

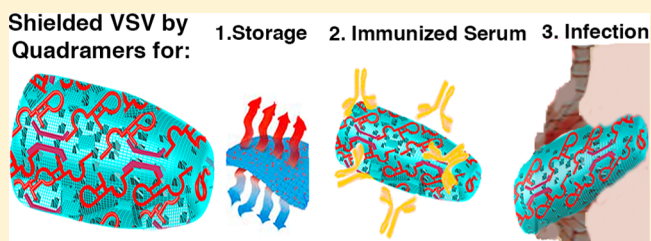
## Aptamer-Facilitated Cryoprotection of Viruses

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**ABSTRACT:** Global vaccination and gene therapy programs have an urgent demand for stabilization of viral vectors at low temperature. We used a quadramer, a bridge-connected DNA tetra-aptamer to antivesicular stomatitis virus (VSV), as a viral cryoprotectant. Results showed that the tetravalent antiviral DNA aptamers protect viral activity during multiple freeze–thaw cycles, shield from neutralizing antibodies, and decrease aggregation of viral particles.

**KEYWORDS:** Aptamer, quadramer, vesicular stomatitis virus, viral stabilizers, cryoprotection



Cryoprotection of viral vectors is essential for the development of vaccines and anticancer therapeutics. Because of temperature sensitivity, many viruses should be stored frozen.<sup>1</sup> Therefore, delivery of active viruses depends on a cold chain, a distribution network set up to maintain optimal low temperatures during the transportation and storage. However, this cold chain system represents a major economic and logistical burden, estimated to be as high as 80% of the entire financial cost.<sup>2</sup> In addition, a viral vector should be infectious in the presence of a host's neutralizing antibodies (nAbs). In the work, we used vesicular stomatitis virus (VSV) as an important viral vector in targeted oncolytic immunotherapies.<sup>3</sup> It is an enveloped RNA rhabdovirus that consists of a lipid envelope enclosing a nucleocapsid and an associated matrix formed by M proteins.<sup>4–6</sup> VSV is innocuous and engineered to target malignant cells and also increase a tumor's susceptibility to chemotherapeutic agents and the host immune response. Furthermore, VSV is a suitable choice for vaccine vectors and also has a key role in immunity and virology research. One of the challenges of utilizing VSV is its limited stability under different handling and storage conditions.<sup>7</sup> Several agents have been employed to increase the low temperature stability of viruses as effective vaccines such as metal ions, albumin, and gelatin.<sup>8–11</sup> However, none of these compounds have provided satisfactory results for oncolytic viruses due to low cryoprotection potency or high cell toxicity. The stabilizing effect of glycoproteins on the VSV surface<sup>12</sup> inspired us to look at VSV-specific aptamers. The goal of this study was to determine whether aptamers can preserve the infectivity of VSV after multiple freeze–thaw cycles and in the presence of nAbs (Scheme 1).

**Results and Discussion.** We used anti-VSV DNA aptamer clones Z23, Z29, S39, and M50 (Table 1) that were previously selected in our laboratory and have shown high affinity to VSV.<sup>13</sup> To increase avidity and serum stability, we designed a quadramer, which was made by connecting four aptamers (equimolar mixture of all above-mentioned aptamers), with an oligonucleotide bridge (Figure 1 and Scheme 1). We measured

the apparent dissociation constant ( $K_d$ ) for monomeric pool and quadramer using the approach described by Sefah et al.<sup>14</sup> The  $K_d$  for monomeric pool of four clones was  $71 \pm 15$  nM, and quadramer had affinity of  $22 \pm 9$  nM. The protective role of the aptamers and quaramers in viral activity was assessed using the plaque-forming and virus aggregation assays after freeze–thaw cycles.

We investigated the dosage effect of an equimolar pool of the aptamer clones and quaramers on VSV stability after 30 freeze–thaw cycles. Dosage effect experiments indicated that increasing the concentration of both the aptamer pool and quaramers above 400 pM improved VSV infectivity (Figure 2). We also studied the effect of the number of freeze–thaw cycles on the infectivity of VSV ( $54 \times 10^5$  plaque-forming units, PFU) with the aptamer pool (1  $\mu$ M), quadramer (0.25  $\mu$ M), and nonspecific DNA library (1  $\mu$ M) and compared these with the pure virus only. To this end, the three VSV groups were subjected to 0, 10, 20, 30, 40, 50, and 60 freeze–thaw cycles in duplicate (Figure 3). The infectivity of quadramer and aptamer pool-protected virus was 1.4 and 0.7 log higher, respectively, than the pure virus after 60 freeze–thaw cycles. Fascinatingly, just adding quaramers to the virus without freezing (0th cycle) increased the virus infectivity by 30% (Figure 3 inset), which can be ascribed to the ability of quaramers to prevent virus aggregation.

The stabilization effect of aptamers was confirmed by viral quantitative capillary electrophoresis (viral qCE),<sup>15</sup> where the intact virus particles were separated from free RNA released during virus degradation (Figure 4) after a cycle of freeze–thaw. The areas corresponding to the intact virus particles were then calculated and displayed in a bar graph (Figure 4B). From the electropherograms, the areas of virus peaks show that

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## Scheme 1. Scheme of a Quadramer and Bridge

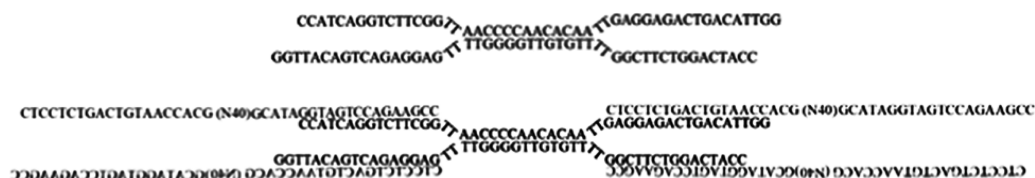


Table 1. Aptamer Sequences, Where F Is CTC CTC TGA CTG TAA CCA CG and cR Is GCA TAG GTA GTC CAG AAG CC

aptamer	sequence
Z-23	F_GGGACCTATCAGGCGATGTGAAAACCTTATACCACTGG_cR
Z-29	F_ACATCTACGTTTGCCACGCGCTACTCCGCCATCTACCC_cR
S39	F_GCACTTCACTTCTCTCTGACTGTAACCACGC_cR
MS0	F_CCATCACCTATTATCTCATTATCTCGTTTTCCCTATGCG_cR

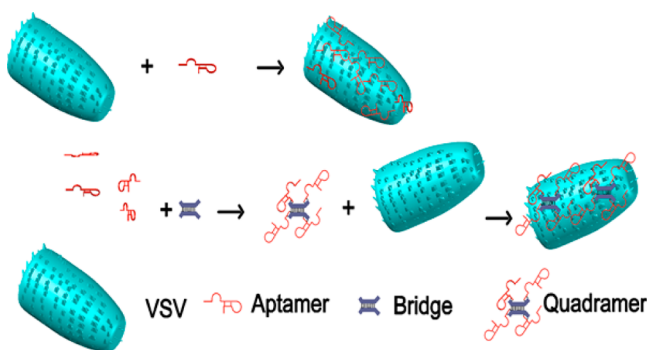


Figure 1. Schematic representation of binding vesicular stomatitis virus (VSV) with an aptamer and a quadramer. The oligonucleotide bridge mediates the formation of the quadramer.

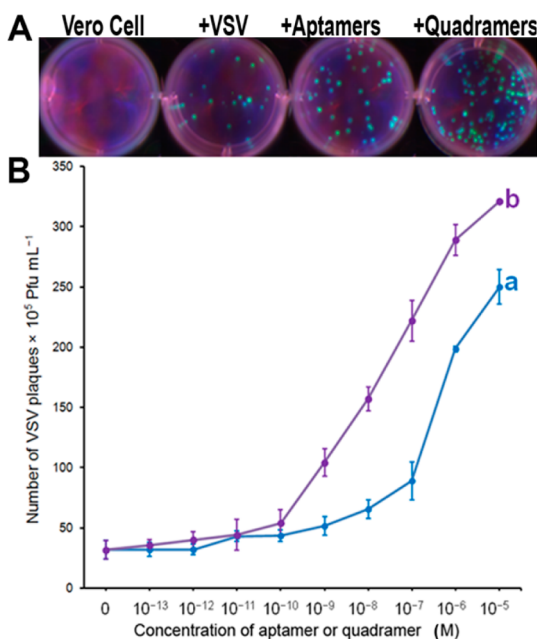


Figure 2. (A) Plaque formation when Vero cells were infected with YFP-coding VSV. (B) Dosage effect assessment. Number of plaques formed by VSV treated with different concentrations of an equimolar mixture of aptamer (a) and quadramer (b), both after 30 freeze-thaw cycles.

treating VSV with quadramers protects about 3 times more efficiently than the untreated virus.

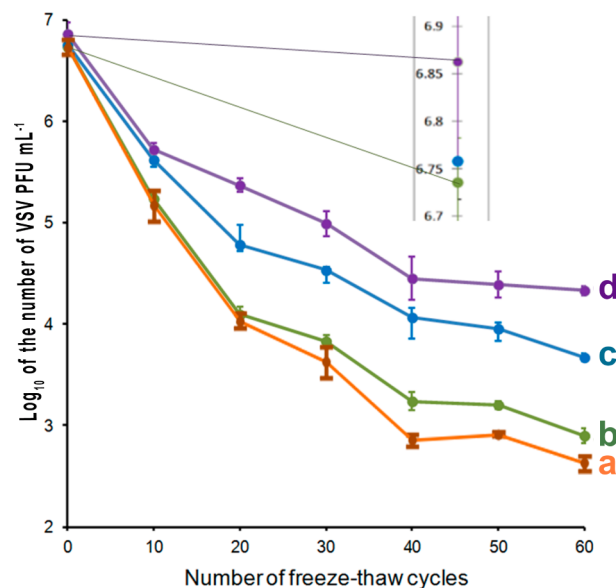
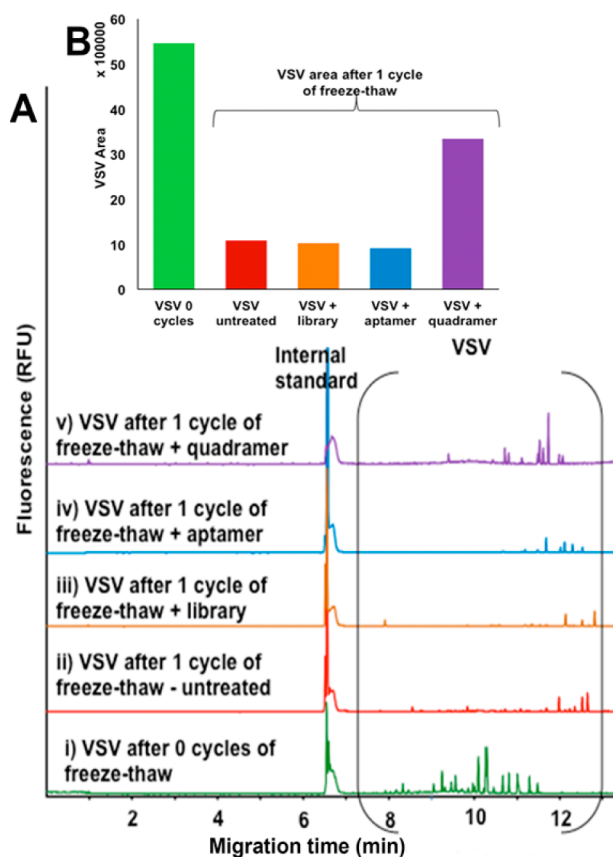
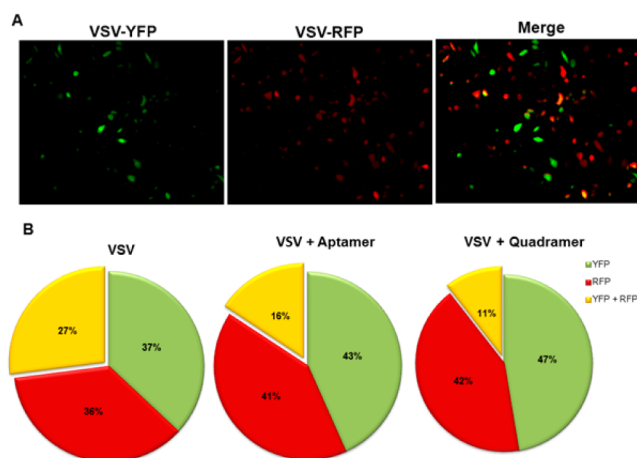


Figure 3. VSV infectivity correlation curve for DNA library (a), pure virus (b), with an aptamer pool (c), and with the quadramer (d) depending on the number of freeze-thaw cycles. The inset represents the initial infectivity before any freeze-thaw (0th cycle).

The effect of quadramers and the aptamer pool on VSV aggregation *in vitro* was also evaluated using a mixture of viruses coding yellow fluorescent protein (YFP) and red fluorescent protein (RFP) and by detecting the expression of the corresponding fluorescent proteins in Vero cells (Figure 5A). Aggregation of virus particles was observed when YFP and RFP were both expressed in the same cells. Overlapping YFP and RFP signals were detected in  $27 \pm 2.1\%$ ,  $16 \pm 1.9\%$ , and  $11 \pm 1.4\%$  of cells infected with pure virus, treated with aptamer pool and quadramer, following one cycle of freeze-thawing, respectively. The results show the relatively low aggregation exhibited in quadramer-treated VSV (Figure 5B). These may be explained by repulsion of virus particles from each other after binding to negatively charged DNA aptamers. The aptamers prevent interaction of surface proteins and keep virus particles suspended in solution. It was previously reported that treating VSV with trypsin in order to remove the sticky part of the glycoprotein leads to a decrease in viral aggregation as well.<sup>16,17</sup> Interesting to note, the aptamer-based coating does not suppress virus capability to infect cells due to the noncovalent and reversible nature of aptamer interaction with the virus.



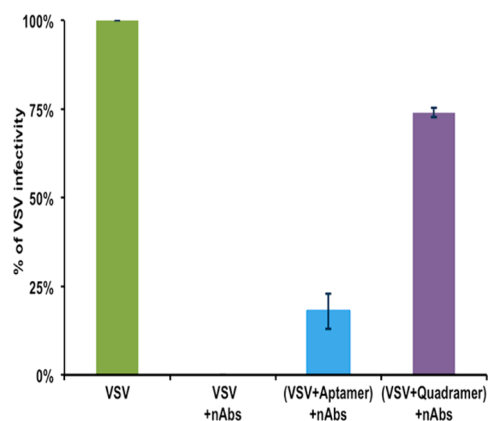
**Figure 4.** (A) Electropherograms for VSV separated by capillary electrophoresis and detected by laser-induced fluorescence. VSV was stained with YOYO-1 dye. CE separations were performed in a 60 cm long capillary under  $250 \text{ V cm}^{-1}$  in 25 mM borax buffer at  $15^\circ \text{C}$ . (i) Fresh, not frozen VSV; (ii–v) VSV after 1 cycle of freeze–thaw, untreated and treated with the library, an aptamer, and a quadramer, respectively. (B) Areas of intact virus peaks for all experiments.



**Figure 5.** Cells infected with a mixture consisting of equal amounts of VSVs coding YFP and RFP. (A) Fluorescent imaging of YFP and RFP in Vero cells after 24 h of virus infection; (B) percentage of cells expressing YFP, RFP, and both after infection with pure VSV and aptamer- and quadramer-treated virus following one cycle of freezing. Less aggregation is observed in the presence of quadramers.

Viral vectors' survival and infectivity in the presence of nAbs are important factors for vaccine efficiency. Therefore, in addition to cryoprotection, we evaluated the aptamer and

quadramer competence to shield VSV from neutralizing antibodies. We measured the infectivity of VSV after incubation with the aptamer pool ( $1 \mu\text{M}$ ) and quadramer ( $0.25 \mu\text{M}$ ) as well as control groups in the presence of rabbit serum containing nAbs on Vero cells. While nAbs neutralized the virus infectivity, incubated VSV with aptamer pool and quadramer retrieved the infectivities of about 25% and 75%, respectively (Figure 6). In order to check the *in vivo* toxicity,  $400 \mu\text{g}$  of both



**Figure 6.** Comparison of the efficiencies of an equimolar mixture of aptamers and quadramer to protect VSV infectivity in the presence of VSV neutralizing antibody.

the aptamer pool alone and the quadramer with VSV were intravenously administered (*i.v.*) through the tail vein two times (day 1 and day 2) into three BL6 male mice (10 weeks old) in two groups, according to the Canadian Council on Animal Care guidelines.<sup>19</sup> They were examined for the external symptoms of toxicity for 5 days after the administration. The mice did not develop diarrhea, lethargy, ruffle fur, loss of appetite, or lose any weight.

**Conclusions.** In conclusion, we have modified anti-VSV aptamers by bridging them together to form tetrameric counterparts and were able to achieve a 1.4 log increase of viral infectivity after 60 freeze–thaw cycles in a plaque-forming assay. Adding these aptamers to VSV prevents viral aggregation and protects its infectivity from neutralizing antibody. The findings of this study opened new doors for aptamer applications as virus cryo- and antibody protectors. These types of aptamers can potentially be applied *in vivo*, in combination with the oncolytic virus, to prevent clearance of the virus and thus increase its oncolytic efficiency without toxic effect.

## EXPERIMENTAL PROCEDURES

**Vesicular Stomatitis Virus Preparation.** The vesicular stomatitis virus was prepared and purified as previously described by Diallo et al.<sup>18</sup> Briefly, Vero African green monkey kidney cells were seeded at  $1 \times 10^4$  cells per  $\text{cm}^2$  in fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD) supplemented with Dulbecco's modified Eagle's medium (DMEM) in 150 mm cell culture Petri dishes. Once they reached around 95% confluence, the cells were infected with the VSV virus at a multiplicity of infection (MOI) of  $\sim 0.01$ . After 24 h, the supernatant was collected, centrifuged, and filtered through a  $0.2 \mu\text{m}$  membrane to remove cell debris. The virus was then pelleted by centrifugation, resuspended in phosphate buffered saline (PBS), and purified with a sucrose gradient.

**Cell and Culture Conditions.** Vero cells were maintained in DMEM supplemented with 10% FBS.



**DNA Aptamers.** All oligonucleotides were synthesized by Integrated DNA Technologies (Iowa, USA). To prepare a pool of different single aptamers, each aptamer was incubated at 95 °C for 5 min, followed by mixing of an equimolar amount of each. For incubation with VSV, the aptamer pool was used at a final concentration of 8  $\mu$ M.

**Quadramer.** The bridge linking the four aptamers together consists of two oligonucleotide strands connected by a central complementary sequence (Figure 1). Each end of the bridge consists of a complementary flank allowing the annealing of an aptamer. To prepare the quadramer, after heating at 95 °C, the sides of the bridge were mixed equally, followed by mixing with the aptamer pool of four clones in equimolar amounts. Subsequently, the complex results in four aptamers annealing to each bridge construct.

**VSV Coating with the Aptamers and Quadramers.** Three groups of VSV were made for assays: untreated VSV as a control and aptamer- and quadramer-treated VSV. For each group, 75  $\mu$ L of virus ( $3 \times 10^9$  PFU) was used together with the aptamer pool (15  $\mu$ L) and the quadramer (15  $\mu$ L), except the control assay, which contained PBS (15  $\mu$ L). The mixtures were incubated for 60 min at 37 °C.

**Freeze–Thaw Cycles.** For testing the dosage effect of the aptamer pool and the quadramer,  $3 \times 10^9$  PFUs of VSV with nine dilutions of the aptamer pool and the quadramer, as well as nine control samples with PBS, were used. The samples were succumbed to 30 freeze–thaw cycles. In order to study the effects of freezing and thawing cycles, after determination of the optimal DNA concentration to be used, seven samples for each VSV group ( $54 \times 10^5$  PFUs) were prepared and exposed to 0, 10, 20, 30, 40, 50, and 60 freeze–thaw cycles. The virus groups were titered for VSV infectivity after each set of 10 freeze–thaw rounds.

**Assessment of VSV Infectivity.** After each set of 10 freeze–thaw cycle experiments, the samples containing the virus were titered using the Vero cell line at  $4 \times 10^5$  per well in 12-well culture plates. After infecting the cells with serial dilutions of each sample in serum free media, the plates were incubated for 1 h at 37 °C in a 5% CO<sub>2</sub> humidified incubator. Thereafter, the medium was removed and the cells overlaid with 1 mL of 0.5% low melting agarose in  $1 \times$  DMEM supplemented with 10% FBS. After 24 h incubation, YFP expressing (fluorescent) cells were visualized using Alfa Innotech Imaging System, version 3.0. Furthermore, a standard plaque assay was performed using the 12-well plates fixed with methanol–acetic acid fixative (3:1 ratio) and stained with Coomassie blue solution, and subsequently, the white plaques were counted.

**Capillary Electrophoresis.** VSV was subjected to one freeze–thaw cycle. Prior to separating each sample by capillary electrophoresis, they were stained with 2.0  $\mu$ M YOYO-1 fluorescent nucleic acid dye (Invitrogen, CA).

Bodipy (Life Technologies, USA) was used as an internal standard. As a control, a VSV sample that did not undergo any freeze–thawing cycle was also subjected to separation by capillary electrophoresis.

A ProteomeLab PA 800 capillary electrophoresis system from Beckman-Coulter, Brea, USA, was used to separate VSV. Fluorescence was induced by a 488 nm Ar-ion laser and detected at  $520 \pm 10$  nm. A bare silica-fused capillary was used, 60 cm in total length with 50 cm from injection to the detection point, an outer diameter of 365  $\mu$ m, and an inner diameter of 75  $\mu$ m. The injections were done by a pressure pulse with hydrodynamic injection volumes of 39 nL. The electric field during the separation was 250 V cm<sup>-1</sup>, with the positive charge at the inlet and ground at the outlet. The capillary temperature was maintained at 15 °C for the duration of the experiment. The run buffer was 25 mM sodium tetraborate at pH 9.84. Prior to each injection, the capillary was rinsed by applying 20.0 psi of 100 mM HCl, 100 mM NaOH, ddH<sub>2</sub>O, and 25 mM Borax for 2.5 min each. 32 Karat software (Beckman-Coulter, Brea, USA) was used for recording the electropherograms.

**Aggregation Test.** Equimolar concentrations of YFP- and RFP-expressing VSV were incubated with the aptamer pool and the quadramer for 1 h at 37 °C and used to infect cells plated on a chamber slide. After 1 h of infection, cells were washed and overlaid with 0.5% low melting agarose with DMEM and supplemented with

10% FBS. After 24 h of incubation at 37 °C in a 5% CO<sub>2</sub> humidified incubator, cells were washed and analyzed by fluorescence microscopy. The cells expressing YFP, RFP, or both YFP and RFP were counted and analyzed (Figure 4).

**In Vivo Toxicity of Aptamers and Quadramers in Mice.** Nine male BL6 mice of 10 weeks of age were obtained and divided into three groups. Two groups (three mice per group) were injected through the tail vein with VSV ( $5 \times 10^4$  PFU) previously incubated with either the aptamer pool or with the quadramer (400  $\mu$ g) in 10  $\mu$ L PBS. Three mice, the third group, were injected with VSV ( $5 \times 10^4$  PFU in 10  $\mu$ L PBS) without the aptamer pool and quadramer as controls. Mice were monitored (5 days) for the development of external symptoms of toxicity, such as ruffle fur, loss of appetite, and body weight loss, according to the Canadian Council on Animal Care guidelines.<sup>19</sup>

**Aptamer Pool and Quadramer Effect on VSV Infectivity in the Presence of nAbs.** Rabbit serum containing nAbs was added to the sample groups with VSV together with the aptamer pool and quadramer for 1 h at 37 °C and were then added to a monolayer of Vero cells. After 1 h, cells were washed, 1% agarose in DMEM was added, and the plates were incubated for 24 h. The infectivity of VSV in each experiment was counted by fluorescent imaging of GFP expression in the infected Vero cells.

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### Notes

The authors declare no competing financial interest.

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