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Nucleic Acid Ligands With Protein-like Side Chains: Modified Aptamers and Their Use as Diagnostic and Therapeutic Agents

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Limited chemical diversity of nucleic acid libraries has long been suspected to be a major constraining factor in the overall success of SELEX (Systematic Evolution of Ligands by EXponential enrichment). Despite this constraint, SELEX has enjoyed considerable success over the past quarter of a century as a result of the enormous size of starting libraries and conformational richness of nucleic acids. With judicious introduction of functional groups absent in natural nucleic acids, the "diversity gap" between nucleic acid–based ligands and protein-based ligands can be substantially bridged, to generate a new class of ligands that represent the best of both worlds. We have explored the effect of various functional groups at the 5-position of uracil and found that hydrophobic aromatic side chains have the most profound influence on the success rate of SELEX and allow the identification of ligands with very low dissociation rate constants (named Slow Off-rate Modified Aptamers or SOMAmers). Such modified nucleotides create unique intramolecular motifs and make direct contacts with proteins. Importantly, SOMAmers engage their protein targets with surfaces that have significantly more hydrophobic character compared with conventional aptamers, thereby increasing the range of epitopes that are available for binding. These improvements have enabled us to build a collection of SOMAmers to over 3,000 human proteins encompassing major families such as growth factors, cytokines, enzymes, hormones, and receptors, with additional SOMAmers aimed at pathogen and rodent proteins. Such a large and growing collection of exquisite affinity reagents expands the scope of possible applications in diagnostics and therapeutics.
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Introduction

In September of 1989, Craig Tuerk got a first look at his sequencing gel that showed a transition from a uniform ladder representing a fully random RNA region of eight contiguous nucleotides, to a converged sequence pattern. Starting from a pool of all possible variants of the short eight-nucleotide segment, this transition was achieved with a few rounds of affinity selection, amplification, and regeneration of the single-stranded library, with bacteriophage T4 DNA polymerase as the target. The evolved pattern, as revealed by cloning, was comprised of two sequences. One was a previously known motif representing a stem-loop region of an autoregulator found on the mRNA that encodes the polymerase. But aside from rediscovering the natural ligand, Craig found a second one, distinct from the native sequence, that bound the polymerase with the same high affinity. It was this second unexpected sequence solution to high-affinity binding that led to the realization that nucleic acid ligands can be identified to any molecule from a collection of randomized single-stranded oligonucleotides.[1]

As it is often the case in science, working independently, Ellington and Szostak[2] arrived at the same conclusion with organic dye molecules as targets. The method of selection was named SELEX (Systematic Evolution of Ligands by EXponential enrichment),[1] and the molecules became known as aptamers.[2]

The wide applicability of SELEX to a multitude of targets soon became clear, beginning with other nucleic acid–binding proteins and expanding to polyanion-binding proteins and proteins not known to bind polyanions, peptides, small molecules,[3,4] and complex targets such as cells and tissues.[5–8] Aside from their high affinities, the exquisite specificities of aptamers suggested binding of precisely folded entities that recognize their targets through shape complementarity. This notion is now corroborated with several crystal structures of aptamer–target complexes.[9–25]

The utility of early aptamers as binding reagents had one major shortcoming: RNA is extraordinarily sensitive to degradation by nucleases and also to alkaline conditions. This is due to the presence of a hydroxyl group at the 2′-position of ribose, which is oriented toward the backbone and can serve as the nucleophile in the enzyme-mediated or base-mediated hydrolysis of the adjacent phosphodiester bond. Replacement of the hydroxyl group of RNA with 2′-fluoro or 2′-amino groups led to analogs with dramatically enhanced stability to RNases.[26,27] As a result of substrate specificity of the most prevalent ribonucleases, such as RNase A, replacement of only the pyrimidine residues with 2′-amino or 2′-fluoro nucleotides led to an improvement in stability in serum of at least three orders of magnitude.[28] Importantly, these modified nucleoside triphosphates could be incorporated into transcripts with T7 RNA polymerase, the workhorse of the RNA research community, with an efficiency of incorporation comparable to that observed with natural nucleotides, so the sequence representation within random libraries was not much compromised.[29–34] The modified transcripts were also substrates for reverse transcriptases and could therefore be converted into cDNA and then amplified by PCR, allowing all the steps of SELEX to be completed.[30]

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Because of the dramatic improvement in nuclease resistance, 2′-aminopyrimidine and 2′-fluoropyrimidine RNA libraries quickly entered the mainstream of SELEX.

Although the performance of 2′-amino and 2′-fluoropyrimidine libraries in SELEX experiments was mostly comparable, the two libraries had several notable differences. The 2′-amino group had some appeal in that it could serve, like the hydroxyl group, as both hydrogen-bonding acceptor and donor. Furthermore, its unusually low pK_a of about 6 (refs. 35,36) could in principle be raised in certain binding environments to a point where this amine would be protonated under physiological conditions, giving RNA libraries a potential for positively charged moieties it otherwise lacked, and therefore bridging part of the diversity gap between nucleic acid–based and protein-based ligands. Nevertheless, in duplexes, 2′-amino substitutions destabilize helices compared with either 2′-OH or 2′-H,[35] potentially raising the entropic cost of binding.[34] In addition, the preference of 2′-amino for the C2′-endo ribose conformation (like DNA), compared with the preference for the C3′-endo ribose conformation in RNA,[37] made extensive post-SELEX substitutions with nuclease resistant 2′-*O*-methyl groups[38,39] that favor the C3′-endo conformation relatively inefficient.[31] In contrast, the 2′-fluoropyrimidine substitution imparts duplex stabilization[40–42] and has the 3′-endo conformation preference,[37] therefore a high degree of 2′-*O*-methyl substitution for the ribopurines could be achieved.[43–45] Most importantly, 2′-fluoro substitution does not require protection–deprotection during solid-phase phosphoramidite synthesis, which generally results in higher synthetic yields. For these reasons, SELEX with 2′-fluoropyrimidine RNA remains one of the most used SELEX libraries today.[39] Libraries that incorporate other 2′-position substitutions, such as the 2′-*O*-methyl group, have also been used. All 2′-*O*-methyl libraries have yielded high-affinity aptamers for at least some of the more accessible SELEX targets[46]; however, lower transcriptional yields have been an impediment and have typically required the use of mutant RNA polymerases.[39,46,47]

Overcoming the 2′-hydroxyl problem intrinsic to RNA could also be achieved by using single-stranded DNA in SELEX. The initial historical bias against DNA libraries[4,48] was based on the assumption that the conformational flexibility of DNA, and therefore shape repertoire of random libraries, was lower for DNA than that for RNA. However, it is now clear that the success rate of SELEX with DNA and RNA, including mixed and partially 2′-substituted DNA/RNA libraries,[46] is comparable. Although the intrinsic stability of DNA is higher than that for RNA, there are of course DNases that can metabolize DNA. Because of the preference of deoxyribose for the C2′-endo conformation, post-SELEX 2′-*O*-methyl substitutions are generally less well tolerated, typically resulting in less substituted and therefore less fully protected ligands. In the balance, our experience has been that starting SELEX with 2′-fluoropyrimidine RNA or DNA results in final optimized ligands of comparable overall performance.

With libraries of this type, the success rate of SELEX against protein targets has been about 30%.[49] Although the entire community of early SELEX researchers resisted this notion for some time, there are in fact difficult targets for SELEX. As a broad generalization, proteins with very low pI values, proteins without cationic regions, proteins lacking conformational definition, or heavily glycosylated proteins have often been problematic. One of the major advantages of SELEX compared with other combinatorial methods is the enormous size of the initial random libraries that can be generated and screened (typically 10^{15} molecules), which is about 100,000 times larger than peptide-based libraries.[50] However, the chemical diversity of nucleic acid libraries is substantially lower compared with protein-based libraries. Not only are there just four bases compared with 20 amino acids, but the bases cover a smaller range of physicochemical properties compared with those available in the 20 amino acids. The modifications at the 2′-position described above aimed at improving nuclease resistance, as well as the incorporation of 5-iodo- or 5-bromo-uracil substitutions introduced for cross-linking to protein targets,[29,51] represent a modest contribution to functional group diversity. The success of SELEX with these types of libraries appears to be driven by the initial library size and therefore a large shape repertoire that offsets to some degree the intrinsic diversity disadvantage of nucleic acids.

Expanded Chemical Diversity

A major enhancement in chemical diversity of nucleic acid libraries has been achieved through the introduction of non-native functional groups at positions oriented away from the hydrogen bonding face of the bases, such as the 5-position of pyrimidines and the 8-position of purines.[29,52–66] Early reports with SELEX using DNA libraries with a hydrophobic pentynyl group at the 5-position of deoxyuridine and thrombin as the target resulted in aptamers with quite poor (close to micromolar) affinities.[57] The incorporation of positively charged primary amines through flexible linkers at the 5-position of uracil in the context of DNA[58,61] or RNA[64] has also been shown to be compatible with SELEX and has led to the identification of aptamers against several small molecule targets, some negatively charged[56,58,59,64] and with affinities in the low micromolar to millimolar range (typical for small molecules).

We have recently focused our efforts on the palladium-catalyzed carboxyamidation reaction that offers a particularly versatile method for the incorporation of side chains through a stable amide linkage.[52,65] Most of the work to date has been done with modified deoxyuridine libraries (that is, one nucleotide out of four is modified) introduced into oligonucleotides either through modified phosphoramidites (for chemical synthesis) or nucleoside triphosphates (for enzymatic incorporation). The use of such libraries makes a dramatic difference in the success rate of SELEX[49,67] (**Table 1**). Empirically, the functional groups that produce the best nucleic acid ligands typically have hydrophobic aromatic character (**Table 1**), which is precisely the kind of functional group most notably absent in natural nucleic acids. These types of side chains are also commonly utilized by antibodies in their recognition of protein antigens.[68,69]

It is important to note that the choice of nucleic acid library used in SELEX determines the unique sequence solutions to high-affinity binding. That is, for a given molecular target, sequences of best ligands obtained from one starting library

Table 1 SELEX library affinities (K_d, nmol/l) with unmodified and modified nucleotides

Target protein	Unmodified DNA	Bn-dUTP	iBu-dUTP	Trp-dUTP
	CH_3			
GA733-1[a]	9	3	5	0.5
Osteoprotegerin[a]	40	5	9	0.2
4-1BB[a]	>100[b]	6	>100	4
B7[a]	>100	10	>100	7
B7-2[a]	>100	>100	>100	6
CTLA-4[a]	>100	>100	>100	1
sE-selectin[a]	>100	>100	>100	2
Fractalkine	>100	>100	>100	0.05
gp130, soluble[a]	>100	6	20	1
HMG-1	>100	>100	20	5
IR	>100	2	10	0.2
PAI-1	>100	0.4	0.9	0.2
P-Cadherin[a]	>100	4	5	3
sLeptin R[a]	>100	2	>100	0.5

[a]The protein used was expressed as a fusion to the F_c of human IgG_1. No detectable binding of the active library to an alternate F_c fusion protein was observed. [b]No detectable binding up to target protein concentration of 100 nmol/l was observed.

are unique and different from those obtained with other libraries. For example, we have observed this previously in SELEX experiments targeting VEGF with libraries containing alternative 2′-positions such as DNA, RNA, 2′-aminopyrimidine, and 2′-fluoropyrimidine RNA.[31,44,70,71] Our more recent work shows that this remains true for libraries with 5-position modifications, even when side chains are closely related in terms of their physicochemical properties, such as the planar, aromatic hydrophobic modified nucleotides utilized in benzyl-dU (Bn-dU), and napthyl-dU (Nap-dU) DNA libraries targeting interleukin 6 (IL-6).[72] We have not yet encountered a deviation from this observation with any nucleic acid library, or with any target. This is consistent with the notion that nucleic acid ligands represent precisely configured macromolecular assemblies with binding-competent conformations that are determined not only by their sequences but also by the aggregate contribution of constituent fragments of individual nucleotides.

Post-Selex Optimization

We have used SELEX libraries with different 5-position modifications, but within each library, every deoxyuridine nucleotide is uniformly substituted with the same modified side chain. This limitation is imposed by the need to know the identity of the modified nucleotide at each sequence position and for every clone in the affinity-enriched pool. In most cases, the affinities obtained from these libraries are quite good, generally in the low nanomolar to sub-nanomolar range,[49] which is sufficient for most applications. When needed, however, and once an individual sequence is defined and truncated, each of the modified nucleotides can be synthetically substituted with other functional groups with the goal of refining the affinity of the original ligand in an exercise akin to affinity maturation in antibodies or to structure–activity relationship optimization of small molecules. For this purpose, we have assembled a collection of variants that include side chains of different sizes, shapes, hydrophobicities, hydrogen-bonding capacities, and distances from the base.[73,74] In designing this diverse set, we made sure to include functional groups overrepresented in antigen–antibody contacts and privileged fragments of small molecule drugs (**Figure 1**). It is clear that substantial affinity improvement can be achieved by this method. In two cases where we applied this method, we have attained affinity improvement of up to 37-fold with a single substitution.[73,74] As expected, some modified positions tolerate substitutions more readily than others, and the identification of positions that are sensitive to changes indicate regions on the interaction surface where optimization of this type is most likely to be fruitful.[73,74] A similar post-SELEX optimization has recently been performed with an unmodified DNA aptamer in which the Bn-dU (**Figure 1**) modified nucleotide was used to replace dT.[75] Smaller but significant enhancement of fluorescence intensity (up to 2.5-fold) attributed to binding of the aptamer to its putative target nucleolin in the membranes of cancer cells has been reported,[75] although direct binding to target has not been confirmed. While replacing one 5-position modification with another will likely be a more efficient way of improving affinity, it is clear that the availability of the library of modifications shown in **Figure 1** will be useful for a broad range of post-SELEX optimizations.

Structures

What are the structural and functional consequences of the modified side chains on Slow Off-rate Modified Aptamers (SOMAmers)? The availability of a large number of synthetic variants resulting from post-SELEX studies has facilitated our efforts to solve several crystal structures of SOMAmers bound to their protein targets.[73,74] These structures have revealed extensive participation of modified nucleotides in both intramolecular interactions that contribute to the folding of the SOMAmer, as well as in making direct contacts with the protein. The latter renders the interaction surfaces between SOMAmers and their protein targets less polar and more hydrophobic compared with those typically observed with conventional aptamers. The analysis of currently available co-crystal structures of eight aptamers and three SOMAmers allows this difference to be quantified in several ways. Compared with aptamers, SOMAmers on average exhibit fewer hydrogen bonds (4.8 ± 1.6 versus 8.5 ± 4.3), fewer charge–charge interactions (2.2 ± 1.6 versus 9.3 ± 4.2), and fewer total polar contacts, defined as the sum of hydrogen bonds and charge–charge interactions (7.0 ± 1.3 versus 17.8 ± 4.2) per 1,000 Å^2 of interface area (**Table 2** and **Figure 2**).[73] Importantly, this comes without a compromise in binding affinity, and rather, there is a trend toward tighter binding for SOMAmers compared with aptamers (average binding free energy, or $-\Delta G$, values of 14.3 ± 0.8 versus 11.1 ± 1.2 kcal/mol, respectively). Values of $-\Delta G$ per nonhydrogen contact atom (or ligand efficiency)[76] are similar for SOMAmers and aptamers (0.16 ± 0.04 versus 0.19 ± 0.13 kcal/mol), as are values of $-\Delta G$ per interface area (12.1 ± 0.9 versus 16.7 ± 9.5 kcal/mol/1,000 Å^2). However, $-\Delta G$ per polar contact values are clearly higher for SOMAmers compared with aptamers

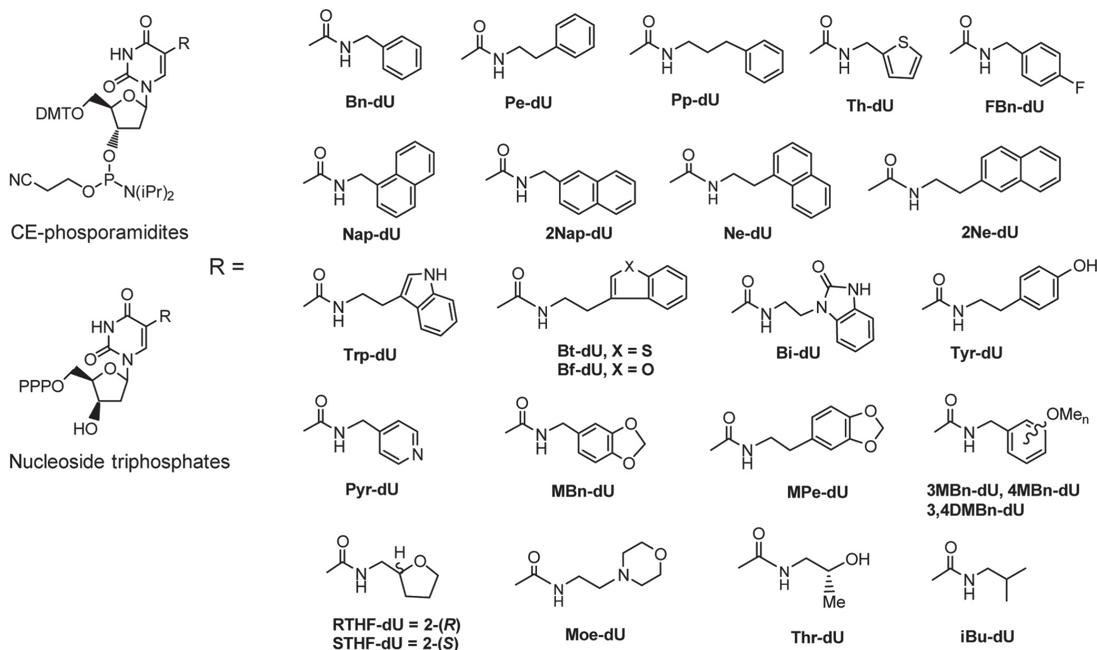

Figure 1 Partial listing of modifications at the 5-position of deoxyuridine available for SELEX and post-SELEX optimization. Side chain abbreviations: Bn, benzyl; Pe, 2-phenylethyl; Pp, 3-phenylpropyl; Th, 2-thiophenylmethyl; FBn, 4-fluorobenzyl; Nap, 1-naphthylmethyl; 2Nap, 2-naphthylmethyl; Ne, 1-naphthyl-2-ethyl; 2Ne, 2-naphthyl-2-ethyl; Trp, 3-indole-2-ethyl; Bt, 3-benzothiophenyl-2-ethyl; Bf, 3-benzofuranyl-2-ethyl; Bi, 1-benzimidazol-2-ethyl; Tyr, 4-hydroxyphenyl-2-ethyl; Pyr, 4-pyridinylmethyl; MBn, 3,4-methylenedioxybenzyl; MPe, 3,4-methylenedioxyphenyl-2-ethyl; 3MBn, 3-methoxybenzyl; 4MBn, 4-methoxybenzyl; 3,4MBn, 3,4-dimethoxybenzyl; RTHF, *R*-tetrahydrofuranylmethyl; STHF, *S*-tetrahydrofuranylmethyl; Moe, morpholino-2-ethyl; Thr, *R*-2-hydroxypropyl; iBu, *iso*-butyl.

Table 2 Protein–nucleic acid ligand interaction parameters for eight crystallized aptamers (with binding affinities better than 100 nmol/l) and three SOMAmers

	Protein target	-Log (K_d)	-ΔG (kcal/mol)[a]	T (K)	H-bonds	Charge–charge contacts	Polar contacts	Interface area (Å^2)[b]	Ligand efficiency (kcal/mol per contact atom)[c]	References
Aptamers	MS2 coat protein	8.6	11.6	296	5	1	6	348	0.464	Talbot *et al.*[20] Convery *et al.*[11]
	Lysozyme	7.7	10.5	296	1	5	6	403	0.187	Padlan *et al.*[16]
	IgG	7.1	9.7	298	6	3	9	477	0.262	Miyakawa *et al.*[14] Nomura *et al.*[15]
	Thrombin	7.6	10.3	296	3	8	11	657	0.166	Pagano *et al.*[17] Russo Krauss *et al.*[18]
	NF-κB	8.3	11.2	296	5	12	17	870	0.196	Huang *et al.*[12]
	vWF	9.2	12.5	296	13	14	27	1,011	0.115	Huang *et al.*[13]
	LDH	7.4	10.1	298	9	7	16	1,276	0.096	Cheung *et al.*[10]
	GlnRS	9.6	13.0	296	22	20	42	2,599	0.064	Bullock *et al.*[9]
SOMAmers	PDGF-BB	10.7	15.2	310	6	1	7	1,225	0.116	Davies *et al.*[73]
	IL-6	9.7	13.7	310	4	5	9	1,248	0.183	Gelinas *et al.*[74]
	Target A	9.9	14.0	310	7	2	9	1,097	0.180	Unpublished

[a]Free energy (ΔG) values were calculated from the published binding affinities for aptamers and SOMAmers at indicated temperatures. [b]Interface area calculations were made with PISA (aptamers)[128] or PyMOL (SOMAmers).[129] [c]Protein contact atoms within 4 Å of each ligand were determined in PyMOL.[129]
GlnRS, Gln tRNA synthase; IgG, immunoglobulin G; LDH, lactate dehydrogenase; PDGF-BB, platelet-derived growth factor B; IL, interleukin; NF, nuclear factor; vWF, von Willebrand factor.

(1.75 ± 0.36 versus 0.97 ± 0.59 kcal/mol), reflecting a larger contribution to binding from hydrophobic interactions in SOMAmers, and as expected in view of the importance of hydrophobic interactions in macromolecular interactions.[77–79]

The structures of SOMAmers described to date exhibit canonical as well as novel structural motifs. The PDGF SOMAmer is composed of two domains, a short stem-loop domain and a very small H-type pseudoknot ("miniknot") domain, connected through a hinge region. The two domains interact with each other through an intricate network of hydrophobic stacking interactions contributed by the aromatic side chains of the modified nucleotides from both domains.[73] All eight of the hydrophobic side chains in the SOMAmer contact the protein, four from each domain, and form an interaction surface that exhibits exquisite shape match with the complementary epitope of the protein (**Figures 3a**,**c** and **4**a–c). The

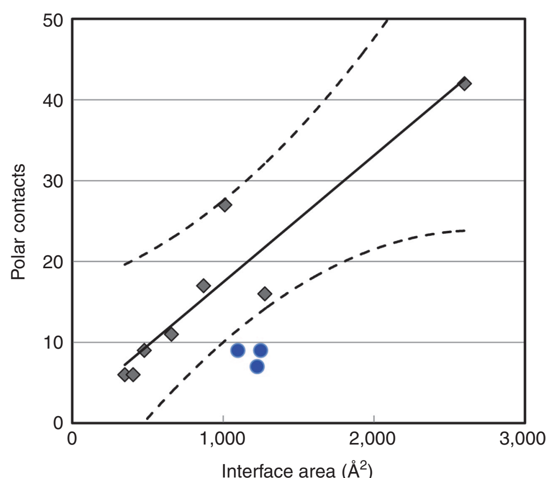

Figure 2 Interface area plotted as a function of the total number of hydrogen bonds and charge–charge interactions (polar contacts) for aptamers (gray diamonds) or SOMAmers (blue circles) bound to their targets (from Table 2). The line represents a linear regression fit to points representing the eight conventional aptamers with an R^2 = 0.88 and a slope of 0.016. Dashed lines represent the 99% confidence intervals of this trend line for the conventional aptamers (the three SOMAmers fall outside those boundaries).

IL-6 SOMAmer is also composed of two domains, a stem-loop domain and a G-quartet domain, where the two domains appear to be essentially independent, and together embrace the protein in a clamp-like manner (Figure 3b,d).[74] From the total of 10 modified nucleotides in the IL-6 SOMAmer, which are also split equally between the two domains, 8 interact with the protein, and again in a manner that defines the contact surface (Figure 4d–f). The combination of known nucleic acid structures and novel motifs comprised of modified nucleotides with protein-like side chains creates hybrid ligands that combine two highly desirable features of nucleic acid–based and protein-based ligands: the enormous conformational flexibility of nucleic acid backbones, which have seven rotatable bonds per monomer compared with two in proteins, augmented with additional side chain diversity available in proteins.

Expanded Range of Epitopes and Sandwiches

We have seen from the crystal structures that the hydrophobic functional groups, in the context of folded structures, engage corresponding epitopes and residues on proteins through both shape and functional group complementarity. Because such interaction surfaces on proteins can include sizeable hydrophobic patches, we reasoned that the range of epitopes for SOMAmers could be larger compared with those preferred by conventional aptamers. One area where multiple epitopes are needed is in the development of nonoverlapping pairs of ligands for sandwich-type diagnostic reagents. With aptamers, there are only a few examples of such pairs, each requiring unique selection conditions.[80–84] This is most likely the consequence of the fact that aptamers tend to favor cationic patches on protein surfaces, resulting in the vast majority of clones from affinity-enriched pools binding to and competing for the same epitope. In contrast, we have recently reported an efficient strategy for identifying SOMAmer pairs that can simultaneously bind to the same protein.[85] In a SELEX method developed for this purpose, which entails the inclusion of non-amplifiable SOMAmers intended to serve as one-half of the sandwich pair, SOMAmer pairs to 7 out of 8 proteins have been identified. We also developed an efficient method of screening the resulting clones for best-performing pairs (Figure 5).[85] The availability of several modified libraries, each with a different 5-position modification, has been very useful for this effort, since each of the modified libraries is likely to have somewhat different preferred epitopes on the protein as a function of its unique physicochemical properties, thus increasing the likelihood of identifying noncompeting pairs. The already high specificity intrinsic to SOMAmers that results from differences in dissociation rates between specific and nonspecific interactions coupled with the use of common polyanionic competitors[49] could be boosted by additional specificity contributed by the second binding event, along with its off-rate differential. SOMAmer pairs may therefore offer a substantial improvement in overall specificity and greater multiplexing capability compared with what is achievable with antibody sandwiches.

Increased Nuclease Resistance

Unmodified oligonucleotides are very sensitive to nuclease degradation, and for most applications, the utility of nucleic acid–based reagents is related to the degree to which this susceptibility can be improved. We have observed that the incorporation of hydrophobic modifications introduced through an amide bond at the 5-position of deoxyuridine nucleotides in DNA imparts a substantial increase in resistance to DNase-mediated degradation.[72] Resistance to both exonucleases and endonucleases is improved (data not shown), although for practical purposes, the resistance to endonucleases is more important, since the 3′–5′ exonuclease activity can be attenuated very effectively with 3′ caps such as inverted (3′–3′-linked) dT nucleotide. The degree of improvement in nuclease resistance is dependent on both the sequence and the nature of 5-position modifications of the SOMAmer in an idiosyncratic manner, but in most cases, the improvement in stability is considerable. For example, in 90% human serum, Bn-dU and Nap-dU IL-6 SOMAmers exhibit about ninefold longer half-lives (50 and 77 hours, respectively) compared with unmodified DNA with the same sequence (5.5 and 8.5 hours, respectively).[72] Overall, the increase in stability to endonuclease degradation appears to be both larger and more consistent than that observed previously with other 5-position modifications.[86–90]

The origin of the enhanced stability to nucleases imparted by the amide-linked 5-position modifications is not clear, however, it is probable that this effect is due to the combination of steric occlusion of some of the backbone phosphodiester bonds by the bulky apolar side chains as well as the overall conformational and physicochemical deviation from native B-form DNA[73,74] that together make SOMAmers poor substrates for DNases. The enhanced metabolic stability provides an important advantage for a broad range of uses that requires exposure of SOMAmers to biological fluids and tissues.

When needed, further nuclease resistance can be achieved with additional substitutions at the 2′-position of deoxyribose,

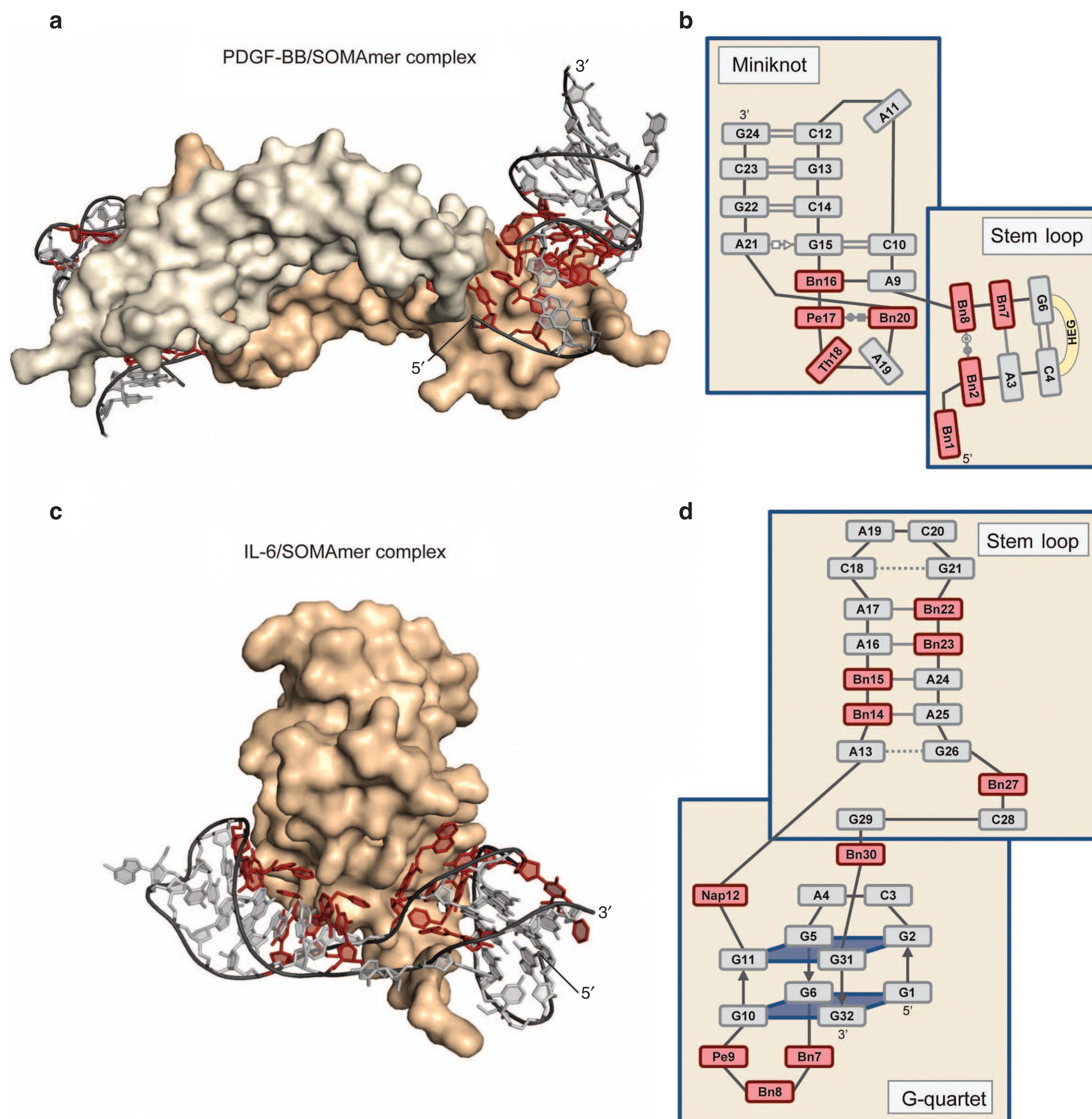

Figure 3 SOMAmers bind their targets with exquisite shape complementarity and utilize hydrophobic modifications at the binding interface. (**a** and **c**) Crystal structures of SOMAmers bound to PDGF-BB and IL-6.[73,74] (**b** and **d**) Schematic representations of the corresponding secondary structures, with base pairs annotated according to the Leonitis and Westhof nomenclature.[130] Modified nucleotides are colored brick red; conventional DNA is colored gray; blue squares in **d** indicate G-quartets.

such as the 2′-*O*-methyl substitution,[72,73] as expected based on a wealth of existing literature on this subject.[39,91] Based on a limited number of examples, the aggregate number of positions that tolerate 2′-*O*-methyl substitution without a compromise in binding affinity is relatively low in SOMAmers. For example, we have been able to substitute 4/23 (17%) nucleotides in the PDGF SOMAmer, and 6/32 (19%) nucleotides in the Bn-dU IL-6 SOMAmer, which is lower than the sum of individual positions that tolerate such substitutions.[72,73] In view of the preference for the C2′-endo sugar pucker in deoxyribose (DNA) and the C3′-endo conformation preference in 2′-*O*-methyl-2′-deoxyribose,[37] this is not surprising. Aptamers arising from SELEX experiments that utilize partial substitution with 2′-modifications favoring C3′-endo conformation in the starting libraries, such as 2′-fluoropyrimidine RNA[43–45,92] or mixed dC, 2′-*O*-methyl-A,-T, and -G libraries,[39,93] clearly tolerate a larger degree of post-SELEX substitution at the 2′-position. Nevertheless, in the context of SOMAmers, and based on a few examples to date, this relatively sparse number of 2′-*O*-methyl substitutions imparts a substantial additional improvement in nuclease resistance with essentially no degradation in 90% serum observed over a period of 2 days.[72]

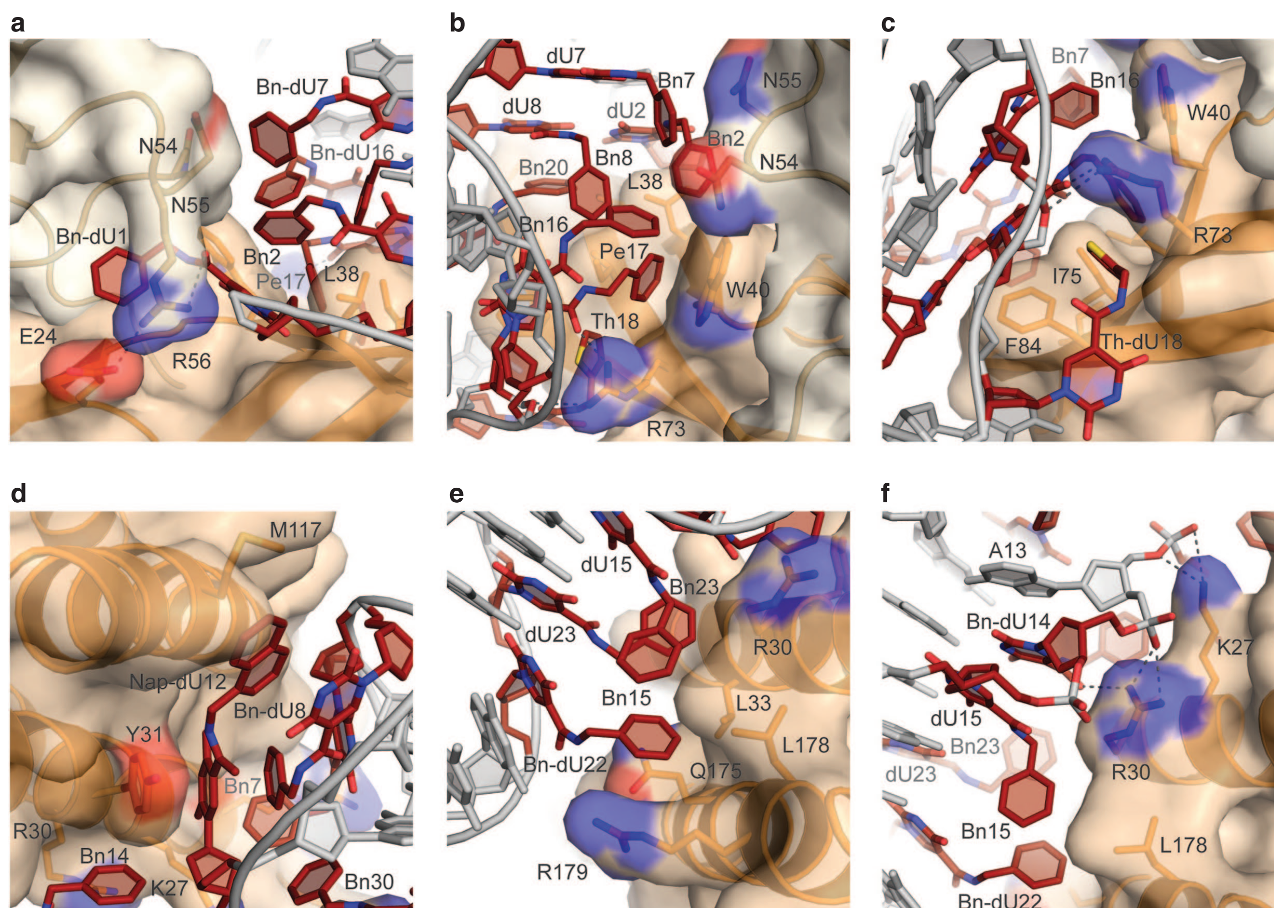

Figure 4 SOMAmer intra- and intermolecular interactions in the PDGF-BB and IL-6 co-crystal structures. Both the PDGF-BB and the IL-6 SOMAmers exhibit striking shape complementarity at the protein/SOMAmer binding interface. (**a**–**c**) All eight modified nucleotides of the PDGF-BB SOMAmer cluster in an extensive arrangement of hydrophobic aromatic interactions that primarily contact the aliphatic side chains of the protein residues. Only seven polar interactions are present at the SOMAmer/PDGF-BB interface. (**d**–**f**) The modified nucleotides of the IL-6 SOMAmer form segregated hydrophobic clusters exhibiting face-to-face and edge-to-face aromatic interactions. With only nine polar contacts, the modified nucleotides mainly stack against the methylene portion of charged residues and hydrophobic amino acid side chains. Bn-dU, 5-(*N*-benzylcarboxamide)-2′-deoxyuridine; Pe-dU, 5-[*N*-(phenyl-2-ethyl)carboxamide]-2′-deoxyuridine; Th-dU, 5-[*N*-(2-thiophene-methyl)carboxamide]-2′-deoxyuridine; Nap-dU, 5-(*N*-(1-naphthylmethyl)carboxamide)-2′-deoxyuridine; dU plus number indicates the uridine ring of specified nucleotide; Bn, Pe, Th, or Nap plus number indicates the modified nucleotide side chain of specified nucleotide; Bn-dU, Pe-dU, Th-dU, or Nap-dU plus number indicates the entire nucleotide. Transparent surface renderings of PDGF-BB and IL-6 are colored gold and wheat; stick renderings of the modified nucleotides are colored brick red.

Applications

The use of modified libraries with enhanced diversity has dramatically expanded the range of protein targets to which high-affinity nucleic acid ligands can be identified,[49] with the current success rate in excess of 80% for our best libraries.[49] For some proteins that fail SELEX with one modified library, very good ligands can usually be identified with another, although the optimal library for a given protein is difficult to predict *a priori*. Proteins with low pI values are clearly accessible with such modified libraries,[49] which is likely the result of expanded range of epitopes accessible to SOMAmers. However, highly disordered proteins or peptides generally remain more difficult targets, reflecting an intrinsic entropic cost of binding to highly flexible molecules.

These improvements to SELEX have allowed us to build a large collection of SOMAmers, currently more than 3,000 and growing. It is worth noting that each of the affinity-selected pools contains a large number, often several thousand, of different clones that represent unique sequence solutions to high-affinity binding, which is now routinely determined by deep sequencing. We are taking advantage of this unique set of binding reagents for use in unbiased biomarker discovery, research tools, diagnostics, and therapeutics.

Biomarkers and Diagnostics

Proteins convey information about the current state of an organism. A person with the same DNA can have substantially altered levels of certain proteins as a function of health condition, age, exposure to external stimuli, and the like. Understanding of protein changes that accompany disease progression is one of the most actionable pieces of information for healthcare.

We have recently developed a SOMAmer-based assay that is both highly multiplexed and highly sensitive. The assay takes

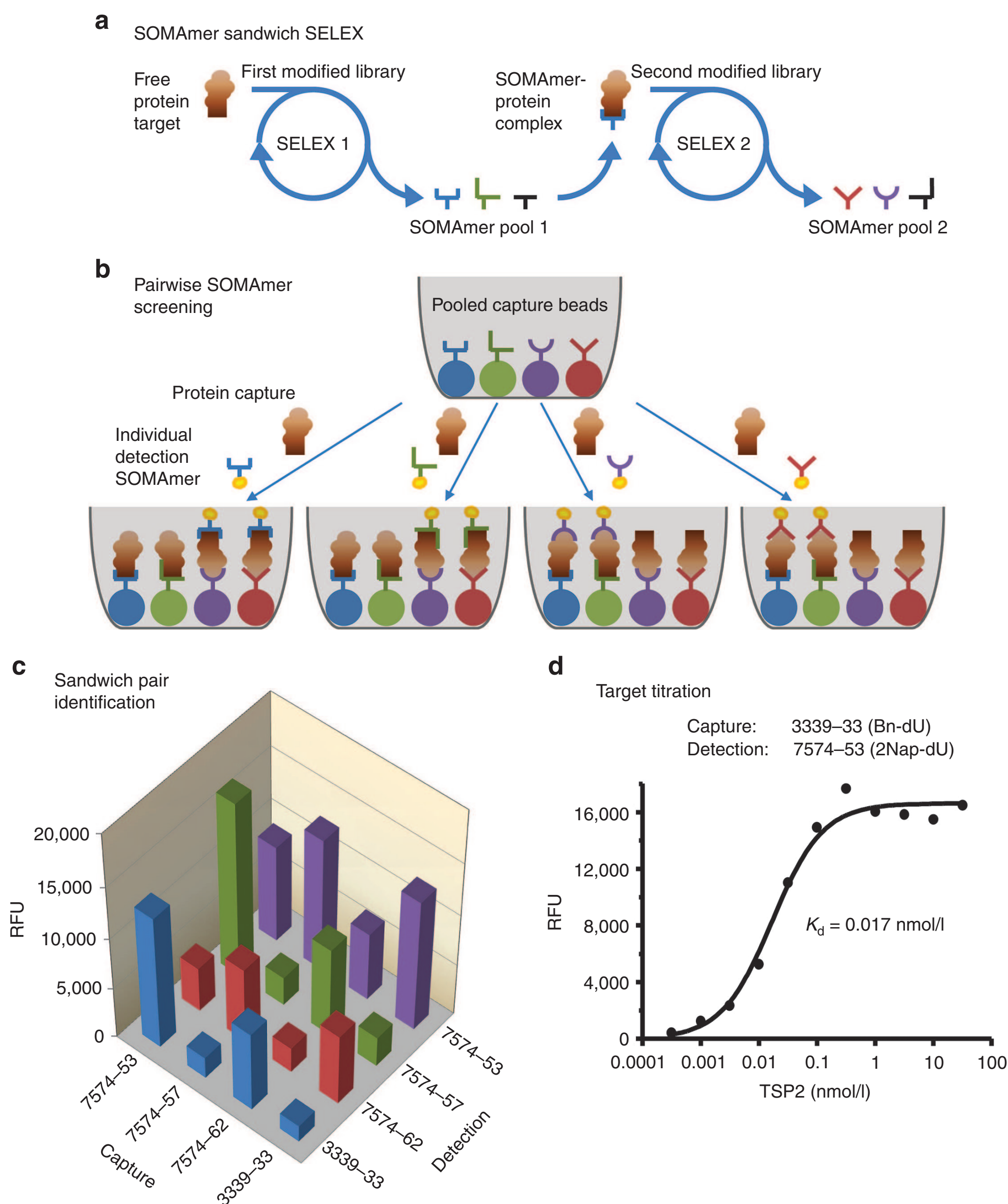

Figure 5 Isolation of SOMAmer pairs for sandwich assays, shown for TSP2 as an example. SOMAmers from a standard SELEX with a first modified nucleotide library (*e.g.*, Bn-dU) were competitive, indicating that they bind to the same epitope. (**a**) SOMAmer pairs were generated via sandwich SELEX using a SOMAmer–protein complex as the target and a second modified library (*e.g.*, 2Nap-dU) that may favor a distinct second epitope. A pairwise screening assay was developed to distinguish truly noncompetitive SOMAmers from SOMAmers with superior binding properties that simply displaced the first SOMAmer from the target during sandwich SELEX. (**b**) Such SOMAmer pairs were identified via attaching each individual SOMAmer as capture agent on a different type of LumAvidin beads, which were then pooled for a multiplexed screening assay on the Luminex platform to test each individual SOMAmer as detection agent. In this assay, a fixed target concentration of 10 nmol/l TSP2 was used, and all SOMAmers carried a 5′ biotin for immobilization of the capture agents on streptavidin beads and for labeling of the detection agents with phycoerythrin–streptavidin conjugate. (**c**) The resulting Luminex signals allowed the identification of the best-performing SOMAmer pairs in this sandwich screening assay. Performance of SOMAmer pair 3339–33 (Bn-dU) for capture and 7574–53 (2Nap-dU) for detection was evaluated via target titration in a Luminex sandwich assay. (**d**) Background signal in the absence of the protein is generally < 10% of the maximum signal and was subtracted from the total signal. Omission of one of the constituents of the sandwich pair reduced the signal to below background.

advantage of the fact that nucleic acid ligands have a dual nature: they spontaneously fold into precise three-dimensional structures that recognize their targets through shape complementarity, but they are also capable of highly specific hybridization to complementary sequences arrayed on surfaces. In our assay, a mixture of SOMAmers, each with a 5′ biotin, a photocleavable group, and a fluorescent tag (in that order from the 5′ end), is immobilized on streptavidin-coated beads and incubated with a protein mixture in a biological sample, and SOMAmer–protein complexes are formed (Figure 6a,b). Following extensive washing and biotin-labeling of immobilized proteins, the complexes are then released from the beads by

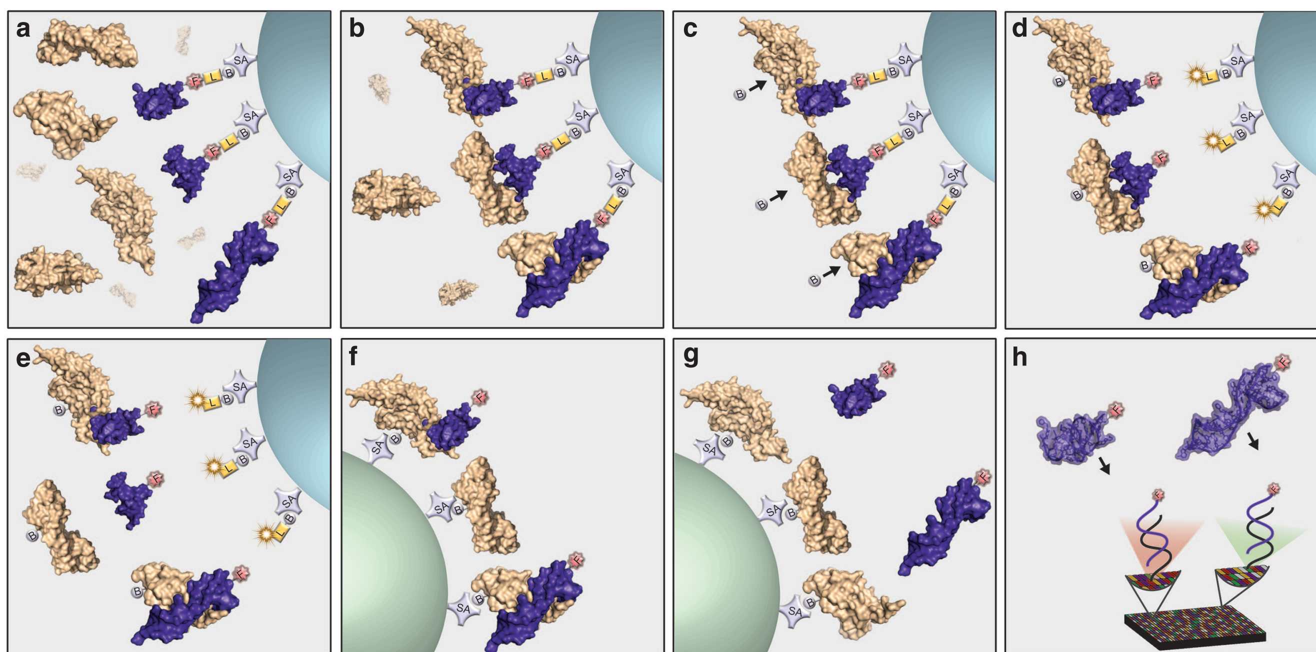

Figure 6 Multiplexed SOMAmer affinity assay. (**a**) SOMAmers labeled with a fluorophore (F), photocleavable linker (L), and biotin (B) are immobilized on streptavidin (SA)-coated beads and incubated with samples containing a complex mixure of proteins (*e.g.*, plasma). (**b**) Cognate (top and bottom) and noncognate (middle) SOMAmer–target protein complexes form on the beads. (**c**) The beads are washed removing the unbound proteins and the proteins are tagged with biotin. (**d**) SOMAmer–protein complexes are released from the beads by photocleavage of the linker with UV light. (**e**) Incubation in a buffer containing a polyanionic competitor selectively disrupts nonspecific interactions. (**f**) SOMAmer–protein complexes are recaptured on a second set of streptavidin-coated beads through biotin-tagged proteins followed by additional washing steps that facilitate further removal of nonspecifically bound SOMAmers. (**g**) SOMAmers are released from the beads in a denaturing buffer. (**h**) SOMAmers are hybridized to complementary sequences on a microarray chip and quantified by fluorescence. Fluorescence intensity is related to protein amount in the original sample.

photocleavage (leaving biotin originally on the SOMAmers behind) and diluted in a buffer containing dextran sulfate, a polyanionic competitor that selectively displaces nonspecific interactions because of their faster dissociation rates (Figure 6c–e). The existence of common polyanionic competitors of nonspecific interactions for nucleic acid ligands, along with the slow off-rate of specific interactions enabled by modified nucleotides is a unique and important feature of our assay that contributes to specificity and allows a high degree of multiplexing. For protein–protein interactions, such a common competitor has not been described, except perhaps the inclusion of carrier proteins such as albumin or casein in some proteomic assay buffers. After this kinetic challenge step, the protein–SOMAmer complexes are captured on a new set of beads, now through biotin on the proteins, and washed again, illustrating the importance of slow dissociation rates (Figure 6f). Sequential immobilization of complexes first through SOMAmers and then through proteins further enhances specificity by altering the nature of surfaces (protein-coated or nucleic acid-coated) to which unbound ligands can interact.[49,67] SOMAmers are released from protein complexes under denaturing conditions (*e.g.*, in a strong base like 0.02 N NaOH) and hybridized to a microarray chip containing the complementary single-stranded DNA (Figure 6g,h). Finally, fluorescence is used to quantify the SOMAmers; the signal intensity correlates to the amount of protein in the original sample (Figure 6h).

Our assay, called SOMAscan, currently measures 1,129 proteins with a dynamic range of 8 orders of magnitude, high sensitivity (median lower limit of detection of 40 fM), and high precision (median intra-run and inter-run coefficient of variance of ~5%). We are working to expand the number of measured proteins by including the SOMAmers from our current collection, after appropriate performance qualifications are completed, with the eventual goal of measuring a substantial fraction of the human proteome. SOMAscan also has high throughput (hundreds of samples per day per instrument system), is readily scalable to accommodate expanded content, and requires small sample volumes (*e.g.*, about 65 µL of plasma). This type of an assay can readily be adapted to plate-based streamlined platforms compatible with smaller panels.[94]

We have applied SOMAscan to discover biomarkers for many diseases and conditions including non-small-cell lung cancer,[95,96] mesothelioma,[97] cancer exosomes,[98] chronic kidney disease,[49] Alzheimer's disease,[99] response to pulmonary tuberculosis treatment,[100,101] aging,[102] and many others (unpublished data).

Although SOMAmers in our current collection have been elicited to human proteins, there is clearly sufficient cross-reactivity to a subset of proteins from other species to allow biomarker discoveries in animal models. One striking example of the use of SOMAscan for such studies is the identification of GDF-11, a protein that reverses the effects of heart hypertrophy when administered to old mice.[103]

Biomarkers identified through the application of SOMAscan can be developed into new diagnostics. We are currently

evaluating multiple platforms that could be suitable for a range of diagnostics applications from point-of-care diagnostics to reference laboratory tests. With affinities comparable to and often superior to those of antibodies, SOMAmers have distinct advantages for such applications, including selection conditions not tied to *in vivo* immunization, thermal and chemical stability, smaller size, ease of manufacturing, reliable supply, and full control of lot-to-lot variability. Nucleic acid chemistry has matured as a field to a point where facile incorporation of a wide variety of functional groups is now feasible with commercially available phosphoramidite reagents including amine, thiol, carboxylate, alkyne modifiers, fluorophores and quenchers, linear and branched spacers, biotin, and photoreactive and photocleavable groups. Introducing such modifications, especially in a site-specific manner, is much more cumbersome with antibodies. Specialty phosphoramidite reagents can also be synthesized with well-established methods. These tools, along with the chemical robustness of nucleic acids ligands and their tolerance to multiple folding–refolding cycles, make SOMAmers excellent candidates for multiple existing diagnostic platforms as well as for use as research tools such as histochemistry probes, cell sorting, and affinity purification.[104–106]

Therapeutics

The possibility of developing aptamer-based therapeutics has been contemplated from the very beginning of SELEX. A number of excellent recent reviews have summarized the developments in this field over the past two decades.[6,47,107–109] The scorecard at this time includes one approved drug (Macugen or pegaptanib),[44,110–112] two compounds in phase 3 clinical trials (Revolixys™ or pegnivacogin and Fovista™ or E10030)[45,113–117] and many candidates in earlier stages of development.[6,47,107] To place this track record in a proper context, we should recall that it took 11 years from the invention of hybridoma technology[118] to the first monoclonal antibody therapeutic, a murine monoclonal to CD3,[119] and more than two decades of major improvements for true blossoming of the technology and full acceptance of antibodies as drugs.[120,121] Nevertheless, facing the remaining challenges and focusing on therapeutic categories where properties of aptamers offer unique advantages, rather than focusing on direct comparison with other classes of high-affinity ligands like antibodies, is crucial to successful development of nucleic acid ligand–based drugs.

Since all nucleic acid ligands are macromolecules with limited oral bioavailability, parenteral delivery has been and will likely remain the only practical option for the foreseeable future. Within parenteral delivery, the therapeutic potential of aptamers is influenced by their pharmacokinetic and biodistribution properties. Because of the difficulties associated with delivering nucleic acid inside cells and into compartments where many drug targets are located, most aptamer therapeutics programs have focused on extracellular targets. Within this category, and as evidenced by the success of antibody-based therapeutics, there are obviously many good targets to consider. Plasma residence time of aptamers is largely determined by metabolism, primarily mediated by nucleases, and size-dependent clearance. As seen with other nucleic acid drug candidates, substantial resistance to nuclease degradation can be achieved with modifications at the ribose 2′-position, conformationally restricted ribose (locked nucleic acids),[122,123] L-ribose (Spiegelmers),[124] other alternative sugars collectively called xeno-nucleic acids,[125] phosphorothioate backbone modifications, and the combination of these modifications.[47,107,126] With SOMAmers, as mentioned above, 5-position deoxyuridine modifications also contribute to enhanced nuclease resistance.[72] Aside from metabolic stability, size-mediated clearance also needs to be addressed, since high-affinity binding of most aptamers is encoded in 20–50 nucleotides, which translates to molecular mass of 7–16 kDa. Molecules in this size range are rapidly cleared by renal filtration, and this has been addressed by coupling aptamers to larger molecules, mostly to polyethylene glycol.[47,107,126] It is worth noting that other than polyethylene glycol, relatively few strategies of increasing the molecular mass of aptamers have been considered beyond early research stage, and this represents a potentially useful area of further research. With extensively nuclease-stabilized aptamers conjugated to 40 kDa polyethylene glycol, a range of plasma half-lives in primates from 9 to 75 h has been observed.[47,123] This is considerably shorter than typical half-lives of antibodies, which is generally measured in weeks, so treatment of chronic conditions in which long-term, sustained systemic exposure to the drug is needed to achieve therapeutic effect is unlikely to be the best choice of indications for the current versions of therapeutic aptamers. Rather, treatment of acute conditions, targeted delivery to specific tissues, and local delivery represent better near-term opportunities, where advantages of aptamers such as high potency, low immunogenicity, high tissue penetration, and the ease of assembling multispecific agents can serve as true and meaningful differentiating factors over other treatment options. This still encompasses a large number of therapeutic areas where new or better therapies are needed, and disciplined focus on the identification and pursuit of such opportunities will be needed to most effectively drive the wider adoption of therapeutic aptamers. At the same time, an ongoing effort is under way in our laboratories to improve the circulating residence time of nucleic acid ligands to a point where treatment of chronic conditions could be reasonably considered.

Conclusions

The field of SELEX has been broadly adopted since its inception. In an effort to continue advancing the functional properties of nucleic acid ligands, we and others have focused our attention in recent years on addressing the intrinsic chemical diversity limitations of nucleic acid libraries.[29,39,49,65,127] The 5-position of deoxyuridine has served as a convenient point of attachment for introducing a variety of functional groups through a conformationally restricted amide linkage that orients the substituent away from the hydrogen-bonding face of the base. Such modifications have exerted a profound effect on the manner in which nucleic acid ligands fold and interact with their targets. The modified nucleotides also represent points at which additional diversity can be explored to optimize ligands for specific purposes. With the expanded range of accessible epitopes on protein targets, this new class of nucleic acid ligands has a broad scope of applications in diagnostics

and therapeutics. Like a pattern that emerges from blurry lanes on a sequencing gel, the role of nucleic acid ligands in biomedical research is becoming more clear.

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