL of 1 N H_2SO_4 were added to each well and the amount of chromogen present was measured spectrophotometrically at 450 nm (Fig. 2).

2.3. Encapsulation of AS-ODNs in EGs

AS-ODNs were loaded into EGs by the hypotonic shock technique as previously described [5], with modifications. AS-ODNs in the naked form or as cationic polymer complexes were used for encapsulation into EGs. Biotin- or FITC-labeled AS-ODNs (1 nmol) were diluted in 20 μL hypotonic sodium phosphate buffer or 0.45% NaCl solution (Table 1) and complexed with linear 25 kDa polyethyleneimine (PEI, Polysciences, Warrington, PA, USA) at an N:P ratio of 10:1. EGs were prepared from whole blood collected from 8-week-old female BALB/c mice (Orient Bio Inc., Seongnam, South Korea). An aliquot (500 μL) of whole blood was mixed with 10 mL PBS and centrifuged at 230 × g for 10 min at 4 °C. The resulting red blood cell (RBC) pellets were suspended in PBS, washed three times (with PBS) and suspended in hypotonic solutions containing PEI/AS-ODN complexes. After incubating for 15 min at room temperature, 100 μL of resealing solution (Table 1) were added and tubes were incubated at 37 °C for 1 h. The resulting AS-ODN-loaded EGs were washed three times with PBS to remove free AS-ODNs. Loading of AS-ODNs into EGs was qualitatively confirmed by observing an aliquot of EGs containing FITC-labeled AS-ODNs under a laser-scanning confocal microscope (LSM 510 META, Carl Zeiss, Germany). In some experiments, EGs loaded with FITC-labeled AS-ODNs were observed by *z*-axis slice processing under the confocal microscope.

2.4. Quantification of AS-ODNs in EGs

AS-ODNs in EGs were quantified by spectrofluorometry and ELISA using FITC-labeled AS-ODN and biotin-labeled AS-ODN, respectively. EGs loaded with FITC-labeled AS-ODNs were placed in black 96-microwell plates (SPL Life Science, Gyeonggi-do, South Korea), and the intensity of each well was read at excitation and emission wavelengths of 485 and 535 nm, respectively, using a UV spectrofluorophotometer microplate reader (Zenith 3200, Anthos Co., Salzburg, Austria). AS-ODN standard curves for spectrofluorometric analysis were prepared using serial dilutions of FITC-labeled AS-ODN. For ELISA-based quantification of AS-ODNs in EGs, biotin-labeled AS-ODN-loaded EGs were lysed using a Triton X-100 solution and serially diluted. EG lysates were added to streptavidin-coated 96-microwell plates, and analyzed as described above. The encapsulation efficiency of AS-ODNs in EGs was expressed as a percentage after dividing the amount of AS-ODN in EGs with the initial amount of AS-ODN before entrapment.

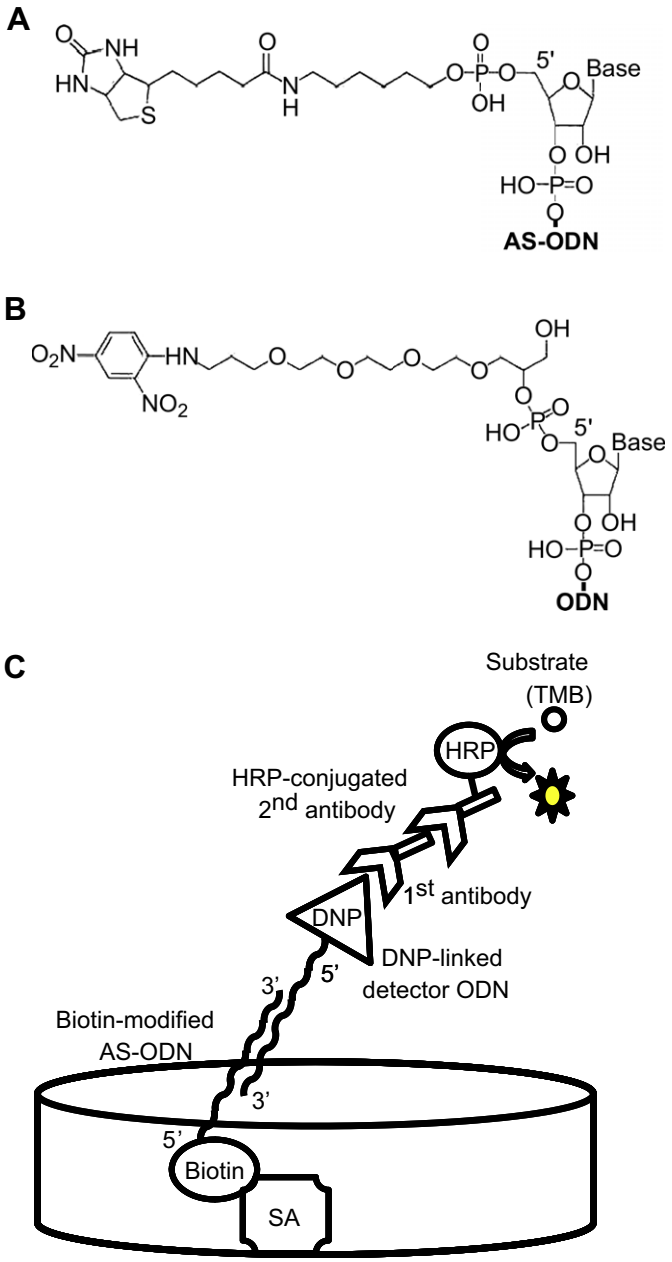

Fig. 1. Modification of ODNs and schematic representation of the ELISA-based assay. (A) The 5′-end of the AS-ODN was biotinylated. (B) The 5′-end of the detector ODN was labeled with DNP. (C) The ELISA reaction scheme (see Section 2 for details). Biotinylated AS-ODNs, added to the streptavidin-coated plate, were detected and quantitated by complementary binding of detector ODNs followed by a colorigenic enzyme–substrate reaction.

2.5. Opsonization and release test

For opsonization, the ODN-entrapped EGs were incubated with rabbit anti-mouse RBC antibody (Fitzgerald, MA, USA; 1/50 dilution) for 1 h at 37 °C, and washed three times with DEPC-PBS. For release test of AS-ODN from opsonized EG, 100 μL of the biotin-labeled AS-ODN-loaded EGs were incubated in 1 mL of PBS for various times. At each time point, the sample was centrifuged for 1 min and the pellets were harvested. The levels of AS-ODN in the pellets were analyzed by the ELISA-based quantification system.

2.6. Stability test against DNase I

To test the entrapment of AS-ODN inside EGs, the stability of AS-ODN to DNase I was measured. Twenty picomoles of AS-ODN in naked form or PEI complexes were incubated with or without 1 unit of DNase I in DNase reaction buffer (Promega, Madison, WI, USA) at 37 °C for 30 min. For comparison, EGs containing 20 pmol of