Functionalized RNA Nanoparticles for Targeted Delivery

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Abstract

Nano-constructs composed of RNA can be employed as scaffolds for delivering functional molecules to cells. Promise has been shown in attaching siRNA for gene silencing to the before mentioned scaffolds. Proven and suitable nano-constructs currently include both a hexagonal nanoring and a nanocube. Both have shown to be able to assemble and deliver siRNAs *in vitro* and *in vivo*[1]. However, this is not all that is required to deliver siRNA for specific gene silencing. Various *in vivo* factors, including degradation and the negative charge of both nano-constructs and cell membranes, hinder the RNA NPs from delivering siRNA to the targeted cells on its own, requiring the aid of delivery agents. Most delivery agents available could impede the function of certain moieties, such as aptamers. In this study, we have evaluated the efficacy of β-CD-P(HMA- *co* -DMAEMA- *co* -TMAEMA)4.8 as an efficient delivery agent of aptamer-functionalized RNA NPs. The aptamer used in these constructs is Sgc8c, a DNA aptamer targeting T-lymphoblastoic cells (CEM cell line). Preliminary results indicate an increased uptake of the functionalized NPs and promising results in delivering siRNAs to targeted cells.

Introduction

Over recent years, the biological molecule RNA has become further characterized and understood, showing great potential in the field of gene therapy. RNA is part of a multitude of cellular processes and regulatory pathways. Notably, small double stranded RNAs, 21 to 25 nucleotides long, are involved in the RNA interference mechanism. These small interfering RNA (siRNA), cleaved by DICER from exogenous double stranded RNA or short hairpin RNAs, are separated into two single stranded RNAs. The guide strand is loaded into the RISC complex, and hybridized with the intended mRNA, interfering with its translation.

Nanoparticles (NPs) incorporating double stranded diceable RNA molecules have shown to be an efficient way of delivering siRNA. RNA NPs can be engineered to pack several siRNAs within the same molecule, minimizing the effect related to individual siRNAs off-target effects, increasing thus the therapeutic potency of these siRNAs introduced with RNA NPs into the cells. Two of the constructs capable of incorporating siRNA, as well as cell specific binding aptamers, are the nanoring and nanocube[1, 2].

Finding a delivery agent which is efficient, has no toxicity or immunogenicity, preserves the functionality of attached moieties, and delivers the NPs mainly to the diseased cells it is not a straightforward task. A novel polymer, which does not bury the RNA, but rather carries them along, has been published[4]. This polymer consists of a beta-cyclodextrin core and several arms composed of an array of hydrophobic, pH-sensitive, and cationic monomers. Each monomer produces corresponding responses to changes in the environment, making it a "smart polymer". Complexation of the "smart" polymer with the functionalized RNA NPs allows their delivery into cells without loss of any attached moieties function, including cell-specific aptamers.
Research has shown that the expression levels of proteins differ between different types of cells. These differences allow for specific aptamer sequences to be selected in order to have high affinity to the targeted cells[6]. The aptamer used in this work is Sgc8c, a 41-nucleotide DNA aptamer, which targets T-lymphoblastoid cells. Preliminary results show an increased uptake of the aptamer-functionalized constructs, and promising results in silencing by siRNAs delivered with these functionalized RNA NPs.

β-CD Mechanism of action

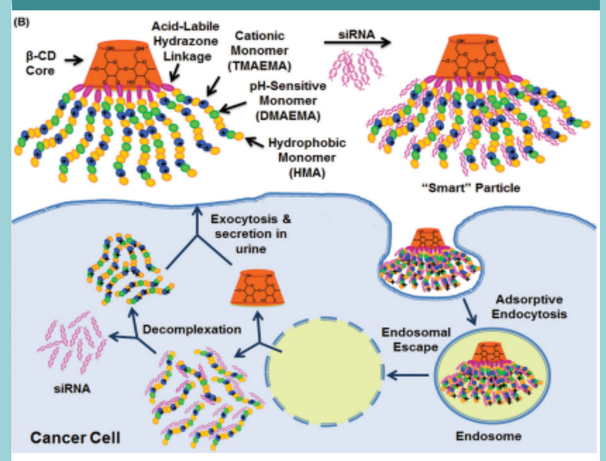


Figure 1. The mechanism by which βC-D delivers siRNA. P(HMA- co -DMAEMA- co -TMAEMA) grafts are released after acid-labile hydrazone linkages are hydrolyzed by the acidic pH. This then ruptures the endosomal membrane releasing the siRNA.[4]

RNA NPs Incorporating siRNA

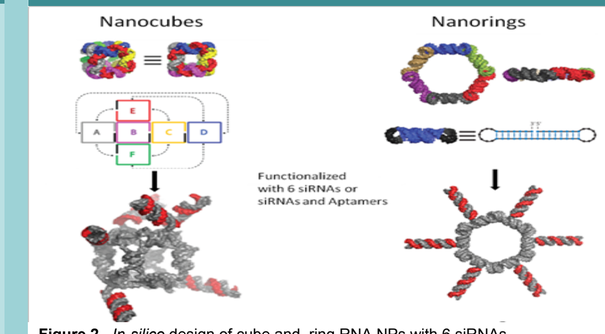


Figure 2. *In-silico* design of cube and ring RNA NPs with 6 siRNAs.

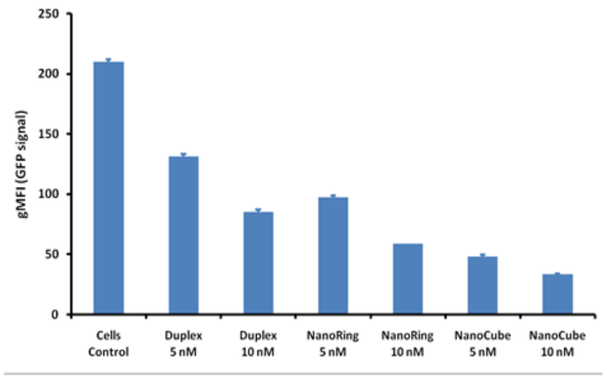


Figure 3. eGFP silencing in breast cancer (MDA-MB 231 eGFP) cells by siRNAs delivered using cube, ring, and corresponding siRNA duplexes, transfected with "smart" β-CD polymer. The amounts indicated correspond to the final concentration of siRNA.

RNA NPs Functionalized with Aptamers

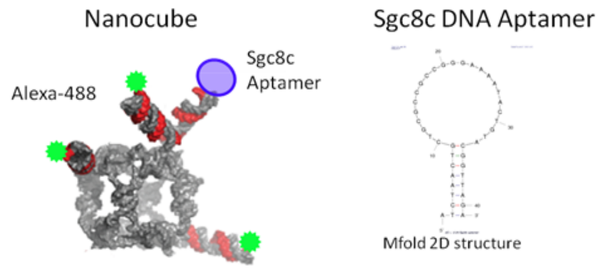


Figure 4. Nanocube decorated with 3 Alexa-488 and functionalized with 1 Sgc8c aptamer, and Scg8c 2D structure.

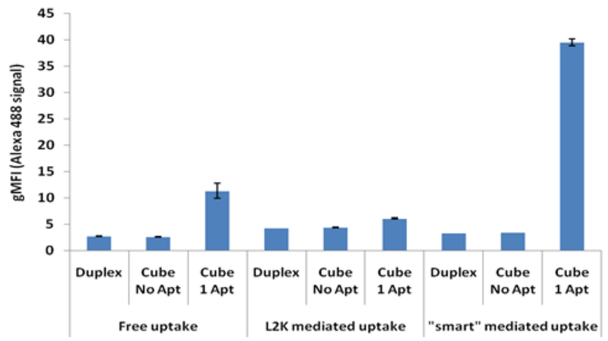
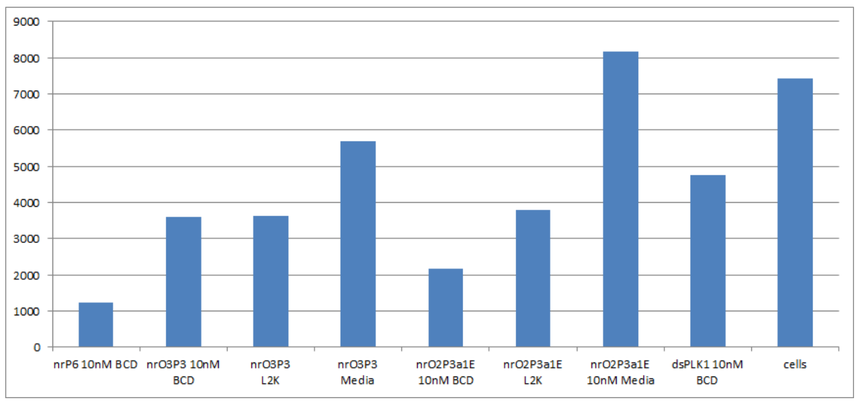


Figure 5. Uptake of siRNA duplex, cubes decorated with 3 Alexa-488 fluorescent probes, and cubes decorated with 3 Alexa-488 fluorescent probed and 1 Sg8c aptamer, mediated by L2K or"smart" β-CD polymer in CEM cells.

β-CD Mediated Transfections



Nanoparticle Annotation

nrP6	Nanoring with 6 siRNAs targeting PLK1
nrO3P3	Nanoring with 3 siRNAs targeting PLK1
nrO2P3a1E	Nanoring with 3 siRNAs targeting PLK1 and one Sgc8c aptamer
dsPLK1	Double-stranded siRNA designed to silence PLK1 production. This siRNA is not attached to a nanostructure.

Figure 6. Silencing of PLK1 with siRNA introduced by dsRNA, nanorings with 6 siRNAs,and nanorings with 1 Sgc8c aptamer, mediated by "smart" β-CD polymer, L2K and free uptake in T-lymphoblastoid (CEM) cells.

Conclusion

- The β-CD "smart" polymer is a more effective delivery agent than Lipofectamine 2000 when transfecting functionalized RNA NPs in CEM cells;
- The Sgc8c aptamer allows for increased targeting of CEM cells, improving the cellular uptake of RNA NPs;
- The structure of the NPs transfected with β-CD affects the degree of uptake and silencing in the MDA-MB 231 and CEM cell lines.

References

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Discussion

Both the nanocube and nanoring constructs were designed *in silico* with the intent to self assemble from individual RNA strands. Each were functionalized with 6 siRNA strands and transfected into cell lines. A novel delivery agent was also tested here, in conjunction with our RNA NPs. The ability of β-CD to transfect NPs of various shape was scrutinized in MDA-MB 231 expressing eGFP. Duplex siRNA, nanorings and nanocubes incorporating siRNAs targeting eGFP were transfected with β-CD into MDA-MB 231 cell line. To capitalize on the aptamer's capability of targeting particular cells, the aptamer has to be readily available to interact with its membrane receptor. Most of the liposome-based delivery agents would enclose the NPs within, making the aptamer's function redundant. The β-CD "smart" polymer complexates with RNA on its arms, potentially leaving the aptamer exposed to interact with the receptors. We assessed the ability of the Sgc8c aptamer to aid in the internalization of the nanocube by comparing the uptake of the duplex siRNAs, the cubes, and the cubes functionalized with aptamer.

Figure 3 shows the ability of the functionalized RNA NPs, transfected with β-CD, to silence the enhanced Green Fluorescence Protein in MDA-MB 231 breast cancer cells expressing eGFP. As seen from the graph, the nanoring-introduced siRNAs silenced better than just the siRNAs, and the siRNA introduced by the nanocube displays the highest degree of silencing. All the RNAs transfected had a 10nM concentration of the final siRNAs.

Figure 5 shows that the β-CD polymer increased the delivery of cube functionalized with the Sgc8 aptamer into the CEM cells. It vastly outperforms lipofectamine regarding the cellular uptake. To confirm these results, the silencing of PLK1 by the siRNA introduced with aptamer-functionalized RNA NPs and transfected with lipofectamine, β-CD or free uptake, were investigated in the CEM cell line. In Figure 6 we present the result of PLK1 silencing in CEM cells by siRNAs delivered by duplexes, nanorings and nanorings with aptamers. The transfection was mediated by β-CD "smart" polymer. The greatest degree of silencing is displayed by the nanoring with six siRNAs attached, followed by the ring with three siRNAs and one aptamer. Given the fact that aptamer-functionalized nanoring has half the concentration of siRNA compared with the nanoring with six siRNAs, the silencing was indeed considerably enhanced.

Methods and Materials

The DNA corresponding to the desired molecules, purchased from IDT, were amplified using PCR techniques and purified with the Zymo Research DNA Clean and Concentration Kit. The purified DNA was transcribed using T7 RNA Polymerase and then purified on 8M urea 8% polyacrylamide denaturing gels. Assembly of NPs were done in 89mM Tris-Borate buffer containing Mg and K. Assembly protocol for nanocubes requires heating to 95°C for 2 minutes for denaturing, followed by annealing at 45°C for 30 minutes in assembly buffer. Five cube and ring variants were used in this experiment. The first was the nanocube without siRNA or aptamers. The second cube was designed to hold six strands of siRNA designed to suppress the PLK1 (Polo-Like Kinase-1) gene, required for several processes in mitotic progression[3] .The third was designed to deliver six siRNA strand to suppress eGFP.. The fourth and fifth cubes had 1 Sgc8 aptamer incorporated, as well as 3 siRNAs targeting PLK1 and eGFP respectively. After assembly of NPs, samples of various concentrations were incubated with β-CD for twenty minutes at room temperature to allow the complexation between the polymer and RNA.

In order to test the effectiveness of the β-CD *in vitro*, free delivery, BCD- and lipofectamine 2000- mediated transfections were compared. NPs incorporating siRNAs designed to target eGFP were transfected into the breast cancer cell line MDA-MB 231 eGFP. The uptake of NPs functionalized with an Sgc8c aptamer and decorated with 3 fluorescent probes was monitored in CEM cells.

The cells were plated 24 hours before transfection on a 96 well plate, at 10,000 cells per well. The effectiveness of the delivery as a function of the fluorescence uptake or the degree of silencing was determined by Fluorescence Activated Cell Sorting (FACS) or cell viability assays. FACS analysis was run three days after the transfection on a FACS Calibur C6 Flow Cytometer. Sampler software analyzed at least 20,000 events. The geometric mean fluorescence intensity (gFMI) was calculated and plotted for statistical analysis. Cell viability assays was done on a Molecular Devices SpectraMax M2e.

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