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RESEARCH ARTICLE

Chemically "barbed" aptamers selected from a base-modified RNA library

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ABSTRACT

Aptamers are capable of binding to target proteins with high affinity and specificity. Recent technologies have focused on increasing the chemical diversity of libraries for the isolation of aptamers with improved binding. For this, DNA has been the nucleic acid of choice, in part due to the ability of certain DNA polymerases to accommodate modified DNA bases. Success with base-modified RNA libraries has been rare to date, despite potential advantages of RNA aptamers over DNA aptamers. Here we further expand the range of base modification in RNA aptamers by a process called RNA-SELMA (SELection with Modified Aptamers). In this process, RNA is displayed by the DNA that encodes it, enabling the selection of post-transcriptionally modified RNA libraries and amplification of their encoding DNA, thus circumventing the need for reverse transcription. In this report, we show that chemical attachment of N-hydroxysuccinimidyl esters to DNA-displayed RNA libraries enables the selection of aptamers capable of bioconjugation with a target protein, streptavidin, at low nanomolar concentration with high specificity.

KEYWORDS: SELEX, mod-SELEX, modified aptamer, RNA-SELMA, covalent aptamer, ethynyluridine

INTRODUCTION

DNA and RNA aptamers bind targets with affinities similar to that of antibodies and are obtained by a process called Systematic Evolution of Ligands by EXponential Enrichment (SELEX) (Blind and Blank, 2015; Sun and Zu, 2015; Maier and Levy, 2016). It has become evident that aptamers favour certain types of targets for high-affinity interaction. In particular, the surface charge of the target protein plays a role in the potential for high-affinity binding, whereby positively charged patches favour strong interaction with the highly negatively charged nucleic acids (Bjerregaard et al, 2016; Gelinas et al, 2016). To circumvent this limitation, researchers have sought to increase the chemical diversity of aptamer libraries, which normally consist of each of the four nucleotides, reviewed in (Lapa et al, 2016; Lipi et al, 2016). The DNA base thymine has proved to be amenable to chemical modification and subsequent amplification, owing to the ability of family B DNA polymerases (Vent, Pfu, KOD) to accommodate the polymerization and copying of thymine-modified nucleotide triphosphates and templates, respectively (Kuwahara et al, 2003). Specifically, the methyl group of thymine, which faces the major groove of double stranded DNA, has been replaced with a wide variety of functional groups with varying linker lengths. The company Somalogic™ has attained success with a short amide linker and planar aromatic functional groups (Gold et al, 2010; Gupta et al, 2014). This enables the formation of strong hydrophobic interactions with targets via the major groove while maintaining a semi-rigid stem loop structure. Recently, a method termed SELMA (SELection with Modified Aptamers) was introduced for obtaining

heavily chemically modified DNA aptamers (MacPherson et al, 2011; Temme et al, 2013; Temme and Krauss, 2015). In SELMA, an unmodified, double-stranded copy of the aptamer remains covalently bound to the single stranded, chemically modified aptamer. SELMA was successfully used to isolate glycosylated DNA libraries with high affinity (~10nM dissociation constant) for a glycan-binding antibody, 2G12 (Temme et al, 2014). Later, a method termed click-SELEX was introduced for the introduction of planar hydrophobic functional groups into DNA libraries (Tolle et al, 2015).

Despite the prevalence of RNA aptamers in the pharmaceutical industry, the increased folding diversity of RNA, and the excellent nuclease resistance of 2' modified RNA, base-modified RNA aptamers are less prevalent in the literature. Traditional RNA SELEX must utilize two enzymatic processes, transcription and reverse transcription, which are distinct from DNA SELEX. For base-modified RNA aptamers to be successfully generated and amplified, transcription and reverse transcription additionally must accommodate the incorporation and copying of base-modified RNA, respectively. To circumvent this obstacle, the DNA-display principle of SELMA was recently extended for use with RNA, where an unmodified double stranded DNA is physically bound to the RNA that it encodes (MacPherson et al, 2017).

Sometimes it may be beneficial for a permanent, covalent interaction between aptamer and target, for example for enabling efficient and robust presentation of targets on DNA nanostructures or other nucleic acid-based nanodevices (Surana et al, 2015). In addition, covalent coupling of aptamer and target proteins enables the use of harsh conditions for separation and quantification of bound aptamers. Somalogic™ has previously used photo-crosslinking chemistry to evolve aptamers that form a covalent bond with their target (Golden et al, 2000).

In this paper, we expand the repertoire of covalent aptamers by using RNA-SELMA. We introduce the concept of the "barbed aptamer", in which a covalent bond is formed upon specific binding of the aptamer to its target. RNA libraries are functionalized with an aromatic N-hydroxysuccinimidyl (NHS) ester, which can react with protein primary amines to form stable amide linkages. We isolate NHS ester-labelled aptamers that specifically and irreversibly react with streptavidin.

MATERIAL AND METHODS

Materials

All enzymes, streptavidin, streptavidin magnetic beads, denatured BSA and natural nucleotide triphosphates used in the study were purchased from New England Biolabs. TOPO-TA cloning kit and non-denatured BSA (RNAse-free) was purchased from Thermo Fisher Scientific. All oligonucleotides were purchased from Integrated DNA Technologies. Ethynyluridine triphosphate (EUTP) was purchased from Jena Biosciences. Sulfo-HSAB was purchased from GBiosciences. Chemicals, electrophoresis reagents and equipment were purchased from VWR, Sigma and Bio-Rad.

RNA-SELMA library construction

Library construction began with a limited (6-cycle) PCR of an 87-base aptamer library (Table S1) using a biotinylated forward primer (85pmol), isodC-containing reverse primer (80pmol), 5pmol template library, Vent-exo polymerase (4 units) and 200µM each dNTP in a 200µl reaction. Thermal cycling was 95 °C for 60sec followed by 6 cycles of 95 °C for 30sec, 57 °C for 30sec, and 72 °C for 10sec. The 200µl PCR product was incubated with 40 units of exonuclease I for 30min at 37°C to digest unused primer. 25µl of 4M NaCl and 5µl 500mM EDTA were added followed by incubation with 0.28mg hydrophilic streptavidin magnetic beads on a rotator for 30min. The beads were washed with 150µl, 125µl and 100µl wash buffer (20mM Tris pH 8.0, 500mM NaCl) with transfer to a fresh tube after each wash, and incubated with 40µl 100mM NaOH for 4min. The supernatant was mixed with 4µl 1M HCl and 1µl 1M Tris pH 8.0 for neutralization. 23µl was added to a new 50µl reaction containing the capture arm primer (40pmol) and 5µl 10x thermopol buffer in a volume of 48µl. The reaction was heated to 95°C for 60sec followed by 57°C for 60sec. 1µl Bst 2.0 warmstart polymerase (8U) and 1µl dNTPs (10mM each) was added, and the reaction was incubated at 60°C for 90sec. The reaction was cooled briefly on ice and loaded onto a Sephadex G-50 spin column equilibrated with 20mM Tris pH 8.0, 4mM $MgSO_4$ and centrifuged to buffer-exchange the library. 14µl of buffer-exchanged library was combined with 2µl T7 RNA polymerase buffer and 10pmol capture arm rigidifier (1µl of a 10µM solution). The mixture was incubated at 50 °C for 5min to facilitate annealing of the rigidifier and incubated with 0.1mg streptavidin magnetic beads to remove biotinylated contaminants. To the supernatant, 2µl EUTP-containing NTPs (5mM each NTP, EUTP replacing UTP), 0.2µl 1M DTT and 5 units T7 RNA polymerase were added and the reaction was incubated at 37 °C for 10min. 10µl of 1X PBS was added to the reaction followed by immediate buffer-exchange into 1X PBS pH 6.0 using a Sephadex G-50 spin column.

Copper(I)-catalyzed azide-alkyne cycloaddition "click" reaction

The copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction was performed as follows: The buffer exchanged library was combined with 3.75µl of 10mM THPTA, 1µl of 25mM $CuSO_4$ and 6µl of 25mM N-hydroxysulfosuccinimidyl-4-azidobenzoate (sulfo-HSAB) (dissolved just prior) in a capless 0.5ml eppendorf tube. To a separate capless tube, 20µl freshly dissolved sodium ascorbate (250mM) was added. The tubes were placed in a two-necked pear-shaped flask (25ml) fixed with rubber septa, 18-gauge needles and tubing to allow the flow of argon into one neck and the flow of circulated argon out of the other neck. Foil was placed over the entire system to minimize light degradation of the sulfo-HSAB. Argon flushing was initiated with high flow rate (~5l/min) for 3min followed by low flow rate (150ml/min) for 30min. At low flow rate, the exhaust septa/needle were removed and the reaction was initiated by transfer of 1µl sodium ascorbate from one capless tube to other capless tube containing the library, THPTA, copper and sulfo-HSAB followed by pipette mixing with care taken to minimize the introduction of air from the pipettes into the flask. The reaction was allowed to proceed under low argon flow for 30min. The reaction

was then buffer-exchanged twice into 1x PBS pH 7.2 using Sephadex G50 spin columns.

Selection

The buffer-exchanged library was immediately exposed to hydrophilic streptavidin magnetic beads for covalent attachment. In round 1 of selection, the library was exposed to 0.2mg of streptavidin magnetic beads for one hour at room temperature on a rotator. These selection conditions were repeated for rounds 2-8. In rounds 9-12 heat-denatured BSA was included and 0.1mg streptavidin magnetic beads were used. In rounds 13-16, non-denatured BSA (rnase-free) was included at increasing concentration (5µM - 15µM) with decreasing reaction time (30min - 5min) and streptavidin bead amount (0.1 - 0.02mg). The beads were washed with 150µl and 100µl of wash buffer (20mM Tris pH 8.0, 500mM NaCl) resuspended with 30µl elution buffer (20mM Tris pH 8.0, 5% (v/v) Tween-20, BSA (150ug/ml), 50mM NaCl) and heated at 95°C in a dry bath incubator for 3min. The supernatant was added to a PCR mix containing 70pmol biotinylated forward primer, 70pmol isodC reverse primer, 200µM each dNTP and 1x thermopol buffer in a total volume of 230µl. 30µl was removed to which 0.6UVent-exo polymerase was added and distributed to 3 tubes which were immediately subjected to thermal cycling. PCR reactions were retrieved at varying cycle intervals and run on 2% (w/v) agarose and visualized with ethidium bromide to determine the optimal cycle number for recovery of the library. Then, 4U of Vent-exo were added to the remaining 200µl reaction and the PCR was allowed to proceed to the optimal cycle number. Library was regenerated as with the initial library to initiate another selection round. Following the 16th round of selection, the library was amplified with unmodified forward and reverse primers for TOPO-TA cloning into plasmid PCR2.1. Clones were obtained with blue-white colony screening, the plasmids isolated and sequenced using M13 forward and reverse primers.

Binding studies

Double-stranded DNA PCR products were generated using the biotinylated forward primer and isodC reverse primer and the total library or isolated clone plasmid as template. The PCR product was used directly in a transcription reaction containing EUTP-containing NTPs (0.5µM each), T7 RNA polymerase in 1x T7 RNA polymerase buffer supplemented with 10mM DTT. Crude RNA products were buffer-exchanged into PBS pH 6.0 with a Sephadex G50 spin column and a portion (100ng) was modified with sulfo-HSAB as described above before the addition of the appropriate amount of RNA for the assay (5nM final concentration). For detailed characterization of clone 4, a large amount of EU-containing RNA was PAGE-purified. ~10ng was modified with sulfo-HSAB. The appropriate amount of streptavidin was mixed with aptamer (~0.5nM) and the reaction was allowed to proceed for 1hr in endpoint determination experiments. Reactions were quenched by addition of 1% (w/v) SDS followed by immediate thermal denaturation at 98°C for 3min after which Tris pH 8.0 was added to a final concentration of 100mM and addition of SDS loading buffer (1x concentration 6% (v/v) glycerol, 1% (v/v) SDS, 20mM Tris pH 8.0, xylene cyanol dye (trace)). In time-point experiments, reactions were stopped at 3min intervals by the above method. For selectivity experiments, aptamer (0.5nM) was incubated with and without 10nM streptavidin and varying concentration of non-denatured BSA. In all gel shift experiments, visualization was accomplished by annealing a 21-base DNA strand (20nM) labelled with a fluorophore (IRDye700) in SDS loading buffer. After an initial denaturation step of 98°C for 3min, samples were cooled to 70°C followed by slow cooling (0.3°C/S) to 37°C. Samples were loaded onto 10% (w/v) polyacrylamide SDS-PAGE mini-gels (19:1 acrylamide:bisacrylamide) and electrophoresed at 200V for 12min. Gels were imaged and quantified with a LI-COR Odyssey system.

RESULTS

SELMA-based selection of bioconjugation-proficient RNA aptamers

A schematic of the library generation and selection strategy is presented in Figure 1. The library was generated by the RNA SELMA method, in which a “capture arm” is added to a typical double-stranded DNA library consisting of a T7 RNA polymerase promoter and a random region (25 bases) flanked by 2 constant regions. When transcription occurs, the capture arm is capable of annealing to the nascent RNA before it dissociates from the T7 RNA polymerase. This is aided by the addition of a non-natural analog isodC at the 5’ end of the template strand. We utilized a uridine triphosphate analog 5-ethynyluridine triphosphate in the transcription reaction to enable copper-catalyzed alkyne-azide cycloaddition, or “click chemistry” modification of the RNA. After transcription, the library was modified with sulfo-HSAB via CuAAC. The library was then exposed to streptavidin magnetic beads, the bound library was amplified by PCR and the library regenerated for the next round of selection. During the 7th round of selection, the PCR cycle number required for optimal recovery decreased from 14 to 10, suggesting enrichment of covalent binders for streptavidin. During selection cycle 9-12, heat-denatured BSA (1-5µM) was included at the binding step to counter-select non-specific binders. A further 4 rounds of selection were performed with non-denatured BSA competitor (5-15µM) and increased stringency. A table summarizing selection conditions and PCR recovery is found in supplementary data (Table S2). After the 16th round of selection, the library was cloned and 10 isolates were sequenced and characterized.

The selected library is enriched for covalently binding aptamers

We detected aptamers and aptamer complexes by annealing a fluorophore-labeled DNA strand to the RNA under SDS-denaturing conditions, which do not substantially affect oligonucleotide hybridization (Rose et al, 2002). Figure 2 is an SDS-PAGE gel shift assay comparing the library after 16 rounds of selection with a naïve library. In the presence of streptavidin, a shifted band (lane 2) can be clearly identified for the selected library but not for the naïve library (lane 4). To rule out the possibility of other factors in the apparent covalent binding, “mock” modified EU-containing RNA were prepared in which sulfo-HSAB was omitted from the CuAAC reaction. In this scenario, no gel shift was observed for either the selected (lane 5) or naïve

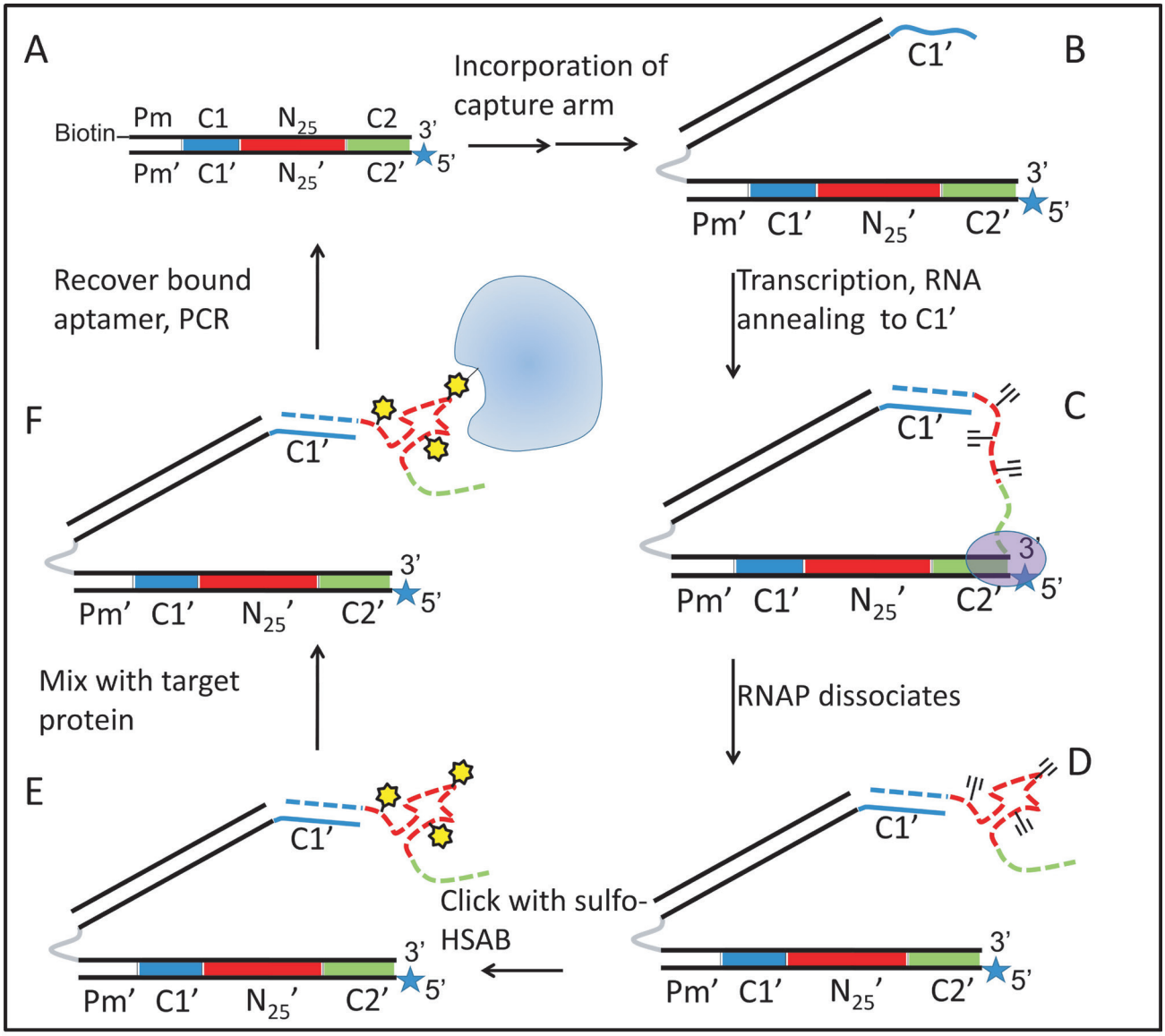


Figure 1. RNA-SELMA library generation and selection scheme. **A.** The library starts as biotinylated double stranded DNA. **B.** The non-biotinylated strand is isolated followed by annealing and polymerase extension of the capture arm, and annealing of the capture strand rigidifier. **C.** Transcription with EUTP-containing NTPs and strand capture. **D.** RNA polymerase dissociates from the DNA/RNA complex. **E.** CuAAC modification of the RNA results in NHS ester functionalization. **F.** The library is exposed to a target protein. Covalent complexes are isolated and bound DNA is amplified to regenerate the double-stranded library.

(lane 6) libraries when exposed to 200nM streptavidin. Lastly, pre-treatment of the sulfo-HSAB-modified selected library with 50mM Tris pH 8.0 for 20min abolishes the band shift (lane 7) whereas addition of the Tris after reaction with streptavidin does not abolish the shifted band (lane 8). Together, these observations confirm the presence of sulfo-HSAB modification-dependent, covalently reactive RNA aptamers in the library.

Sequence convergence of clones from the library

From the library, 10 clones were sequenced and characterized. A list of the clone sequences and their covalent binding activity is found in Table 1. A conserved motif (E/A)EAACE(G/A), where E represents 5-ethynyluridine) can be identified from nine of the ten sequences. Clone pairs (4 and 5) and (6 and 10) contained identical sequences. The last sequence (clone 1) does not appear to have any sequence similarity with the other nine clones. Individual clones were characterized by their ability to cause a denaturing gel shift upon incubation with streptavidin (Figure S1 and Table 1). Clone 1, which does not contain the conserved motif, did not show observable covalent binding activity. 8 of the 10 clones (2, 4, 5, 6, 7, 8, 9, 10) form a shifted band in the presence of streptavidin. Clones 6 and 10, which have an identical sequence, showed two bright bands even in the absence of streptavidin, indicating higher-order species most likely resulting from dimerization of the aptamers. However, with streptavidin incubation, a shifted band is evident just below the higher order band and at the top of the gel. It appears that the banding pattern at the top of the gel is of dimers of aptamers with either one or two streptavidin monomers bound. Clone 3, which differs from clone 7 by a deletion of a semi-conserved ethynyluridine as well as a C→E substitution away from the conserved region, did not cause a gel shift while clone 7 did. This observation may be explained by a recent loss of function of clone 3, where mutation late

Selected (S) or Naïve (N) Library	S	S	N	N	S	N	S	S
Sulfo HSAB modified	+	+	+	+	-	-	+	+
Pre-quenched	-	-	-	-	-	-	+	-
SA (200 nM)	-	+	-	+	+	+	+	+
Lane #	1	2	3	4	5	6	7	8

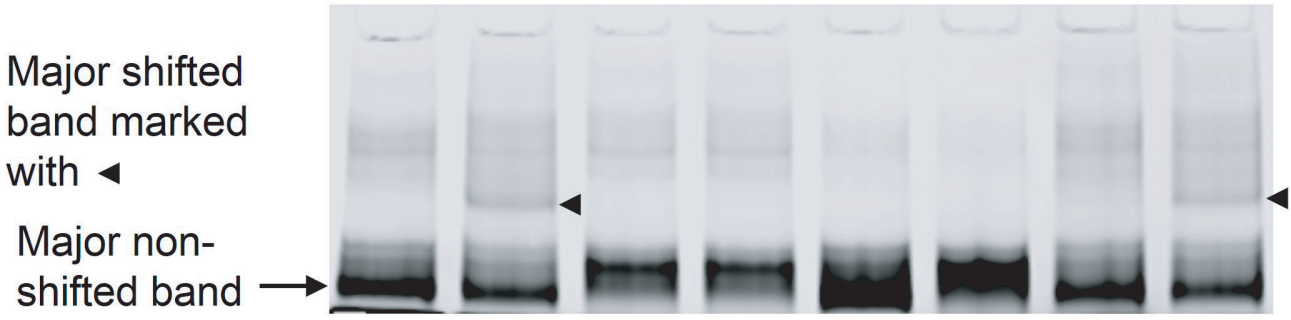


Figure 2. SDS-PAGE gel shift assay comparing the library after 16 rounds of selection with a naïve library. Lanes 1 and 2) The library after round 16 of selection shows a distinct gel shift upon incubation with streptavidin (SA). Lanes 3 and 4) The naïve library does not show a gel shift upon incubation with streptavidin. Lanes 5 and 6) NHS ester-functionalization is required for the gel shift as evidenced by the lack of a shift in the absence of sulfo-HSAB in the CuAAC reaction. Lane 7 and 8) Pre-incubation of the library with Tris buffer (primary amine) abolishes the gel shift, indicating the NHS ester is required.

in the selection experiment occurred (most likely during the last amplification step prior to cloning).

Characterization of clone 4

We chose clone 4 for further characterization. An endpoint binding assay was performed in which the aptamer was mixed with varying concentrations of streptavidin and assessed for covalent complex formation by SDS-PAGE gel shift. Figure 3A shows the binding curve indicating half-maximal covalent complex formation at <10nM streptavidin. One might expect the covalent reaction to proceed to completion for all functioning aptamers, regardless of target concentration. However, nucleophilic attack by hydroxyl ion is a known side-reaction of NHS esters. Apparently at low streptavidin concentrations (10-20nM), the aptamer is competitively quenched by hydroxyl ion such that lower reactivity occurs, compared with higher streptavidin concentration.

NHS ester reactions are typically dependent on pH. We characterized the ability of clone 4 to form a covalent complex at varying pH (Figure 3B). For all reactions in which a shift was observed, a second more slowly migrating shifted band was also observed, and the relative intensity of the second band increased with increasing pH (supplementary Figure S2). The most probable explanation of the second band is the reaction of a single aptamer with two streptavidin monomers (of the functional tetramer). It is likely that after the initial covalent reaction, a second NHS ester is positioned near an adjacent streptavidin monomer such that it can react with a primary amine from that monomer. Accordingly, we included the second shifted band in our calculation of total reacted aptamer. Similar binding plots were observed at all pH levels tested (6.0-8.0). To determine if initial reaction rates varied with pH, we measured complex formation as a function of time at pH 6.0 and 8.0 and 10nM streptavidin. We observed slightly faster rates of reaction at pH 8.0 (Figure S3). Therefore, the endpoint data can be explained by slower reaction rate at lower pH for a longer duration (lower quenching rate) and faster reaction rate at higher pH for a shorter duration (higher quenching rate).

Clone 4 was also assessed for reaction specificity. To this end, we incubated the NHS ester-modified clone 4 with varying concentrations of bovine serum albumin (BSA). BSA contains 30-35 lysine residues capable of reaction with NHS esters. In comparison, streptavidin has 12-16 lysine residues capable of reaction with NHS esters. NHS ester-modified clone 4 (~0.5nM) and streptavidin (10nM) were incubated with increasing concentrations of BSA (0-32µM) at pH 7.2. A decrease in the streptavidin-shifted band corresponded with an increase in a slower-migrating BSA-shifted band,

Table 1. Sequences from 10 clones from the library after 16 rounds of selection and their corresponding covalent reactivity. Consensus sequence is in bold. Ethynyluridine bases are in red.

Clone	Sequence from random region (aligned)	Covalent complex?
10	CCGGGEAGCC**EEAACEG**AC--GCGCEE	+
6	CCGGGEAGCC**EEAACEG**AC--GCGCEE	+
7	AGGGCAAAAEEE**AACEG**AC--AAGCEE	+
3	AGGGEAAAAEEE**AACEG**AC--AAGCE-	-
8	EGGAAACGCA**EEAACE**A**A**C--AAGCEE	+
5	--GGGACAGC**EEAACEG**ACGAAAACEE	+
4	--GGGACAGC**EEAACEG**ACGAAAACEE	+
2	--AGGCACGE**A**E**AACEG**ACAAACGCEE	+
9	AEAACGGCAEEGAGC**EEAACEG**EAA-------	+
1	EAGCACCEAACAEEECEEGAAAEGA	-

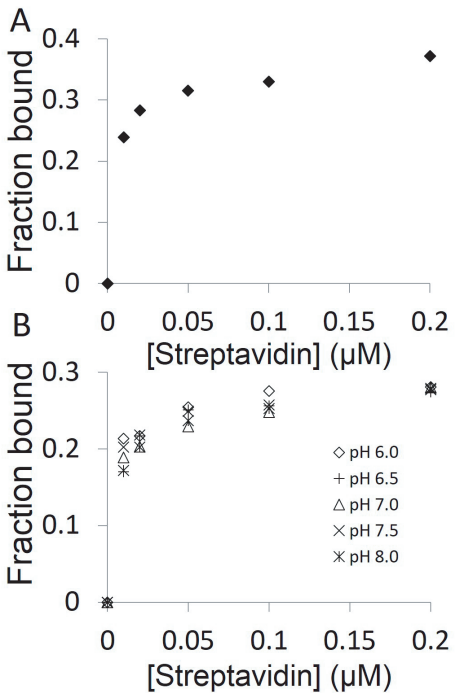


Figure 3. A. SDS-PAGE gel shift data for clone 4 at pH 7.2. **B.** SDS-PAGE gel shift data for clone 4 at varying pH. Sulfo-HSAB-modified clone 4 was prepared as described in the materials and methods and reacted with increasing concentrations of streptavidin at the designated pH, denatured with SDS and annealed to a fluorophore-conjugated oligonucleotide, electrophoresed on SDS-PAGE and quantified by fluorescence.

with equal band intensities at 16μM-32μM BSA, suggesting a specificity coefficient of >1000 for streptavidin (Figure 4).

DISCUSSION

This study represents the first application of RNA-SELMA, a concept that was introduced recently for reverse transcription-free isolation of chemically modified RNA aptamers (MacPherson et al, 2017). RNA-SELMA entails the display of RNA by the dsDNA that encodes it, enabling chemical modification and selection of the RNA library with no influence on its polymerase-based amplification. Similar to DNA-SELMA, modification is facilitated by incorporation of the non-natural base analog 5-ethynyluracil, followed by CuAAC with organic azides. In this study we armed RNA libraries with an NHS ester for covalent modification. While we did not explicitly select for covalent linkage, we used harsh washing conditions (high-salt wash buffer: 500mM NaCl), to remove non-covalently bound library fractions. After 16 rounds of selection, the library contained mostly covalently binding aptamers. This indicates that under the selection conditions, the simplest chemical solution is covalent binding, instead of salt-resistant binding. However, salt-resistant binding is not ruled out for minor constituents of the enriched library.

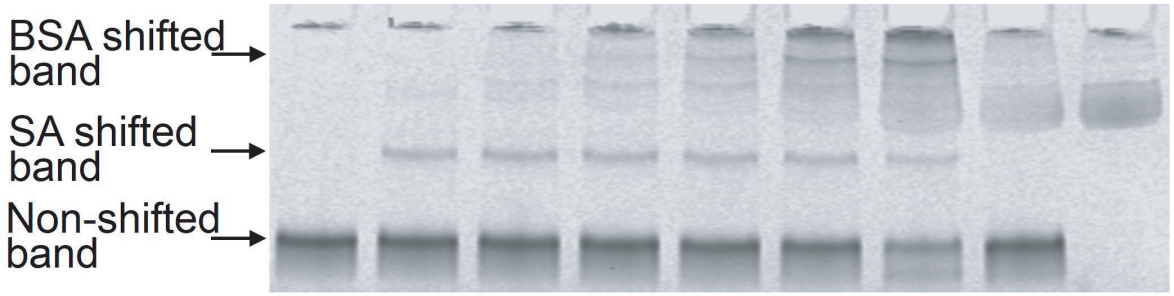
During selection, careful optimization of PCR recovery each round by way of pilot PCR on a small fraction of eluted library has two benefits. First, it is a convenient way to observe library enrichment as indicated by a decrease in the PCR cycle number required for efficient amplification of the library. Second, by not over-amplifying the recovered library, the accumulation of PCR artefacts that can overtake a library (library dimers, for example) is minimized. In our experience, propagation of PCR artefacts by over-amplification is a major barrier to successful SELMA. Compared with conventional SELEX libraries, the increased complexity of SELMA libraries (capture arm addition) may increase the likelihood of PCR artefacts.

Covalent binding curves presented here are related to the affinity of clone 4 for streptavidin, however they cannot be used to determine the dissociation constant (K_D) because the binding is irreversible. Although the curves resemble traditional aptamer binding curves, they are the result of several factors including on/off rates, covalent reaction kinetics once the complex is formed, and quenching rate by hydroxyl ion. We did attempt gel shift assays with native PAGE using clone 4 containing either 5-ethynyluridine or the hydrolyzed CuAAC product but did not observe a gel shift with up to 10μM streptavidin (supplementary figure S4). It is likely that streptavidin binding requires NHS ester modifications for initial binding.

NHS ester-labelled RNA libraries enable the covalent linkage of the aptamer to its binding partner once a precise complex is formed. Here we report a >1000 fold specificity of clone 4 aptamer for streptavidin over BSA. PhotoSELEX, a method developed by Somalogic™, has a key requirement for covalent linkage- aptamer/target complex formation at the time of photocrosslinking (Golden et al, 2000). Therefore, the technology requires high-affinity aptamers in addition to proper spatial positioning of a photoreactive nucleotide analog to an electron donor on the protein surface. Given the strict criteria, it may be difficult to obtain photoaptamers for all proteins. Other studies have attempted to couple

A

RNA	+	+	+	+	+	+	+	+	-
Pre-quenching	-	-	-	-	-	-	-	+	-
[SA] (μM)	0	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0
[BSA] (μM)	0	0	2	4	8	16	32	32	32



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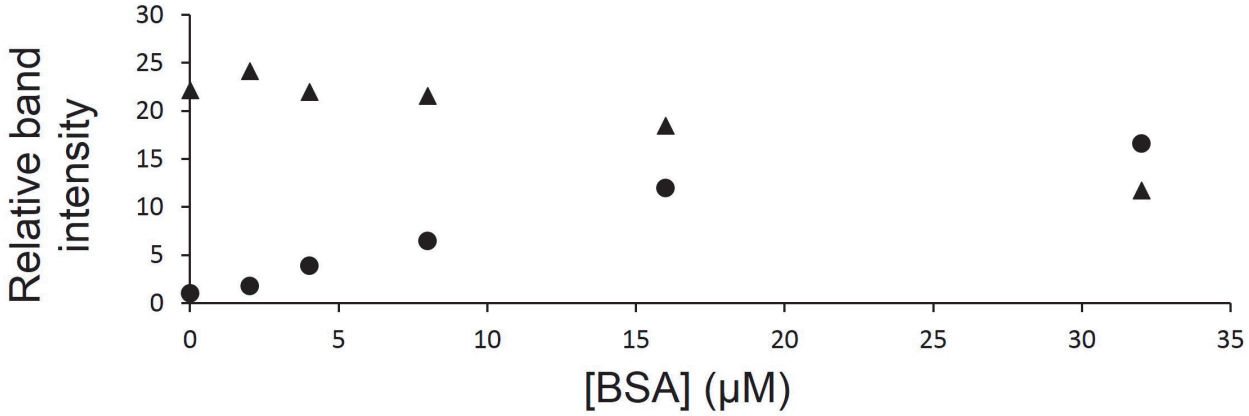

Figure 4. **A.** Specificity of clone 4 as determined by BSA competition assay. Increasing concentrations of BSA were included in the reaction of NHS ester-modified clone 4 with streptavidin (10nM). Relevant controls (last 2 lanes) were included to show the impact of quenching the NHS ester and the effect of high quantities of BSA in the absence of RNA on the banding pattern. **B.** Plot representing RNA-streptavidin covalent product (triangles) and RNA-BSA covalent product (circles) as a function of BSA concentration.

the binding specificity of aptamers with covalent reactivity (Vinkenborg et al, 2012; Zhang et al, 2014; Wang et al, 2016). Generally, aptamers are first evolved against a protein target, followed by attachment of a covalent reactive species with a linker varying in length and composition. This approach depends on a nearby moiety on the protein that reacts efficiently with the aptamer functional group containing an efficient linker. In some cases, the linker is tens of angstroms in length. This increases the possibility of non-specific reaction because of the increased availability of the reactive group to the bulk solution. Therefore, the approach requires substantial optimization and can still yield aptamers with non-specific reactivity. In contrast to the alternative methods above, NHS ester-modified aptamers with dissociation constants in the micromolar range (binding half-life typically on the order of tens of seconds) can result in specific complex formation. This increases the probability of obtaining covalent aptamers for any given protein.

CONCLUSIONS

In this report, RNA-SELMA has been shown to yield RNA with novel functionality. In applications where protein-nucleic acid conjugates are desired in a highly reproducible fashion (sensing nanostructures, antibody-oligonucleotide conjugates for protein quantification, etc.), NHS ester-modified "barbed" aptamers may provide the desired constructs. If covalent aptamers are required in large quantities, they may be generated by solid-phase synthesis using ethynyluridine phosphoramidites followed by CuAAC with sulfo-HSAB. NHS ester modifications are labile and therefore the aptamers would require cool, dry storage to maintain reactivity.

While covalent binding was the primary goal in this study, non-covalently binding modified RNA aptamers can be selected in a similar manner. Specifically, SOMAmer™ technology, which relies on the modification of DNA libraries with planar aromatic groups, should be directly applicable to RNA-SELMA and will be the subject of future investigation. Ribozyme selection can potentially benefit from RNA-SELMA for the introduction of bulky cofactors for enzymatic activity. Thus, we expect RNA-SELMA to contribute to the continually expanding repertoire of aptamer and ribozyme discovery methods.

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COMPETING INTERESTS

None declared.

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