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Method for Confirming Cytoplasmic Delivery of RNA Aptamers

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Abstract

RNA aptamers are single-stranded RNA oligos that represent a powerful emerging technology with potential for treating numerous diseases. More recently, cell-targeted RNA aptamers have been developed for delivering RNA interference (RNAi) modulators (siRNAs and miRNAs) to specific diseased cells (e.g., cancer cells or HIV infected cells) in vitro and in vivo. However, despite initial promising reports, the broad application of this aptamer delivery technology awaits the development of methods that can verify and confirm delivery of aptamers to the cytoplasm of target cells where the RNAi machinery resides. We recently developed a functional assay (*RIP assay*) to confirm cellular uptake and subsequent cytoplasmic release of an RNA aptamer which binds to a cell surface receptor expressed on prostate cancer cells (PSMA). To assess cytoplasmic delivery, the aptamer was chemically conjugated to saporin, a ribosome inactivating protein toxin that is toxic to cells only when delivered to the cytoplasm (where it inhibits the ribosome) by a cell-targeting ligand (e.g., aptamer). Here, we describe the chemistry used to conjugate the aptamer to saporin and discuss a gel-based method to verify conjugation efficiency. We also detail an in vitro functional assay to confirm that the aptamer retains function following conjugation to saporin and describe a cellular assay to measure aptamer-mediated saporin-induced cytotoxicity.

Keywords

Aptamers; Cytoplasmic delivery; Saporin; Cytotoxicity

1 Introduction

RNA aptamers are single-stranded nucleic acids whose binding properties are similar to those of antibody/antigen interactions (thus are often referred to as *nucleic acid antibodies*) [1, 2]. Aptamers have been developed for a variety of technical and therapeutic applications [1–3]. Therapeutic RNA aptamers are modified with fluoro or *O*-methyl groups at the 2'-position of their sugar moiety, which renders them resistant to degradation by serum nucleases and significantly reduces their immunogenicity. Thus, these aptamers can be used in preclinical as well as clinical applications [2]. Their development as therapeutics has primarily involved their tendency to inhibit their targets upon binding [4–7], although

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aptamers have also been developed to activate cell-surface receptors [8]. In 2006 came the first description of aptamers for delivery of therapeutic siRNAs to target cells [9, 10]. Since then, multiple in vivo studies involving low-dose (<1 mg/kg), systemic administration of aptamer-targeted siRNA conjugates have reported efficacy, highlighting the promise of this approach for future clinical applications [11–16]. Despite these encouraging studies, which demonstrated aptamer-mediated delivery of functional RNAi to target cells in vivo, the broad application of the aptamer delivery technology awaits the identification of aptamer sequences capable of internalizing into target cells and delivering the siRNA cargo to the cytoplasm where the RNA-induced silencing complex (RISC) resides.

In a recent study, we described a functional assay (RIP assay) for assessing cell internalization and subsequent release of aptamers into the cytoplasm of target cells [17]. The RIP (ribosome-inactivating protein) assay is based on targeting a RIP (e.g., saporin) to the cytoplasm of target cells *via* conjugation to a ligand. Saporin is a so-called RIP due to its N-glycosidase activity which, leads to a single adenine base being removed from the ribosomal RNA of the large subunit of the ribosome. RIPs are some of the most toxic molecules (poisons) known. Notorious examples of these toxins include ricin and abrin [18]. These toxins contain a signal sequence that functions to insert the RIP into a cell, leading to cytoplasmic localization and subsequent enzymatic inactivation of ribosomes and inhibition of protein synthesis. This results in efficient cell death and eventually causes death of the victim. Unlike ricin and abrin, saporin lacks this signal sequence and thus, is unable to internalize into cells and is safe to handle. However, if given a method of entry into the cell (*via* conjugation to a ligand/aptamer), saporin becomes a very potent toxin, since its enzymatic activity is among the highest of all RIPs [18].

We previously employed this property of saporin to confirm cytoplasmic delivery of an RNA aptamer to prostate-specific membrane antigen (PSMA), a cell surface receptor expressed on prostate cancer cells [17]. Below, we describe the chemistry used to conjugate the PSMA RNA aptamer to the saporin toxin and propose an agarose gel-based method to quickly assess conjugation efficiency and purity. We also detail an in vitro functional assay (NAALADase assay) to confirm the function of the aptamer-saporin conjugate post-conjugation. In addition to being highly expressed on the surface of prostate cancer cells, the extracellular portion of PSMA is known to have multiple catalytic activities, including *N*-acetylated alpha-linked acidic dipeptidase (NAALADase), folate carboxypeptidase, and dipeptidyl peptidase IV functions [19, 20]. Importantly, PSMA's enzymatic activity has been linked to increased cellular migration and activation of oncogenic pathways [21]. RNA aptamers to PSMA have been shown to inhibit the enzymatic activity of the protein [22, 23] and thus, can be evaluated for function using this assay. Finally, we describe a standard cellular assay (MTS assay) to measure saporin-induced cytotoxicity. This assay is based on the principle that NAD(P)H-dependent cellular oxidoreductase enzymes within cells can be a measure of the number of viable cells present within a culture. These enzymes are capable of reducing the tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS] to its insoluble formazan, which has a purple color and can be quantitated using a plate reader [24]. Tetrazolium dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity

(shift from proliferative to resting status) of potential medicinal agents and toxic materials (e.g., saporin) [25].

In conclusion, the methodologies and assays described herein, with the exception of the NALAADase assay, can be broadly applied to evaluating other aptamers with cell internalizing properties and are poised to expedite the identification of aptamer sequences capable of delivering therapeutics (e.g., RNAi modulators) to the cytoplasm of target cells. Functional assays (e.g., binding assays, inhibition assays) to assess aptamer function post-conjugation should be developed and optimized for each aptamer and used in place of the NAALADase assay.

2 Materials

2.1 RNA-Saporin

1. Biotinylated RNA aptamers.
2. Streptavidin-ZAP.
3. FGF-Saporin.
4. 10× binding buffer: 200 mM HEPES, pH 7.4, 1.5 M NaCl, 20 mM calcium chloride, and 0.1 % BSA.
5. 10× Binding buffer: 10 mL of 1 M HEPES, pH 7.4, 15 mL of 5 M sodium chloride, and 0.5 g of bovine serum albumin. Bring to a final volume of 50 mL with UltraPure H₂O. Store at room temperature.
6. Molecular biology-grade agarose.
7. TAE buffer.
8. 6× gel loading dye.
9. 100 base pair DNA ladder.
10. 7 × 10 cm Minigel System.
11. PowerPac Basic Power Supply.
12. SYBR Gold Nucleic Acid Gel Stain.
13. Molecular Imager Gel Doc XR System.

2.2 Reagents for NAALADase Assay

1. 200 mM Tris–HCl, pH 7.5: Add 200 mL of UltraPure 1 M Tris–HCl buffer, pH 7.5 to 800 mL of UltraPure H₂O.
2. 10 mM CoCl₂: Dissolve 1189.6 mg of cobalt (II) chloride hexahydrate in 500 mL UltraPure H₂O.
3. Recombinant human PSMA/FOLH1/NAALADase I.
4. 100 μM NAAG.

5. [Glutamate-3,4- H^3]-NAAG.
6. AG 1-X8 Resin.
7. Flint glass Pasteur pipettes, 9 in.
8. Custom-made rack to hold 9 in. Pasteur pipettes in place and in alignment with rack scintillation vials (see Fig. 1.).
9. Borosilicate glass beads, 3 mm diameter.
10. 0.1 M Na_3HPO_4 : Add 5.7 g of Na_3HPO_4 (anhydrous) to double-distilled H_2O . Bring to a final volume of 400 mL with double-distilled H_2O , and store at 4 °C until ready to use.
11. 1 M formic acid: Add 383 mL of double-distilled H_2O to 17 mL of 88 % formic acid.
12. Bio-Safe II scintillation fluid.
13. Polyethylene scintillation vials.
14. Beckman LS6500 scintillation counter.

2.3 Reagents for MTS Assay

1. RPMI medium 1640.
2. 22Rv1.7 cells [11].
3. Fetal bovine serum: premium select.
4. 100× MEM nonessential amino acids solution.
5. Nunclon delta-treated 150 mm cell culture dishes.
6. 0.25 % Trypsin-EDTA.
7. CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS Assay).
8. 96-Well dishes.
9. Thermomax Microplate Reader.

3 Methods

3.1 RNA-Saporin Conjugation

1. Resuspend the biotinylated aptamer in ultrapure H_2O at a concentration of 100 μM , aliquot, and store at $-80\text{ }^{\circ}C$.
2. Fold the biotinylated aptamer in 1× binding buffer (obtained by doing a 1:10 dilution of the 10× binding buffer) at a final concentration of 5 μM . The folding protocol is as follows: incubate aptamer solution at 65 °C for 10 min, followed by incubating at 37 °C for 10 min. The aptamer can be freeze-thawed several times after the folding protocol and still retain its activity.

3. Use a 1:4 molar ratio of streptavidin-ZAP and the folded biotinylated aptamer. Incubate at room temperature for 30 min in 1× binding buffer with a final concentration of 2.5 μM of the RNA.
4. The purity and efficiency of the conjugation of biotinylated aptamer to streptavidin-ZAP will need to be confirmed by running the aptamer-saporin conjugates on a 1 % agarose gel (see **Note 1**). For the 1 % agarose gel, dissolve 0.7 g of agarose into 70 mL of TAE buffer by heating in a microwave for approximately 2 min. Pour the agarose gel mixture into the Minigel system, and place the well combs into the gel, and allow the gel to cool and set for 30–45 min. After the gel has solidified, turn the gel so the wells are closest to the anode, and fill the apparatus with enough TAE to cover the gel. Mix 10 μL of the 2.5 μM biotinylated aptamer alone, streptavidin-ZAP alone, or biotinylated aptamer bound to saporin with 2 μL of the 6× gel loading dye. Carefully pipette the RNA/loading gel solution into the wells. Run the gel for 30 min at 100 V.
5. Stain the gel with 1× SYBR Gold for 30 min and perform UV imaging. The SYBR Gold stock solution is 10,000×, so dilute 5 μL of the 10,000× stock SYBR Gold solution into 50 mL of 1× TAE. Cover the gel with the 1× SYBR Gold solution and gently rock at room temperature in the dark for 30 min. Image the gel in a UV imager similar to the Molecular Imager Gel Doc XR System.

3.2 Activity of PSMA-Saporin Conjugate

1. A functional assay should be performed in order to confirm that the activity of the aptamer is not inhibited following conjugation to saporin (see **Note 2**). The functional assay we describe here measures PSMA enzymatic activity (NAALADase). The NAALADase assay is performed as follows (120 μL per sample replicate): A concentration of 0.333 μM is used for each aptamer, aptamer-saporin conjugate, and/or streptavidin-ZAP. 1× binding buffer alone is also included as a control.
2. Generate a Tris/CoCl₂ master mix by adding 500 μL of 200 mM Tris–HCl, pH 7.5 to 250 μL of 10 mM CoCl₂. Add 60 μL of the Tris–HCl/CoCl₂ master mix to each sample.
3. Upon receiving the rhPSMA, aliquot it into 2 μL aliquots under sterile conditions and store the aliquots at –80 °C. The rhPSMA aliquots should be diluted by adding 375 μL of ultra-pure H₂O and 125 μL of 200 mM Tris–HCl, pH 7.5 to the 2 μL of the PSMA solution before using in the NAALADase assay. Add 10 μL of rhPSMA

¹Conjugation problems: It is possible that conjugating the aptamer to saporin could ablate the activity of the aptamer. An aptamer-specific functional assay to verify activity of aptamer-saporin conjugate, such as the NAALADase describe for the PSMA aptamer, is recommended. Prior to conjugation to saporin-streptavidin (ZAP), it is advisable to determine that the biotinylated aptamer binds to its target and/or retains function. In the event that placing a biotin on the 5' end of an aptamer disrupts its function, we advise positioning the biotin on the 3' end. Additional linking chemistries could also be explored [26–29].

²Variability in the expression of cell-surface receptors: The expression of cell-surface receptors being targeted with aptamers can vary from cell line to cell line and can also depend on passage number and growth conditions. We recommend verifying cell-surface protein expression levels for any given aptamer target receptor prior to performing the cell-based cytotoxic assay. Flow cytometry using antibodies specific to the receptor target should be performed.

mixture to the RNA mixtures, and mix by pipetting. Incubate at 37 °C for 5 min, and start the timer after adding PSMA to the first sample.

4. Make a NAAG solution consisting of 992 μL of ultrapure H_2O , 5.5 μL of 100 μM NAAG, and 2.5 μL of ^3H -NAAG. Add 10 μL of the NAAG solution to each sample and incubate at 37 °C for 15 min. Mix the reaction after 7.5 min has past. Start the timer after adding the NAAG solution to the first sample.
5. Stop the reaction by adding 200 μL of ice cold 0.1 M Na_3HPO_4 to each sample.
6. Prepare columns by placing as many Pasteur pipettes as needed into the plastic rack (Fig. 1). Place one 3 mm borosilicate glass ball into each pipette.
7. Make a 1:1 mixture of AG 1-X8 resin and ultrapure H_2O to create a resin slurry. Add 2 mL of the resin slurry to each Pasteur pipette. Once all of the liquid has left the columns, add 2 mL of double-distilled H_2O to the columns, and allow this to flow through and discard.
8. Once all of the liquid has drained from the columns, place the scintillation vials under the columns. Add 190 μL of each reaction mixture to separate columns, and allow it to completely soak into the resin.
9. Add 2 mL of 1 M formic acid to each column. Allow the liquid to completely flow through the columns and collect 2 mL of eluent into each scintillation vial.
10. Add 10 mL of Bio-Safe II scintillation fluid to each scintillation vial and count in a liquid scintillation counter.

3.3 Measurement of Aptamer-Saporin Cytotoxicity

1. 22Rv1.7 cells are grown in RPMI medium 1640 with 10 % FBS and 1 % non-essential amino acids, and are propagated in 150 mm culture dishes (*see Notes 3 and 4*). The cells are split 1:3 when they are approximately 80 % confluent by trypsinizing with 0.25 % trypsin-EDTA for no more than 4 min for further propagation. After trypsinizing the cells, plate 1000 cells per well in a 96 well plate with 100 μL of media. Allow the cells to attach overnight.
2. Incubate the RNA-saporin conjugate, RNA alone, streptavidin-ZAP alone, or 1 $\times$ binding buffer alone on the cells. Vary the time and treatment amounts in order to determine the EC_{50} and optimize treatment efficiency for each cell line. As a positive control for saporin-induced cytotoxicity, treat cells with FGF-saporin conjugate. The optimal amount of FGF-saporin needed to kill cells may have to be determined a priori. In our experience, the EC_{50} of FGF-saporin is anywhere from 0.15 to 1.4 nM depending on the cell line being tested.
3. Mix the cellular growth media in a 5:1 ratio with MTS (e.g., 100 μL of media and 20 μL of MTS per well in a 96-well plate). Remove media from cells and add 120

³Because modified nucleic acids are susceptible to degradation by mycoplasma nucleases [30], it is advisable to ensure that all cell cultures are free of mycoplasma contamination prior to performing the cell-based cytotoxic assays.

⁴Cell-type variability: Different cell types have different sensitivities to saporin-induced toxicity. The streptavidin-ZAP kit contains saporin conjugated to FGF (saporin-FGF) as a positive control to confirm sensitivity to saporin-induced cell death.

μL of the 5:1 media:MTS mixture to each well, followed by incubating for 1 h at 37 °C. Record the absorbance at 490 nm with a 96-well plate reader such as a Molecular Devices Thermomax microplate reader.

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Fig. 1. NAALADase assay apparatus. Pictured is a custom-made plastic rack used to hold 9 in. long flint glass Pasteur pipettes in alignment with scintillation vials for measuring NAALADase activity. The *red arrow* points to a Pasteur pipette filled with 2 mL of AG 1-X8 resin