

Glucan Particles as an Efficient siRNA Delivery Vehicle

E. Soto, G. Ostroff*

Program in Molecular Medicine, University of Massachusetts Medical School, Worcester MA,
gary.ostroff@umassmed.edu

ABSTRACT

RNA interference (RNAi) is a new, promising approach to selectively regulate gene expression through modulating mRNA stability and translation. However, the efficient delivery of dsRNA molecules (siRNA) to affect RNAi remains a challenge. Here we report on the development of a new delivery technology based on the *in situ* layer by layer synthesis of siRNA containing nanoplexes encapsulated within hollow, porous glucan particles (GPs). GPs provide for receptor-mediated oral bioavailability and macrophage targeting of nanoplexed cargos, such as siRNA.

Keywords: glucan particles, siRNA delivery, layer by layer synthesis, encapsulated nanoparticles.

INTRODUCTION

RNA interference (RNAi) is a powerful process to regulate gene expression. It is based on short (19-27 nucleotides), double-stranded RNA molecules (small interfering RNA or siRNA) that silence gene expression by inducing a sequence-specific degradation of messenger RNA [1-3]. Application of RNAi has the potential of becoming a breakthrough for the development of gene specific disease therapies. The current limitation to realize the promise of RNAi is efficient siRNA delivery, as highly negatively-charged siRNAs on their own are unstable and not efficiently taken up by most cells. A variety of delivery technologies have been studied both *in vitro* and *in vivo*; including lipid-based transfection formulations, nanoparticle based drug delivery systems, hydrodynamic injection, fusion proteins, and methods derived from chemical modification of siRNA (i.e. covalent binding of the passenger strand of siRNA to cholesterol to facilitate uptake through cell-surface LDL receptors) [2-3]. These delivery methods have propelled RNAi to become a transformative research tool and initial clinical development efforts have focused on applications not limited by efficient delivery [reviewed in 3].

The formation of stable complexes between negatively charged genetic material and cationic polymers is the most commonly studied approach to develop non-viral delivery agents for DNA-based therapies [4]. A similar approach is applicable to siRNA, as cationic polymers are capable of

protecting siRNA during delivery to cells, and to release them inside the endosome. Previously, we have reported on a DNA delivery method based on the use of DNA nanocomplexes encapsulated inside glucan particles (GPs) [5-6]. GPs are hollow, porous 2-4 micron microspheres prepared from Baker's yeast cell walls, composed primarily of beta 1,3-D-glucan and low levels of chitin. GPs are used as an encapsulation system to cage polyelectrolyte-DNA nanocomplexes providing for efficient intracellular DNA delivery, release, and gene expression. Formation of the caged polyelectrolyte nanocomplexes follows a layer-by-layer (LbL) synthetic approach, with the different components assembled through electrostatic interactions by diffusion through the porous glucan shell. In this communication we report the application of the glucan particle delivery technology to accomplish efficient co-delivery of DNA and siRNA. The advantages of the GP drug delivery system include high payload capacity, multiplexable DNA and siRNA delivery, oral bioavailability and beta 1,3-D-glucan receptor targeting to macrophages and dendritic cells, and cell trafficking to the reticuloendothelial system and sites of macrophage accumulation [6].

MATERIALS AND METHODS

1. Materials

Ribonucleic acid from *Torula* yeast, Type VI, (tRNA), cetyl trimethylammonium bromide (CTAB), and high molecular weight water-free polyethylenimine (PEI, 25 kDa) were purchased from Sigma Aldrich (Allentown, PA) and used as received. gWizGFP plasmid DNA expressing green fluorescent protein (GFP) was obtained from Genlantis (San Diego, CA), and Alexa488 labeled Negative Control and gWizGFP targeted Stealth™ siRNAs (CGG CAA CUA CAA GAC ACG UGC UGA A) were supplied by Invitrogen (Carlsbad, CA). Scrambled Dy547 siRNA were supplied by Dharmacon (Lafayette, CO). Saline for irrigation was purchased from Baxter Healthcare (Deerfield, IL). Solvents and buffers solutions were purchased from Sigma Aldrich or VWR and used without further purification.

2. Methods

2.1 Preparation of Glucan Particles (GP): GP were prepared by suspending *Sacharomyces cerevisiae* (100 g Fleishmans Bakers yeast, AB Mauri™ Food Inc., Chesterfield, MO) in 1 liter 1 M NaOH and heating to 80 °C for 1 hour. The insoluble material containing the yeast cell walls was collected by centrifugation at 2000x g for 10 minutes. This insoluble material was then suspended in 1 liter of water and brought to pH 4-5 with HCl, and incubated at 55 °C for 1 hour. The insoluble residue was again collected by centrifugation and washed once with 1 L water, four times with 200 ml isopropanol and twice with 200 mL acetone. The resulting slurry was placed in a glass tray and dried at room temperature to produce 12.4 g of a fine, slightly off-white powder.

2.2 Fluorescein labeling of glucan particles: Glucan particles (1 g) were suspended in 100 ml 0.1 M carbonate buffer (pH 9.2), collected by centrifugation at 2000x g for 10 minutes and resuspended in 100 ml 0.1 M carbonate buffer (pH 9.2). DTAF at a concentration of 1 mg/ml in DMSO was added to the buffered glucan particle suspension (10% v/v) and the reaction was mixed at room temperature in the dark overnight. Tris buffer (2 ml 1M, pH 8.3) was added and the reaction mixture was stirred for additional 15 minutes at room temperature to quench free fluorescent labeling reagent. The fluorescently labeled glucan particles were collected by centrifugation at 2000x g for 10 minutes and washed with sterile pyrogen-free water until the color was removed. The glucan particles were then dehydrated by four washes with absolute ethanol, two washes with acetone and dried in the dark at room temperature. The resulting powder was ground to a fine bright yellow powder to produce ~ 1 g of fluorescent-Glucan particles (f-GP).

2.3 Preparation of glucan particle encapsulated cationized cores: Dry GP or f-GP was mixed with a volume of the anionic core polymer tRNA (10 mg/ml in 50 mM Tris HCl pH 8, 2 mM EDTA and 0.15 M NaCl (TEN)) to minimally hydrate the particles and incubated for 2 hours to allow the particles to swell and adsorb the tRNA solution. Neutral PEI (2 mg/ml in TEN) was added in excess to form GP or f-GP encapsulated tRNA nanocomplexes, and the particles were resuspended by homogenization or sonication. PEI adsorption and nanocomplex formation was allowed to proceed for at least one hour. The suspension was centrifuged and particles were resuspended in CTAB (20 mg/mL in 0.9% saline) and incubated for 20 minutes. The suspension was centrifuged and particles were resuspended in 70% ethanol to sterilize the particles, and washed three times in 0.9% saline for irrigation, aseptically resuspended, counted with a hemacytometer, diluted to 1×10^8 GP/mL in 0.9% saline for injection, and stored at -20°C.

2.4 Transfection experiments: GP-tRNA/PEI-CTAB or f-GP-tRNA/PEI-CTAB ($10 \mu\text{L}$, 1×10^8 particles/mL) suspensions were mixed with 250 ng of gWizGFP plasmid DNA per 1×10^6 particles, the indicated amount of siRNA over a concentration range from 0 to 50 pmoles, and 0.9% saline to a final volume of 75 μL . DNA and siRNA were bound to the GP-tRNA/PEI-CTAB cationic nanoparticle surfaces for 2 hours at room temperature. Then, 25 μL of 0.01% neutral PEI in 0.9% saline for irrigation was added to coat the bound DNA and siRNA for 20 minutes. Dulbecco's Modified Eagles Medium (DMEM medium with 10% fetal calf serum, 1% penicillin-streptomycin and 1% glutamine) was added and the final mixture transferred to 24-well plates containing 5×10^5 cells/well of the murine fibroblast cell line NIH3T3-D1, capable of ingesting glucan particles via the dectin-1 receptor [7]. The plates were incubated at 37°C under 5% CO₂ overnight, the growth medium was replaced and the plates incubated for an additional 16-24 hours. After 40-48 hours, the growth medium was removed, cells washed once with phosphate buffer saline (PBS), fixed with 250 μL of 0.5% formalin in PBS, and scored for GFP expression by manual counting of the proportion of fluorescent cells following visualization with a fluorescence microscope.

RESULTS AND DISCUSSION

Previous work has shown the effective use of the glucan particle delivery system for *in vitro* DNA delivery [5-6]. These experimental results demonstrated that gWizGFP plasmid DNA (125-250 ng DNA) delivered as caged nanocomplexes in glucan particles to NIH-3T3-D1 cells (1×10^6 particles/ 5×10^5 cells) resulted in transfection frequencies of 50% or higher. The glucan particle delivery system is also useful to deliver payloads other than DNA. The results in this paper extend the usefulness of the glucan delivery system to the effective delivery of siRNA, and co-delivery of siRNA and DNA. The versatility of the GP encapsulated nanoparticulate system allows for the incorporation of siRNA as part of a nanocomplexed core or adsorbed onto the surface of a nanocomplexed core as shown in Figure 1. GPs provide an efficient receptor targeted delivery system to cells bearing beta 1,3-D-glucan receptors (*i.e.*, dectin-1) including macrophages and dendritic cells. Figure 2 shows efficient *in vitro* delivery of fluorescently labeled siRNA into J774A.1 cells, a murine macrophage cell line.

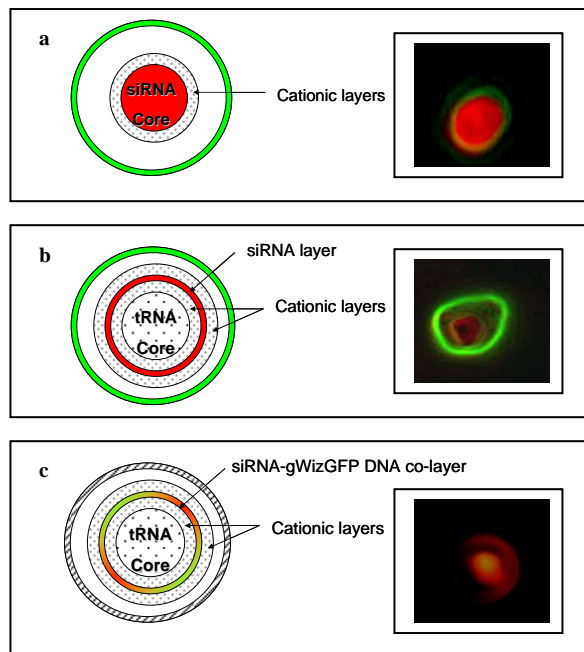


Figure 1: Schematic representation and overlaid fluorescent photomicrographs showing localization of fluorescently labeled siRNA and DNA as part of the GP encapsulated nanocomplexes:

- Dy547 labeled scrambled siRNA as part of the nanocomplexed core inside fluorescent particles: F-GP Dy547 Scr siRNA/PEI
- Dy547 labeled scrambled siRNA adsorbed onto the surface of F-GP-tRNA/PEI nanocomplexed cores: F-GP tRNA/PEI-Dy547 Scr siRNA/PEI
- Co-delivery of gWizGFP plasmid DNA and Dy547 Scr siRNA adsorbed onto the surface of GP-tRNA/PEI nanocomplexed cores: GP tRNA/PEI (gWizGFP-DNA+Dy547 siRNA)/PEI



Figure 2: Glucan particle mediated delivery of Alexa488 Negative Control siRNA (GP tRNA/PEI /Alexa488-siRNA/PEI) into J774A.1 cells

A model system was developed in which both gWizGFP plasmid DNA and a scrambled negative control siRNA, or an siRNA targeting GFP were assembled as a co-layer adsorbed onto tRNA/PEI-CTAB nanocomplexed cores caged inside glucan particles. CTAB is a cationic surfactant that is used for core formation as it facilitates endosomal release of DNA. An optimal gWizGFP DNA concentration

was used to achieve maximum transfection efficiency (~50%) for the control experiments. Negative control scrambled and gWizGFP specific siRNAs were used to test the efficiency of this co-delivery system to down regulate transient GFP expression. Titration experiments were carried out at constant DNA concentration while varying amounts of siRNA to determine the required siRNA concentration for optimal knock down of GFP expression.

RNAi by GFP sequence-specific siRNA significantly decreased the transfection frequency to ~5% using 3.3 pmoles of siRNA, and to less than 1% of control levels when using 33 pmoles of siRNA (Figures 3 and 4).

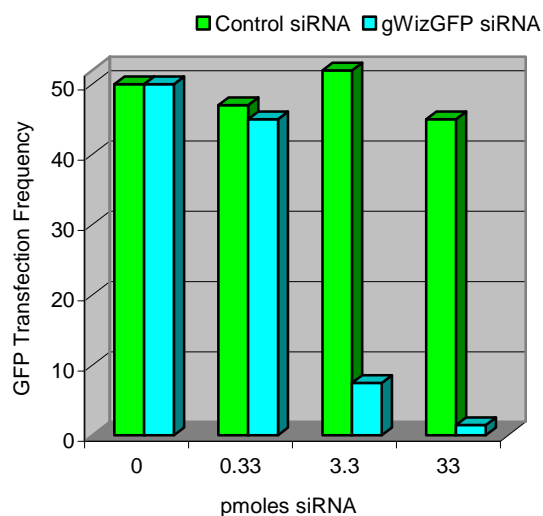


Figure 3: Effect of siRNA on gWizGFP Transfection Frequency

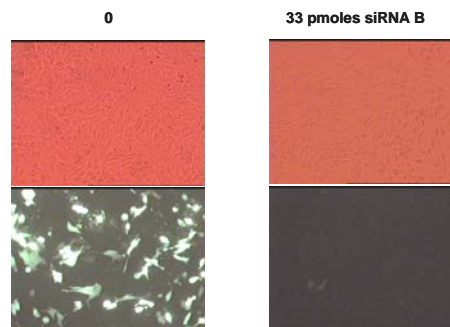


Figure 4: Co-delivery of gWizGFP DNA and siRNA showing effective knock down of GFP expression

The results from Figure 3 demonstrate the ability of the GP delivery system to effectively load, protect, deliver and release siRNA into cells for effective sequence-specific RNAi. As observed for DNA delivery, one hallmark feature of the GP delivery system is the efficient delivery of siRNA. Optimal DNA transfection by the GP delivery system requires 125-250 ng of DNA for 5×10^5 cells and

transient GFP expression is down regulated by 2-20 ng (3.3-33 pmoles) of sequence-specific siRNA per 5×10^7 cells. The GP delivery system offers the advantage of effective control of gene expression using small amounts of siRNA, as the siRNA is effectively protected inside the nanocomplexed core during particle uptake. These results also demonstrate the multiplexed features of the GP delivery system documenting the efficient co-delivery of DNA and siRNA.

CONCLUSIONS

Glucan particles containing tRNA/PEI-CTAB nanocomplexed cores were shown to be an efficient *in vitro* delivery system for siRNA. The use of glucan particles provides a convenient method to form encapsulated layer-by-layer nanocomplexes that can deliver multiplexed payloads to cells. Current work is focused on *in vivo* DNA and siRNA delivery.

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