Effect of Chemical Modifications on Aptamer Stability in Serum

Christina Kratschmer and Matthew Levy

There is increasing interest in the use of aptamers for the development of therapeutics. However, as oligonucleotides, aptamers are susceptible to nuclease degradation; poor serum stability is likely to negatively affect *in vivo* function. Modified nucleotides have been used to thwart nuclease degradation. However, few studies report the serum stability of selected aptamers. In this study, we examined the effect of various chemical modifications (2'-deoxy, 2'-hydroxyl, 2'-fluoro, and 2'-O-methyl) on the stability of a control oligonucleotide sequence following incubation in frozen human, fresh mouse, and fresh human serum. We also assessed the effect of the 3' inverted dT cap on stability. Surprisingly, we found that fYrR (2'-fluoro RNA) is only roughly as stable as DNA (2'-deoxy). Interestingly, the inclusion of a 3' inverted dT cap had only a modest effect on serum stability, if any. In one instance, the addition of a 3' inverted dT cap rendered a molecule composed of DNA more stable than its fYrR counterpart. By far, fully modified oligonucleotides (100% 2-O-Methyl or 2'-O-methyl A, C, and U in combination with 2'-fluoro G, termed fGmH) had the longest half-lives. These compositions demonstrated little degradation in human serum even after prolonged incubation. Together these results support the need for using fully modified aptamers for *in vivo* applications and should encourage those in the field to exploit newer polymerase variants capable of directly generating such polymers.

Keywords: aptamer, serum stability, chemical modification

Introduction

APTAMERS ARE SINGLE STRANDED oligonucleotides which bind tightly and specifically to a variety of targets, including proteins, sugars, small organic compounds, and even whole cells. There is increasing interest in using aptamers for the development of both therapeutics and diagnostics. However, aptamers, as oligonucleotides, are susceptible to nuclease degradation which may affect *in vivo* function.

Aptamer stability is a function of both structure and chemical composition. Tightly folded molecules are expected to be more resistant to degradation because of limited solvent exposure. However, because aptamer structure is intrinsically related to the affinity for its target, it is difficult if not impossible to modify. Aptamer chemistry on the other hand, in particular, the identity of the sugar moiety, can be easily modified postselection to dramatically improve oligonucleotide stability. However, these modifications can effect sugar conformation and care needs to be taken to ensure that changes in chemistry do not affect aptamer structure and, thus, function.

Both the naturally occurring 2'-deoxy (DNA) and, to a greater extent, 2'-hydroxyl (RNA) nucleic acids are susceptible to ubiquitous serum nucleases. The 2' hydroxyl group, in

particular, can become deprotonated, enabling nucleophilic attack on the adjacent phosphorus of the phosphodiester bond. This forms a 2', 3' cyclic phosphodiester which can become a 2' or 3' nucleotide when hydrolyzed. In serum, reported half-lives for 2'-hydroxy (RNA) nucleic acids are in the order of seconds. This is prolonged slightly, to minutes, when double stranded [1,2]. Reported half-lives of DNA are typically longer, ~1 h [3,4]. The addition of a 3' inverted dT residue can extend this to several hours [5–7]. Modifications to the 2' position of ribose, in particular, 2'-fluoro, 2'-amino, and 2'-O-methyl modifications further increase serum stability [8]. The use of 2'-O-methyl and 2'-fluoro ribose modifications is key to the development of aptamers that have moved toward or through the clinic (reviewed in Maier and Levy [9]).

For the selection of therapeutic aptamers and, in particular, those developed by academic laboratories, the field has come to be dominated by molecules composed of either DNA or 2'-fluoro modified RNA, a combination of 2'-fluoro pyrimidines and 2'-hydroxyl purines (fYrR). DNA aptamer selections, which make use of commonly available DNA polymerases (eg, Taq), are readily accessible to most laboratories and are inexpensive to setup and perform. Just as important, DNA aptamers selected can readily and reliably be chemically synthesized by almost any commercial source.

fYrR RNA chemistry, which renders RNA resistant to RNase A (and RNase A-like enzymes), is generally touted as being “nuclease stable RNA.” It can be readily generated in high yield using the Y639F variant of T7 RNA polymerase [10]. The enzyme and triphosphates are now available from commercial sources as a kit (eg, DuraScribe, Epicentre) which has provided a simple means for laboratories to utilize and adopt this composition for selection. As the molecules contain 2'-hydroxyl groups, chemical synthesis and deprotection are somewhat more complicated than for the production of DNA, a factor reflected in the cost, which has likely prevented this chemistry from being more widely adopted.

There is often much debate between “selectionologists” as to which platform to use for selection. Both DNA and fYrR RNA selections have led to the development of therapeutic aptamers which have progressed to clinical trials. For example, Fovista, an anti-platelet derived growth factor aptamer, was originally selected as a DNA aptamer and subsequently modified to enhance its chemical stability through the incorporation of backbone modifications [11]. Similarly, Macugen, an anti-vascular endothelial growth factor aptamer, and REG1, an anti-Factor IXa aptamer, were both selected from fYrR libraries, but underwent postselection substitution to incorporate 2'-O-methyl nucleosides with the goal of increasing serum stability [12–15].

However, for many groups performing preclinical studies, the additional stabilization that would likely be required to move toward the clinic is not performed. This is likely a consequence of the fact that the process of postselection stabilization is nontrivial. The incorporation of a single backbone modification can ablate function; thus hundreds of different combinations need to be made and tested (see eg: prostate specific membrane antigen [PSMA] patent [16]).

Since the most common library backbone modifications used by the field are DNA or fYrR modified RNA, in this study we examined the half-lives of a control sequence, C36, a molecule that was designed and is predicted to fold into an aptamer-like structure, composed of these two nucleotide compositions in pooled frozen human serum, fresh human serum, and fresh mouse serum. We compared these to fully modified constructs composed of either 100% 2'-O-methyl

RNA or a combination of 2'-fluoro G with 2'-O-methyl A, C, and U RNA (fGmH). In addition, we assessed the effects of the addition of a 3' inverted dT residue on stability. Surprisingly, we observed only a small difference in serum stability between DNA and 2'F pyrimidine modified RNA. Similarly, the effect of the 3' inverted dT was also modest, less than twofold. In the case of the 100% backbone modified RNAs, the stability of these molecules push the limit of what can reliably be determined under our assay conditions. Together, by systematically comparing the serum stability of these constructs in three different serums, our results should help standardize comparisons and guide future work.

Materials and Methods

Chemical synthesis of oligonucleotides

Oligonucleotide synthesis was performed in-house using solid phase synthesis on an Expedite 8909 DNA synthesizer (BioLytic Lab Performance, Fremont, CA). Phosphoramidite monomers were purchased from ChemGenes (Wilmington, MA). All other synthesis reagents were purchased from Glen Research (Sterling, VA). A9.min, an anti-PSMA aptamer [16,17], was synthesized along with eight variants of C36, a nontargeting control (Table 1 and Fig. 1A). It should be noted that although C36 is designed and is predicted to fold into an aptamer-like structure, it is not in fact an aptamer. The sequence of A9.min is 5S GGG ACC GAA AAA GAC CUG ACU UCU AUA CUA AGU CUA CGU UCC C(t) and C36 is 5S GGC GUA GUG AUU AUG AAU CGU GUG CUA AUA CAC GCC (t) where “5S” indicates a 5' thiol and (t) indicates an inverted dT. The predicted folds for A9 and C36 are shown in Fig. 1B. All oligonucleotides were synthesized bearing a 5' thiol modification using a thiol modifier C6 S-S phosphoramidite and a final dimethoxytrityl group on to facilitate purification. Following cleavage and deprotection, oligonucleotides were purified by reverse-phase high performance liquid chromatography (HPLC) on a 10×50 XBridge C18 column (Waters, Milford, MA) using a linear gradient of acetonitrile in 0.1 M triethylammonium acetate (TEAA) at pH 7.0.

Thiolated oligonucleotides were labeled with DyLight 650 (Thermo Fisher, Waltham, MA). Oligonucleotides in 0.1 M TEAA were reduced with 10 mM tris (2-chloroethyl) phosphate

TABLE 1. CHEMICAL COMPOSITION OF SYNTHESIZED OLIGONUCLEOTIDES

Identifier	5' Modifications	2' Ribose nucleotide modifications				3' Nucleotide
		A	C	G	T/U	
DNA T	S	H	H	H	H	Inverted dT
DNA C	S	H	H	H	H	2'-Deoxy C
fYrR T	S	OH	F	OH	F	Inverted dT
fYrR C	S	OH	F	OH	F	2'-Fluoro C
fGmH T	S	OMe	OMe	F	OMe	Inverted dT
fGmH C	S	OMe	OMe	F	OMe	2'-O-methyl C
OMe T	S	OMe	OMe	OMe	OMe	Inverted dT
OMe C	S	OMe	OMe	OMe	OMe	2'-O-methyl C
A9.min	S	OH	F	OH	F	dT

We synthesized A9.min, an anti-PSMA aptamer, as well as eight variants of C36, a nontargeting control sequence. Their 5' and 3' end and 2' ribose modifications are as indicated. S indicates a 5' thiol modifier C6 modification. Note that C36 oligonucleotides synthesized with an inverted dT column contain 37 nucleotides while those synthesized on a C column contain 36 nucleotides. fYrR C was synthesized on a universal support column because of the lack of availability of 2'-fluoro C columns. After cleavage from the column, the first 3' nucleotide is 2'-fluoro C.

PSMA, prostate specific membrane antigen.

FIG. 1. Synthesized oligonucleotides and chemical modifications of nucleotides. **(A)** Chemical structures of the 2' ribose and 3' inverted dT modifications used in aptamer synthesis. These include: 2'-Deoxy (H), 2'-hydroxyl (OH), 2'-Fluoro (F), and 2'-O-methyl (OMe) at the 2' position of the ribose sugar, as well as the inverted dT at the 3' end of the oligonucleotide. **(B)** Structures of C36 and A9.min predicted using mFold.

(TCEP) by heating to 70°C for 10 min and cooling for 1 h at room temperature. Reduction was confirmed by HPLC. TCEP was removed by buffer exchanging into phosphate buffered saline (PBS)(-) using a 10K concentrator (Millipore). Reduced oligonucleotides were incubated for 1 h at room temperature with a fivefold molar excess of maleimide-labeled DyLight 650 (Thermo Fisher). Conjugation was confirmed through HPLC and routinely proceeded to 100%. Excess dye

was removed by buffer exchanging into PBS(-) using a 10K concentrator (Millipore).

The identity of the labeled oligonucleotides was confirmed by liquid chromatography mass spectrometry (LCMS), which was performed on an Agilent 1200 HPLC coupled to a 6130 Single Quad Mass spectrometer. Analysis was performed at 60°C on a 2.1 × 50 XBridge C18 column using 5 mM dibutylammonium acetate and a linear gradient (20%–100%) of acetonitrile. The resulting mass spectra were deconvoluted using MagTran [18]. The reported masses can be found in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/nat).

Serum collection

Serum was obtained from three sources. Frozen, pooled normal human serum was purchased from Innovative Research, Inc. (Peary Court Novi, MI). To obtain fresh human serum whole blood was taken by venipuncture from a deidentified healthy human volunteer who provided informed consent in accordance with Institutional Review Board approval obtained from Albert Einstein College of Medicine. Approximately 20 mL of whole blood was taken by venipuncture into a serum plus blood collection tube. To obtain fresh mouse serum, whole blood was collected from female C57BL/6J mice by terminal cardiac puncture. In both instances, blood was allowed to clot at room temperature for 20 m, and serum was separated by centrifuging at 2,000g for 15 min.

Serum stability assay

Stability assay was performed as previously described [17]. Ten microliters of 25 μM DyLight 650 labeled oligonucleotide was added to 90 μL of serum and incubated at 37°C for up to 7 days. Oligonucleotides were recovered by methanol chloroform extraction [19]. In short, 10 μL of serum was diluted in 50 μL PBS. One hundred microliters of methanol and 50 μL of chloroform were added, and the solution was vortexed. Then, 50 μL of water and 50 μL of chloroform were added, the solution was vortexed again, and the aqueous phase was resolved by centrifuging at 500g for 20 m. Samples were stored at -80°C. An aliquot of the aqueous phase was diluted 1:1 in loading dye and run on a 12% polyacrylamide denaturing gel. Gels were scanned on a LI-COR Odyssey Infrared Imaging System (LI-COR, Lincoln, Nebraska) and quantified using LI-COR Lite software. First order degradation rates were determined using GraphPad Prism 7 software.

Assays performed using fresh mouse serum were conducted in serum pooled from two C57b/6 mice. This was done on three separate occasions, spaced days apart. Reported data are an average of these three replicates. Assays performed using fresh human serum were conducted using serum collected from a single, deidentified male donor on three separate days over the course of a month.

For assays performed comparing direct visualization of the fluorophore to staining with SYBR Gold, 75 μL of the aqueous phase was concentrated by lyophilization and resuspended in 7.5 μL water. Sample was diluted 1:1 in loading dye and run on a 12% polyacrylamide denaturing gel. Gels were scanned on a LI-COR Odyssey Infrared Imaging System on Channel 1 (ex 685/em 700), then stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Carlsbad,

CA), and scanned again using a Storm 840 imaging system (GE Healthcare, Marlborough, MA).

Results

To systemically assess the effects of chemical modifications on serum stability, we chemically synthesized eight variants of C36, a nontargeting control, designed, aptamer-like sequence (Table 1). The predicted folds for both of these molecules are shown in Fig. 1B.

To assess aptamer stability, we used 5'-fluorophore labeled aptamers (to facilitate analysis) and denaturing electrophoresis. Because the loss of the dye molecule alone could result in a misrepresentation of the rates of degradation of the full length aptamer, we initially compared degradation rates calculated from monitoring the DyLight 650 labeled aptamer directly to degradation rates following staining using the nucleic acid stain SYBR Gold. Briefly, control oligonucleotide C36, composed of fYrR RNA bearing a terminal 3'-fluoro cytidine (Table 1; fYrR C), was incubated in frozen human serum acquired from a commercial vendor. Following collection and processing at the specified time points, samples were frozen and stored for subsequent analysis by denaturing gel electrophoresis. To compare methods, a single gel was first scanned using a LI-COR to detect the DyLight 650 label (Fig. 2A; top). [We note that while the excitation

(685 nm) and emission (700 nm) channel on this instrument are not optimal for this fluorescent dye, the machine still proves to be highly sensitive, capable of detecting as little as 250 pg of DyLight 650 labeled oligonucleotide, Supplementary Fig. S1.] The same gel was subsequently stained using SYBR Gold and analyzed on a Storm 840 imager using an excitation of 450 nm and a 520 nm long pass filter (Fig. 2A; bottom). Importantly, there is no cross talk between these readings; the 650-labeled oligonucleotides cannot be detected with the Storm imager unless stained with SYBR Gold. As shown in Fig. 2B, when rates are calculated from the two different methods of analysis, very similar values are obtained. Similar results were observed when experiments were performed in fresh human and mouse serum (data not shown). Thus, direct fluorescent detection of the 5' fluorophore can be used as a proxy for molecular stability of the full length molecule.

An additional consideration for our assay was the stability of the serum itself; the nuclease activity is expected to decrease with time. To quantify the decreasing potency of serum, we compared the stability of C36 composed of fYrR RNA bearing either a terminal 3'-fluoro cytidine (fYrR C) or a 3' inverted dT residue (fYrR T) in fresh mouse, fresh human, or freshly thawed frozen human serum with serum that had been preincubated for 5 days at 37°C. Oligonucleotides were incubated in serum and samples were collected and processed at the indicated time points and analyzed by denaturing gel electrophoresis. Rates were calculated based on detection of DyLight-650. As shown in Fig. 3, the rates of degradation for both oligonucleotides decreased significantly when comparing experiments performed in preincubated to fresh serum.

A summary of the degradation half-life for each condition is shown in Table 2. Frozen human serum and fresh human serum experienced approximately fivefold increases in half-life over time, fYrR T in frozen human serum: 9.9–53 h, fYrR C in frozen human serum: 11–53 h, fYrR T in fresh human serum: 12–56 h, and fYrR C in fresh human serum: 10–59 h. The increase in half-life of fYrR T incubated in mouse serum experienced a similar increase, approximately fourfold, from 2.5 to 9.9 h. Interestingly, fYrR C incubated in mouse serum experienced a much less dramatic change in degradation rate over time, less than twofold, from 2.2 to 3.7 h. Together these data suggest that care needs to be implemented when assessing serum stability as degradation rates are significantly slowed during prolonged incubation. Thus, although subsequent assays were taken to 5 days (120 h), all fits to determine rate of degradation were performed using data points collected within 24 h except where indicated.

Having established parameters and validated our approach for assessing serum stability, we measured the stability of all of the molecules listed in Table 1 in frozen human serum, fresh human serum, and fresh mouse serum. Again, molecules bearing a 5' DyLight 650 fluorescent tag were incubated in serum samples at 37°C after which time samples were collected, processed, frozen, and analyzed by denaturing gel electrophoresis. Gels were imaged by detection of the DyLight 650 and rates of degradation determined based on a fit of the data (Fig. 4). A summary of the half-life of degradation for each condition is shown in Table 2.

When composed of DNA, the control oligonucleotide proved to be the least stable of the molecules tested. In human

FIG. 2. Degradation of fYrR C in frozen human serum by direct visualization of the DyLight 650 fluorophore and by SYBR Gold Nucleic Acid Stain. **(A)** DyLight 650 labeled fYrR C was recovered from frozen human serum and run on 12% polyacrylamide gels as described in the methods. The fluorophore was visualized by scanning on a LI-COR infrared imaging system (*top*). The gel was then stained with SYBR Gold and reimaged by scanning on a Storm 840 (*bottom*). **(B)** The fraction of intact oligonucleotide was determined from a plot of the area corresponding to the full-length oligonucleotide on the gel. Half-life calculations are based on a minimum of two independent experiments and assume 100% degradation. Measurements taken after 24 h were excluded from the calculations because of the decreasing potency of serum over time. The half-life for each condition is as indicated.

FIG. 3. Potency of fresh versus preincubated frozen human serum, fresh human serum, and mouse serum. The fraction of intact oligonucleotide remaining after incubation of (A) fYrR T in freshly thawed frozen human serum (*dark gray*) and preincubated frozen human serum (*black*), (B) fYrR C in freshly thawed frozen human serum (*dark gray*) and preincubated frozen human serum (*black*), (C) fYrR T in fresh human serum (*light gray*) and preincubated human serum (*black*), (D) fYrR C in fresh human serum (*light gray*) and preincubated human serum (*black*), (E) fYrR T in fresh mouse serum (*light gray*) and preincubated mouse serum (*black*), and (F) fYrR C in fresh mouse serum (*light gray*) and preincubated mouse serum (*black*). Half-life calculations are based on a minimum of two independent experiments. Half-lives are reported in Table 2.

serum, the half-life ranged from ~5 to 16 h. Interestingly, the presence of the inverted dT had only a modest effect on stability, improving the stability in human serum (both fresh and frozen) by approximately twofold (Table 2; DNA T Frozen Human Serum: 16 h, DNA C Frozen Human Serum:

6 h, DNA T Fresh Human Serum: 8.2 h, and DNA C Fresh Human Serum: 4.9 h). In mouse serum, the half-life for degradation was faster, ~1.7 h. Surprisingly, the inverted dT had essentially no effect on stability (Table 2; DNA T Mouse Serum: 1.8 h, DNA C Mouse Serum: 1.6 h).

TABLE 2. DEGRADATION HALF-LIVES OF OLIGONUCLEOTIDES IN SERUM

Identifier	Hours (95% confidence interval)					
	Frozen human	Fresh human	Fresh mouse	Preincubated frozen human	Preincubated fresh human	Preincubated fresh mouse
DNA T	16 (12–23)	8.2 (7.2–9.4)	1.8 (1.6–1.9)			
DNA C	6 (4.8–7.6)	4.9 (4.4–5.4)	1.6 (1.4–1.9)			
fYrR T	9.9 (6.8–15)	12 (9.3–15)	2.5 (2.1–2.8)	53 (34–110)	56 (34–13)	9.9 (6.7–14)
fYrR C	11 (9.9–13)	10 (8.4–13)	2.2 (2.0–2.4)	53 (40–74)	59 (52–68)	3.7 (3.3–4.3)
fGmH T	>240	>240	180 (150–250)			
fGmH C	>240	>240	110 (85–150)			
OMe T	>240	>240	230 (130–830)			
OMe C	>240	>240	75 (60–99)			
A9.min	8.0 (6.1–11)	3.8 (2.7–5.3)	2.5 (2.4–2.7)			

The degradation half-life of A9.min and C36 constructs in frozen human, fresh human, and fresh mouse serum presented in h, including the 95% confidence interval. Preincubated serum was heated to 37°C for 5 days before addition of oligonucleotides. The fraction of intact oligonucleotide was determined from a plot of the area corresponding to the full-length oligonucleotide on the gel. Half-life calculations are the result of a minimum of two independent experiments. The potency of fresh and frozen human serum begins to decrease after 24 h; hence half-life calculations are based on DyLight 650 measurements taken in the first 24 h assuming 100% degradation over time. Mouse serum retains its potency longer; half-lives in mouse serum are based on DyLight [650 measurements taken over 72 h (fresh mouse serum)] or 48 h (preincubated mouse serum).

For constructs composed of 2'-fluoro RNA the calculated stability half-lives ranged from 2.2 h in mouse serum, without a 3' inverted dT, to 12 h in fresh human serum, with a 3' inverted dT (Table 2; fYrR T and C). Here again, the effect of the inverted dT on stability was essentially nonapparent; fYrR C demonstrated a longer half-life than fYrR T although the difference is not significant.

As a comparator, we measured the stability of the anti-PSMA aptamer, A9.min, a fYrR aptamer synthesized with a 3' inverted dT (Table 2; A9.min). The molecule demonstrated the greatest stability when assayed in pooled frozen human serum, 8.0 h, and was somewhat less stable in fresh human serum, 3.8 h. Consistent with the other oligonucleotide compositions, the molecule was least stable in fresh mouse serum, demonstrating a half-life of ~2.5 h, a value similar to that previously reported by us [17].

Consistent with the literature [20], when composed of 100% OMe RNA, the resultant oligonucleotides demonstrated significant nuclease resistance, with only trace levels of degradation detected when assays were performed in pooled, frozen human serum or fresh human serum (Table 2; OMe T and OMe C). Unfortunately, because of the decreasing potency of fresh and frozen human serum an accurate calculation of half-lives was not possible. Similarly, assays performed on constructs composed of 2'-fluoro G in combination with 2'-O-methyl A, C, and U (Table 2; fGmH), a recently reported nucleotide mixture [21] proved quite stable in human serum, with little degradation observed over the course of the assay. Again, however, the relatively slow degradation rate relative to the rate at which serum potency decreases prevented us from quantifying the half-lives of fGmH C or T. In addition, this prevents us from drawing conclusions about the effect of the inverted dT on the OMe and fGmH oligonucleotides in human serum. However, half-life calculations were possible in mouse serum. Incubation in mouse serum resulted in slightly faster rates of degradation for these molecules (Table 2; OMeT: 230 h, OMe C: 75 h, fGmH T: 180 h, fGmH C: 110 h). The more stable activity

of this serum (Fig. 3) afforded the ability to confidently fit these data out to 72 h (although a fit of the data through only 24 h did not significantly alter the rates). In this study, the presence of the inverted dT had a modest approximately threefold effect on stability.

Discussion

There are a variety of different chemistries available for aptamer development, including 2'-deoxy, 2'-hydroxyl, 2'-fluoro, 2'-O-methyl, and 2'-amino. While the earliest reported selections utilized either DNA [22] or RNA [23], later selections focused on identifying ligands composed of nuclease stabilized variants of RNA composed of 2'-fluoro pyrimidines and 2'-hydroxyl purines (fYrR; so called 2'-fluoro RNA) or 2'-amino pyrimidines and 2'-hydroxyl purines (aYrR; so called 2'-amino RNA) (see eg, Green *et al.* [24]). However, for most aptamers which have progressed toward the clinic (Fovista, REG1) [11,12] or have been FDA approved (Macugen) [25], additional modifications to further enhance stability have been necessary (reviewed in Maier and Levy [9]). Most recently, through the optimization of reaction conditions and development of novel polymerases [26–28], it has become possible to more efficiently generate oligonucleotides with 100% modified sugars (see eg: Burmeister *et al.* [20] and Friedman *et al.* [21]). However, the use of these novel polymerases has yet to really catch on, and DNA and fYrR selections predominate. This is especially true in academic laboratories which to a large extent are focused on developing aptamers for therapeutic purposes, necessitating more stable chemistry. Few studies, in fact, report the serum stability of selected molecules, a potentially critical feature especially for assessing preclinical *in vivo* studies. To our knowledge there have been no systematic studies of the effect of composition on aptamer serum stability.

In this study, we examined the serum stability of four different aptamer chemistries: the commonly used DNA and fYrR, 100% 2'-O-methyl, and a more recently reported

FIG. 4. Half-life of DyLight 650 labeled aptamers in pooled, frozen human serum (**A**); fresh human serum (**B**); and fresh mouse serum (**C**). Plots shown on the *right* represent the first 24 or 12 h of those on the *left* and display only the data for constructs composed of DNA and fYrR. Ten microliters of 25 μ M DyLight 650 labeled aptamer was incubated in 90 μ L of serum at 37°C for the indicated time. Data are presented in hours, including the 95% confidence interval and are the result of a minimum of two independent experiments. Because of the decreasing potency of serum over time, the half-life of aptamers in pooled, frozen human serum and fresh human serum was calculated based on measurements taken in the initial 24 h, assuming 100% degradation over time. However, the fraction of intact aptamer past 24 h is included for reference. Mouse serum retains its potency much longer; the half-life of aptamers in mouse serum was calculated based on measurements taken in the initial 72 h, assuming 100% degradation over time. Again, measurements past 72 h are included for reference. Data shown for fYrR T and fYrR C are replicated from Fig. 3. Color images available online at www.liebertpub.com/nat

fGmH composition [21]. For our comparison, we utilized a nonfunctional control aptamer-like sequence, C36, which our laboratory has previously used as a control for targeting experiments [17,29,30]. Stability experiments were performed using fresh human serum from a donor, frozen pooled serum from a commercial source and fresh mouse serum to compare how the serum source affected stability. In addition, we assessed the effect of adding a 3' inverted dT to each molecule, a common method for inhibiting 3' exonuclease activity in serum.

In initial experiments, we found (perhaps unsurprisingly) that the potency of the nucleases in serum decreases with extended incubation time. Using our fYrR T and fYrR C constructs, we compared the rate of degradation of oligonucleotides following incubation in fresh serum to serum pre-incubated for 5 days at 37°C. After 5 days, the potency of both human serums decreased by nearly fivefold based on the observed decrease in degradation rates. Mouse serum, on the other hand, was more robust, showing only a modest (less than twofold) decrease in activity. As a result of these findings,

although time courses for experiments shown in Fig. 4 were taken to 5 days, rates were not calculated using all of the time points collected. For experiments performed in human serum using both DNA and fYrR constructs, rate calculations were limited to points collected within the first 24 h. Rates calculated using these points are, in fact, in good agreement ($\sim 15\%$ variation) with values calculated from limiting the analysis to data collected within the first 12 and even 6 h, reaffirming that the loss of potency in this time frame is not significantly affecting our analysis (data not shown). For experiments in human serum performed with the more stable nucleoside compositions, OMe and fGmH, an accurate assessment of the stability is simply not possible.

For our studies in mouse serum, fits for DNA and fYrR constructs were performed over 72 h although data fit to an earlier time point, for example, 24 h yielded similar results (data not shown). In the case of the more stable compositions (OMe and fGmH), fits were also performed using data points out through 72 h. Fitting the data with only a single point at 24 h yielded comparable results (data not shown), indicating that any decrease in potency of the serum was not adversely affecting these calculations.

It is also important to consider that although the sequence of our test molecule, C36, was designed to and is predicted to fold into an aptamer-like structure (Fig. 1B), it is not an aptamer. Aptamers are selected to fold into specific functional structures. Our designed control sequence, therefore, might not be expected to adopt as stable of a structure, especially within the nonpaired loop regions which may be stabilized by canonical pairings in selected molecules. As such, these data should be considered a lower limit for stability. Although we note that when we performed similar analyses using a bona fide fYrR aptamer, A9.min, the serum half-lives under all conditions tested were quite similar to that of C36 composed of the same nucleotide mixture.

Other aptamers may behave differently, a likely consequence of their primary, secondary, and tertiary structures. For comparison, a fYrR modified anti-thrombin/prothrombin aptamer, R9D-14T, has a half-life of >6 h (although <24 h) in human serum [31], well in line with the results that we obtained. However, a fYrR variant of the anti-trypanosome aptamer, 2-16, is predicted to form a stable pseudoknot and has a much longer half-life, 81 h [32]. Similarly, a conjugate composed of an siRNA linked to the highly structured fYrR EpCAM aptamer, which is predicted to form a very tight hairpin, has a reported half-life of >36 h, although these assays were only performed in 50% human serum [33]. Structural effects apply to other backbone and sugar chemistries as well. For example, the ssDNA oligonucleotide, T30175, which is predicted to form a G-quadruplex demonstrated a half-life of ~ 5 h while a point mutant of the sequence, which abolished this structure, demonstrated a half-life of only ~ 3 m [34].

Surprisingly, in our analyses we found that the fYrR chemistry was in general only slightly more stable than DNA chemistry. In frozen human serum, the stability of C36 DNA T actually proved to be greater than its fYrR counterpart.

An additional surprise finding was the level of protection afforded by the addition of a 3' inverted dT residue which is reported to inhibit exonuclease activity [6], a primary driver

of serum degradation [35]. For the fYrR construct, there was essentially no effect on stability; for the DNA construct the effect was modest: approximately two- to threefold in both human serums. Dass *et al.* reported that this modification enhanced the half-life of a DNA deoxyribozyme from 70 min to 22 h [5], whereas Shaw *et al.* reported an increase from <5 min to ~ 4 h [7]. In addition, Beigelman *et al.* reported that the inverted T residue improved stability 30–50-fold depending on the particular chemistries involved [36]. In all of these examples, the constructs tested were predicted to be unpaired at the 3' end, suggesting that this modification only affects the stability of oligonucleotides with unpaired ends, unlike our construct. A similar modest enhancement in stability (two- to threefold) was observed for this modification on the fully modified fGmH and OMe oligonucleotides in mouse serum (assessment of the stability of these oligonucleotides in human serum is not possible).

To date, aptamer selections with fYrR and DNA chemistries have predominated due to the availability of polymerases which could directly incorporate their nucleotides during Systematic Evolution of Ligands by EXponential Enrichment. However, these chemistries provide, at baseline, half-lives of less than ~ 10 h in fresh human serum. Although it is possible to select fYrR or DNA aptamers with structural features to impart improved nuclease resistance, this is not guaranteed. Until recently, incorporating nucleosides which impart greater stability (2'-O-methyl or purine 2'-fluoro) generally required tedious postselection modifications. However, with the availability of newer polymerases, it is now possible to directly incorporate these chemistries into the selections [20,21,26,27].

We note that the needed stability of an aptamer will depend on its clinical purpose. For example, without pegylation aptamers *in vivo* will be rapidly cleared, and as such, excessively stable aptamers may not be necessary. However, because postselection modification is nontrivial and polymerases to select fully modified aptamers have been reported, we urge researchers to consider more stable chemistries for selection libraries moving forward. While exceptionally stable molecules are not a prerequisite for many applications, it is significantly easier to adapt these molecules for clinical indications than it is to impart nuclease resistance on a less stable one.

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Address correspondence to:

Matthew Levy

Department of Biochemistry

Albert Einstein College of Medicine

Bronx, NY 10461

E-mail: matthew.levy@einstein.yu.edu

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