An Assay that Predicts In Vivo Efficacy for DNA Aptamers that Stimulate Remyelination in a Mouse Model of Multiple Sclerosis

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Multiple sclerosis (MS) is a debilitating disease for which regenerative therapies are sought. We have previously described human antibodies and DNA aptamer-streptavidin conjugates that promote remyelination after systemic injection into mice infected by Theiler’s murine encephalomyelitis virus. Here, we report an in vitro assay of myelin binding with results that correlate with remyelination outcome in vivo, as shown for data from a set of DNA aptamer complexes of different size and formulation. This in vitro assay will be valuable for future screening of MS regenerative therapies targeting remyelination.

INTRODUCTION
Multiple sclerosis (MS) is a chronic, debilitating disease with prevalence as high as 0.1% in northern latitudes.1 Although the cause of MS is largely unknown, the disease etiology is considered to be an immune-mediated attack on myelin, a specialized membrane that insulates nerves of the CNS. MS is characterized by CNS lesions accompanied by localized inflammation with common presenting symptoms of numbness, weakness, gait disturbances, and dizziness, with disability often accumulating over time.1,2

Modern treatments for MS address the immune response,3 stimulate repair of existing lesions, or slow disease progression,4 but no current therapies are considered curative. Disease progression and patient disability correlate with axonal damage,5 and evidence suggests remyelination may partially protect neurological function.5 Thus, a potential avenue to address current treatment disparities includes neuro-regenerative therapies exemplified by cell transfer of oligodendrocyte progenitors6 or a recombinant human immunoglobulin M (IgM) autoantibody (rHIgM22) that has been shown to stimulate remyelination in multiple animal models of MS.8 To address limitations associated with biologics, we have identified a DNA aptamer-based alternative that drastically reduces the size and synthetic cost of the stimulatory molecule. This DNA aptamer is the product of in vitro selection (SELEX) against murine myelin suspension as a selection target.1 The myelin binding aptamer sequence of interest, 3064 (Figure 1A), is a guanosine-rich 40-nucleotide sequence characterized by G-quadruplex-forming secondary structures (Figure 1B).9 When functionalized with a 3’ biotin tag and conjugated to streptavidin in a 4:1 molar ratio, the formulation (termed Myaptavin-3064, or 3064-B5 here) exhibits myelin-regenerative properties in the Theiler’s murine encephalomyelitis virus (TMEV) mouse model of MS,10 a model characterized by chronic demyelination.11

Because streptavidin is a bacterial protein, the aptamer-streptavidin complex has the potential to be immunogenic in mammals, potentially limiting its therapeutic efficacy. This prompts the desire to identify other effective aptamer formulations. In the current work, we describe how the lack of activity of a rationally designed streptavidin-free aptamer-presenting DNA complex led to the observation that in vitro myelin binding is a surrogate for in vivo remyelination induction. Here, we describe the design of a rapid and reproducible myelin-binding assay and demonstrate its utility in qualifying novel alternative aptamer formulations by demonstrating the correlation between in vitro assay performance and in vivo remyelination.

RESULTS AND DISCUSSION
Preparation of Protein-Based and Protein-free Aptamer Complexes
Streptavidin-based conjugates studied here involve aptamer sequences shown in Figure 2A. 3’ biotinylated versions of each of these oligonucleotides were mixed in a 4:1 ratio to produce streptavidin-oligonucleotide conjugates (Figure 2B), as described previously.10 Because streptavidin is a bacterial protein, with immunogenicity that may limit its therapeutic efficacy, we therefore sought to develop streptavidin-free aptamer formulations that could overcome this possible limitation.
We designed protein-free multivalent aptamer candidates using NU-PACK software,\(^{12}\) leveraging principles of catalytic hybridization to achieve a dynamic structural equilibrium favoring assembly of aptamer-presenting four-way-junction cruciform structures (“4WJ”). This approach was inspired by previously published work demonstrating programmed biomolecular self-assembly of oligonucleotide systems.\(^{13}\) The design of our programmed biomolecular self-assembly process involved a system of metastable aptamer-presenting hairpin structures (Figure 2D), which can be triggered to sequentially hybridize into higher order complexes (Figure 2E) by the addition of an initiator oligonucleotide (Figure 2C). Versions of protein-free multivalent aptamer 4WJ candidates were also synthesized with locked nucleic acid modified sugars at 5’ and 3’ termini (Figure S1; “3064-4WJ-LNA”) to reduce susceptibility to exonuclease degradation and potentially extend serum half-life.\(^{14}\)

For the previously described streptavidin-aptamer conjugates, conjugation was analyzed by native acrylamide gel electrophoresis revealing a mixture of complexes, dependent on the sequence and folding of the biotinylated aptamers (Figure 3A). 3064 assembly was compared with a second G-rich DNA sequence (3060; Figures 2A and 3A) that does not bind myelin and a T\(_{40}\) negative control DNA sequence (3202; Figures 2A and 3A). All sequences were functionalized with a 3’ biotin tag and a 5’ 6-FAM for visualization. Consistent with our previous work, the results suggest a heterogeneous ensemble of complexes with 1–4 aptamers per streptavidin tetramer.

Protein-free aptamer conjugate assembly was analyzed using agarose gel electrophoresis (Figures 3B and 3C). Stepwise isothermal assembly of multiple hairpin structures into a higher order complex was confirmed, as was the dependence of complex assembly on the presence of the initiator oligonucleotide. Additionally, the prepared protein-free aptamer complexes were observed to be prepared with relatively high homogeneity.

**In Vivo Testing of Protein-free Aptamer Formulations**

Protein-free complexes 3064-4WJ and 3064-4WJ-LNA were tested in the TMEV mouse model of MS to assess whether either of these reagents possesses remyelination-stimulating properties that are comparable to streptavidin-based formulations. Chronically TMEV-infected mice (>180 days post-infection) were dosed with protein-free aptamer formulations and remyelination scores calculated, comparing to streptavidin-based aptamer formulations and buffer control. Remyelination scores following treatment (Table 1) indicate that neither 3064-4WJ nor 3064-4WJ-LNA was as effective at stimulating remyelination as streptavidin-based complex 3064-BS, though both 3064-4WJ and 3064-4WJ-LNA were more effective than 3064-B. Statistical analysis suggests no significance in efficacy between 3064-B and 3064-4WJ or 3064-4WJ-LNA (Table 1). This failure of a rationally designed protein-free multivalent aptamer formulation indicates the complexity of designing remyelinating agents. We hypothesized that the failure of protein-free 3064-4WJ and 3064-4WJ-LNA related either to reduced biodistribution properties or reduced myelin binding affinity. We therefore investigated whether significant differences exist in these parameters that would explain the failure of these agents in in vivo remyelination experiments. The results inspired us to identify an in vitro assay that could be deployed during formulation development for prediction of in vivo remyelinating activity.

**Pharmacokinetics of Protein-Based and Protein-free Aptamer Formulations**

Using a previously devised qPCR method,\(^{15}\) we assessed whether protein-free anti-myelin aptamer formulations 3064-4WJ and 3064-4WJ-LNA demonstrate biodistribution and pharmacokinetic properties similar to streptavidin-aptamer conjugates (3064-BS) or unconjugated biotinylated aptamer (3064-B). Measured serum levels suggest that protein-free aptamer formulations reach the circulation...
following intraperitoneal injection (Figure 4, right) and gain access to the CNS, suggesting they can reach myelin targets in vivo (Figure 4, left and middle panels).

To evaluate whether altered biodistribution influenced the observed difference in therapeutic activity between formulations, we quantitated area under the curve (AUC) using the gamma variate summation model to characterize tissue exposure. We found that AUC values were comparable between each formulation in brain. Biodistribution varied among formulations in spinal cord (Table 2; Figure S2), with 3064-4WJ and 3064-B having greater exposure than 3064-4WJ-LNA and 3064-BS. These trends in biodistribution do not explain the observed remyelination scores (Table 1), where 3064-BS exhibited pronounced remyelination activity compared to the other formulations. Likewise, the difference in tissue exposure of 3064-4WJ and 3064-4WJ-LNA does not explain their similar remyelination scores. This suggests that the observed biological responses in the remyelination experiment are not solely determined by tissue exposure. We hypothesized that remyelination activity may also reflect target binding activity.

We investigated this hypothesis using a novel in vitro myelin binding assay.

**In Vitro Myelin Binding Assay**

We developed a method that recapitulates the original myelin binding selection used to identify remyelinating aptamer 3064. In this method, fluorescein-tagged aptamer samples are incubated with sonicated murine myelin suspensions, followed by separation of bound and unbound aptamer fractions by centrifugation. The resulting myelin pellet is washed prior to re-suspension in PBS (Figure S3), and the fraction of aptamer fluorescence in the bound fraction is measured (Figure 5). This approach controls for potential differences in 5’ 6-FAM labeling efficiency at the time of synthesis and for known heterogeneity among different aptamer complexes. We calculated the effect size of myelin binding relative to 3064-B. Intriguingly, streptavidin-aptamer complex 3064-BS exhibited the greatest myelin binding, with the other protein-based aptamer formulations each showing reduced levels of myelin binding (i.e., negative effect sizes; Table 1). The protein-free aptamer formulations displayed positive effect sizes but smaller in magnitude than 3064-BS (Table 1). When comparing...
the two highest scoring formulations from this assay, 3064-BS and 3064-4WJ-LNA using a Tukey contrast test, a statistically significant difference exists (p = 1.68e⁻⁵). This suggests that myelin binding may be a surrogate for in vivo remyelination and that reduced myelin binding may explain the failure of protein-free aptamer formulations 3064-4WJ and 3064-4WJ-LNA to promote remyelination in animal experiments.

Correlation of In Vitro Myelin Binding and In Vivo Remyelination

We thus hypothesized that in vitro myelin binding of an aptamer formulation corresponds with remyelinating activity in vivo. Indeed, analysis of our data shows this to be the case. In vivo remyelination scores for each aptamer formulation plotted against the fraction bound to myelin in vitro revealed a strong correlation (0.83; p value: 0.02; Figure 6A). We conclude that this simple in vitro myelin binding assay serves as a valuable screening tool capable of prioritizing aptamer formulation candidates for future in vivo studies.

Deviations from expected remyelination scores based on the correlation plot are expected to be influenced by differential tissue exposure in vivo. For example, AUC values in spinal cord tissue are different between formulations, with greater exposure to 3064-4WJ relative to 3064-4WJ-LNA and 3064-BS (Figure S2). Although 3064-4WJ exhibited lower affinity to myelin in vitro relative to 3064-4WJ-LNA (Table 1), 3064-4WJ-LNA did not reach spinal cord tissue to the same extent as 3064-4WJ (Figure S2), resulting in similar remyelination scores. Conversely, although 3064-BS displayed lower spinal cord

Table 1. Comparison of In Vivo Remyelination Score and In Vitro Myelin Binding Assay Result for DNA Aptamer Formulations

| Formulation | Remyelination In Vivo | SEM | p Value Relative to 3064-B | Fraction Bound In Vitro | SD | Effect Size Relative to 3064-B | p Value Relative to 3064-B |
|-------------|-----------------------|-----|--------------------------|------------------------|----|-----------------------------|--------------------------|
| 3064-B      | 8.5 \(^a\)            | 3.4 \(^b\) | –                        | 0.085                  | 0.012 | 0                           | –                        |
| 3064-BS     | 34.9 \(^b\)           | 6.1 \(^b\) | 0.0039                  | 0.194                  | 0.027 | 8.69                        | <0.001                   |
| 3060-B      | 10.3 \(^b\)           | 3.4 \(^b\) | 0.9997                  | 0.052                  | 0.004 | –2.64                       | <0.001                   |
| 3060-BS     | 8.5 \(^b\)            | 4.5 \(^b\) | 0.9999                  | 0.026                  | 0.002 | –4.71                       | <0.001                   |
| 3202-B      | not tested             | –   | –                       | –                     | 0.010 | 0                           | –                        |
| 3202-BS     | 4.2 \(^b\)            | 2.3 \(^b\) | 0.9863                  | 0.026                  | 0.004 | –4.73                       | <0.001                   |
| 3064-4WJ    | 14.0                  | 6.0  | 0.9189                  | 0.108                  | 0.013 | 1.83                        | 0.0048                   |
| 3064-4WJ-LNA| 13.3                  | 4.8  | 0.9692                  | 0.160                  | 0.021 | 6.01                        | <0.001                   |

\(^a\)SD for n = 9 in vitro measurements.
\(^b\)Data previously published.\(^{10}\)
tissue exposure relative to other formulations, its increased myelin affinity resulted in greater remyelination activity. Therefore, we corrected the remyelination score for the effect of spinal cord tissue exposure, i.e., AUC relative to 3064-BS. The results revealed an even stronger correlation (0.96; p value: 0.04; Figure 6B). Thus, optimization of both tissue exposure and myelin affinity is required for maximal remyelination in vivo.

Our observation of a correlation between apparent myelin binding affinity in vitro and stimulated remyelination in vivo gives some insight into the currently unknown mechanism of DNA aptamers that provoke myelin regeneration. It is reasonable to hypothesize that some molecular feature of myelin, perhaps an exposed epitope of a protein, such as myelin basic protein, is recognized by anti-myelin aptamers. The probability and duration of binding and cross-linking of such targets by multivalent aptamer complexes is enhanced by higher affinity. How cross-linking of such targets then signals a regenerative response to oligodendrocytes remains unknown.

Future experiments will also seek to understand how the structural differences between the 3064-BS protein conjugate and the protein-free 3064-4WJ formulations account for altered myelin binding affinity. For example, the 4WJ series formulations are characterized by a rigid and negatively charged core, quite different from the streptavidin core of BS series formulations.

MATERIALS AND METHODS

Aptamer-Streptavidin Conjugate Assembly
DNA aptamer oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) with 3′ biotin- triethylene glycol (TEG) modifications. Fluorescent aptamers were functionalized with an additional 5′ 6-FAM fluorescein modification. Oligonucleotide stock concentrations were determined using a spectrophotometer with molar extinction coefficients estimated by the manufacturer. Negative control oligonucleotide sequences are identified using number codes 3060 and 3202.

Streptavidin purchased from Genscript (Piscataway, NJ; Z02043-5; 5 mg) was re-suspended in 1 mL PBS. Concentration (mg/mL) was estimated by dividing the A280 value (measured using a Nanodrop 100 spectrophotometer) by a factor of 3.2. Molar concentrations were then calculated using the molecular weight of tetrameric streptavidin (54 kDa).

A solution of 1 μM aptamer carrying 3′ biotin-TEG modification in PBS supplemented with 1 mM MgCl2 was incubated in an 85°C water bath for 5 min to disrupt secondary structures and snap cooled on ice for 15 min. A volume of streptavidin solution was added to a final streptavidin concentration of 0.25 μM, giving a 4:1 molar ratio of aptamer to streptavidin. The aptamer/streptavidin solution was mixed and incubated at 37°C for 1 hr to allow conjugate assembly. The distribution of conjugated species was assessed by electrophoresis through 8% native polyacrylamide (29:1 acrylamide: bisacrylamide) gel at 10 V/cm in 0.25× Tris/ borate/EDTA (TBE) buffer. Gels were stained for 1 hr with 1× SYBR Green dye solution in 0.25× TBE buffer and imaged using a Typhoon FLA 7000 imager. Here, complexes are described using codes 3064-BS, 3060-BS, and 3202-BS whereas free biotinylated aptamers are described using codes 3064-B, 3060-B, and 3202-B.
with one-tenth molar equivalent initiator oligonucleotide and incubating in a slow-cooling water bath from 94
modiﬁed and synthesized by IDT. Sequences containing locked-nucleic acid 
thermally annealed on a 2% agarose gel with 1
in equimolar concentrations (4WJ and 4WJ-LNA, respectively) were designed using

Protein-free Complex Assembly
Hairpin-forming oligonucleotide sequences for protein-free complexes with and without modified locked nucleic acid bases (4WJ and 4WJ-LNA, respectively) were designed using NUPACK 3.0. Standard DNA oligonucleotide sequences were synthesized by IDT. Sequences containing locked-nucleic acid modifications were synthesized by Exiqon (German town, MD) at 1 μm scale. One oligonucleotide was synthesized by IDT with a 5’ 6-FAM modiﬁcation. Oligonucleotides were received from the manufacturers as lyophilized pellets and suspended in water to approximate 1 mM concentrated stock solutions. Prior to complex assembly, each hairpin-forming oligonucleotide was diluted 1:100 in water, and the absorbance was measured at 260 nm using a Nanodrop 100 spectrophotometer. Beer’s law was used to calculate actual concentrations, and 2.4 μM working stocks were created. The hairpin-forming oligonucleotides were folded by incubating in a slow-cooling water bath from 94°C to room temperature over 2 hr. The folded oligonucleotides were combined in equimolar concentrations (ﬁnal concentration of 0.55 μM) with one-tenth molar equivalent initiator oligonucleotide and incubated for 3 hr at 37°C. Each assembly reaction was analyzed on a 2% agarose gel with 1× Tris/acetate/EDTA (TAE) buffer containing ethidium bromide. Electrophoresis was at 130 V for 50 min. DNA bands were visualized by scanning gel on Typhoon ﬂuorimeter using ﬂuorescence ﬁlters in ethidium bromide mode.

In Vivo Remyelination in the TMEV Mouse Model
Spinal cord morphometry and remyelination data in TMEV-infected mice compare previously published experiments (3064-BS, 3064-B, 3060-BS, 3060-B, 3202-BS, and 3202-B) and new experiments (3064-4WJ, 3064-4WJ-LNA, and PBS) performed by the methods previously described. All studies conformed to Mayo Clinic and NIH animal use guidelines and were reviewed and approved by the Mayo Clinic Institutional Animal Care and Use Committee as protocol A29509.

Eight-week-old female Swiss Jim Lambert (SJL/J) mice experienced demyelination by 6 months of chronic TMEV infection. Groups of 6–10 mice received 500 μL intraperitoneal injections of the various aptamer formulations (1 μM ﬁnal concentration of aptamer) in Calcium-free D-PBS (Invitrogen) supplemented with magnesium chloride (1 mM). Injections were twice per week for 5 weeks. Mice were euthanized with sodium pentobarbital and perfused intracardially with Trump’s ﬁxative (phosphate-buffered 4% formaldehyde/1% glutaraldehyde [pH 7.4]). Spinal cords were removed, cut into 1 mm blocks, and every third block ﬁxed and stained with osmium tetroxide and embedded in araldite plastic (Polysciences, Warrington, PA). One-micrometer-thick cross-sections were cut from each block, mounted onto glass slides, and stained with 4% paraphenylenediamine to visualize myelin. Cross-sections (10–12) represent samples from the cervical, thoracic, lumbar, and sacral spinal cord. Neuropathology to characterize extent of lesion remyelination was
performed according to a blinded protocol. Slides were first coded by numbers and then randomized across all experimental groups such that slides from one animal were not grouped consecutively together but appeared to the examiner for grading in a blinded and random manner. Two investigators (M.R. and A.E.W.) examined the sections independently. For grading, each spinal cord section was divided visually into four quadrants based on morphological symmetry of the coronal section and examined by bright-field microscopy at 100× and 200× total magnification using an Olympus Provis microscope. Demyelinated areas were characterized by denuded axons and inflammatory cell infiltrates. In contrast, demyelinated areas with remyelination were characterized by thin myelin sheaths compared with the thicker, intact myelin sheaths. The spinal cord white matter was scored as normal, demyelinated with no remyelination, or demyelinated with remyelination. Partial quadrants were excluded. Lesions were judged to be remyelinated when the lesion was 75%–100% repaired. Remyelinated lesions below this threshold were scored as negative. Data were not assembled into treatment groups until all slides in a given study were graded. Demyelination for each mouse was calculated as a percentage based on the number of spinal cord quadrants with demyelination, which includes those quadrants with demyelination and repair, divided by the total number of quadrants scored. Remyelination for each mouse was calculated as a percentage based on the number of demyelinated quadrants above threshold remyelination divided by the number of quadrants with demyelination. Data for percentage spinal cord demyelination and remyelination were compared. p values were calculated by performing one-way ANOVA with multiple comparisons using Graphpad Prism 7.0a for Mac OSX (GraphPad, La Jolla, CA, USA; https://www.graphpad.com).

Pharmacokinetics of Protein-free and Protein-Based Aptamer Complexes in Mice
Pharmacokinetic measurements were made as previously described. Four mice were included for each time point, with one mouse left untreated as a negative control. Aptamer administration, tissue preparation, aptamer extraction, and qPCR were performed as previously described, and the pharmacokinetic modeling results are given in Table 2. Parameter estimates are reported from pharmacokinetic modeling along with uncertainty estimates from fitting 1,000 simulated datasets as previously described. Total tissue exposure to aptamer was estimated by AUC analysis.

Myelin Binding Assay
Murine myelin was prepared from SJL mice as previously described. The in vitro myelin binding activities of different aptamer formulations were measured as previously described. The fluorescent counts measured from the re-suspended pellets were divided by the sum of all fluorescent counts for each sample. Three independent biological replicates of each formulation were tested in three separate trials before pooling “fraction bound” values (n = 9). Error bars indicate SEM.

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Figure 5. Fraction of Aptamer Sample Associated with Myelin Pellet in a Binding Assay
Fluorescent counts were measured from the re-suspended pellet, supernatant, and washes. The fluorescent counts measured from the re-suspended pellets were divided by the sum of all fluorescent counts for each sample. Three independent biological replicates of each formulation were tested in three separate trials before pooling “fraction bound” values (n = 9). Error bars indicate SEM.

Figure 6. Relationship between Aptamer Binding to Myelin In Vitro and Remyelination Stimulation In Vivo
(A) Remyelination scores from in vivo studies are plotted against fraction bound values from in vitro binding assays. Error bars indicate errors given in Table 1. Data for 3064-B, 3064-BS, 3060-BS, 3060-B, and 3022-BS are from previously published data. Open symbols indicate formulations for which there is pharmacokinetic data. (B) Spinal cord tissue exposure corrected score plotted against fraction bound values is shown. Error bars indicate the propagated error of the corrected score, which is the quotient of the remyelination score and AUC for spinal cord relative 3064-BS.
formulations were determined by incubating 230 nM aptamer in the presence of 0.2 μg/μL murine myelin suspension. Excess sheared salmon sperm DNA (20-fold by mass) was included as a competitor to suppress nonspecific binding. Final sample volume was 100 μL. Samples were incubated at 37°C for 90 min and then pelleted by centrifugation (microcentrifuge; 13,000 rpm; 1 min). The supernatant was collected and placed in a clean microfuge tube. The pellet was washed twice with PBS (with centrifugation) before being re-suspended in 100 μL PBS. The supernatant and wash samples were pooled. Pellet and non-pellet fractions were adjusted to an equal volume and placed in wells of a black 96-well microplate (Grenier Bio-One). The fluorescent aptamer signal was quantified using a plate reader (Analyst AD 96-384). Fraction bound values were calculated by determining the fraction of total fluorescent signal from the pellet sample. Control experiments showed that myelin did not quench oligonucleotide fluorescence. The effect size of myelin binding relative to 3064-B was calculated as follows: (mean of aptamer oligonucleotide)/(SD of 3064-B).

SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
Conceptualization, L.J.M., M.R., J.A.S., and A.E.W.; Methodology, L.J.M., M.R., J.A.S., R.M.H., and B.W.; Validation, R.M.H., J.A.S., and B.W.; Formal Analysis, R.M.H., J.P.P., and H.N.L.; Investigation, R.M.H. and H.N.L.; Resources, L.J.M. and M.R.; Writing – Original Draft, R.M.H. and H.N.L.; Writing – Review & Editing, R.M.H., L.J.M., and M.R.; Visualization, R.M.H. and J.P.P.; Supervision, L.J.M. and M.R.; Funding Acquisition, L.J.M., M.R., and A.E.W.

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