



Beyond antibodies: New affinity reagents to unlock the proteome

Bridget Lollo, Fintan Steele and Larry Gold

SomaLogic, Inc., Boulder, CO, USA

Antibodies have been the workhorse reagents of protein capture and quantification since their 1959 debut in the RIAs developed by Yalow and Berson. However, there are technical challenges to the use of antibodies in highly multiplexed arrays aimed at measuring hundreds or even thousands of proteins at one time. We describe here a recently developed class of synthetic protein-binding reagents (slow off-rate modified aptamer). We discuss the chemical makeup and protein binding specifications of slow off-rate modified aptamer reagents, compare them to traditional aptamers and antibodies, briefly describe the novel proteomic assay that takes advantage of their unique properties, and provide several examples of their multiple applications to biomarker discovery and validation across a range of biomedical science questions.

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1 Aptamers as molecular affinity reagents

When thinking about molecular affinity reagents, antibodies are usually the first and perhaps only reagents that come to mind. Antibodies were first described as a life sciences' tool in the 1950s and used successfully by Yalow and Berson in a RIA for insulin in 1959 [1]. A leap forward in their utility came in 1975 when Kohler and Milstein introduced monoclonal antibodies [2], without intellectual property restriction [3], thereby spawning whole industries in sensitive in vivo-derived antibody-based diagnostics and therapeutics.

Concurrently, nucleic acid manipulating technologies were advancing. In 1990 an in vitro display of the antibody-binding variable region on phage was described [4], followed by selection of intact antibody fragments (Fabs) using phage display in 1991 [5, 6]. In vitro display provided several advantages over monoclonal antibodies, the most notable being

that the gene sequence was attached to the affinity reagent. At the same time a new molecular recognition element called the aptamer was introduced [7, 8]. Aptamers rely on the natural 3D folding of nucleic acids (RNA and then later single-stranded DNA) to generate specific structures that bind to their respective protein targets. These molecules were selected from a large library of molecules using an iterative in vitro selection method, analogous to that for phage display, resulting in identification of the best binder or set of binders. Aptamers took the technology of affinity reagents one step further: the affinity reagent was the nucleic acid sequence.

Although aptamers are generally very good molecular recognition elements, with affinities comparable to high quality antibodies [9], they have been largely ignored in favor of the more established and studied antibody-based industry for several reasons. First, aptamers were relatively new molecules compared to in vivo-derived antibodies and techniques for their use were not as well established. Second, although aptamers to a large number of proteins were rapidly discovered, the generation of high-affinity aptamers across a wide range of proteins was challenging [10]. Third, the perception that intellectual property issues surrounding the selection procedure for high quality aptamers limited their widespread adoption [11], a concern that was also raised for in vitro display-derived antibodies [3]. Nevertheless, in the nearly two dozen years since their introduction, aptamers have gained

Correspondence: Dr. Bridget Lollo, SomaLogic, Inc., 2945 Wilderness Pl., Boulder, CO 80304, USA
E-mail: BLollo@somallogic.com
Fax: 303-449-1057

Abbreviations: SOMAmer, Slow Off-rate Modified Aptamer; K_D , dissociation constant

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numerous supporters, particularly as advances in nucleic acid technologies have made large random library generation relatively easy, and multiple innovations have overcome earlier difficulties [12–16].

2 Beyond aptamers

A 2007 compendium of 41 published RNA and DNA aptamers described a range of dissociation constants, K_D , from 0.05 to 1200 nM, with 20% demonstrating K_D s of ≤ 1 nM for binding to proteins [17]. These are respectable affinities, comparable to high quality antibodies [18, 19], and can be useful in many applications. However, probing the depth and breadth of the plasma proteome, which requires detecting some analytes at very low concentrations (e.g. in the fM range), demands even higher affinities. Fortunately, several innovations in aptamer generation have led to higher affinity aptamers. For example, a recently described automated microfluidic device has improved throughput and the ability to select aptamers in the low pM affinity range for basic proteins, and more modest affinities for the neutral or acidic forms of the proteins [20]. In addition, numerous efforts have been undertaken to make chemical modifications to aptamers to improve nuclease resistance [12] or to improve the affinity and range of targetable proteins [10, 12, 21, 22]. While these approaches are interesting, the most successful innovation to generate high-affinity aptamers for use as protein binding reagents—and the subject of the remainder of this article—has been the incorporation of modified nucleotides that conferred more protein-like properties on the nucleic acid-based aptamer [10].

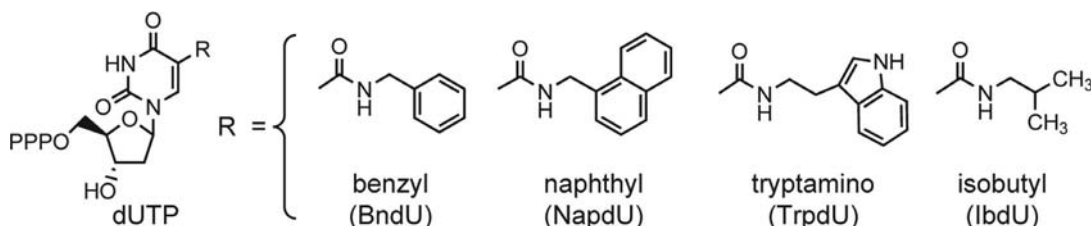


Figure 1. Modified nucleotides incorporated into libraries for aptamer selection. Nucleotide triphosphate analogs are modified at the 5-position (R) of uridine (dUTP): 5-benzylaminocarbonyl-dU (BndU); 5-naphthylmethylaminocarbonyl-dU (NapdU); 5-tryptaminocarbonyl-dU (TrpdU); and 5-isobutylaminocarbonyl-dU (IbdU).

3 SOMAmer reagents: “next generation” aptamers

By the addition of benzyl, naphthyl, tryptophan, or isobutyl moieties to the 5 position of deoxyuridine (Fig. 1) and the incorporation of those modified nucleotides into the aptamer, Gold et al. demonstrated the ability to generate aptamers to 84% of proteins attempted, 55% of which had $K_D < 1$ nM [10]. Nucleic acids with modifications at this position are still recognized by many polymerases and retain the ability to hybridize to complementary DNA sequences, absolute requirements for in vitro selection [22]. Selection using large libraries of $>10^{14}$ molecules containing these modified nucleotides also improved the ability to identify reagents that bind to targets with affinities in the picomolar range [10].

Another important feature for a binding reagent is specificity, particularly when using only one reagent for a measurement. The high specificity of traditional aptamers has been demonstrated using reagents selected to distinguish theophylline from caffeine, molecules that differ by only one methyl group [23], and the Ras-binding domain of Raf-1 from that of the RGL protein [13, 24]. The generation of aptamers with such high specificities often, but not always [25], requires unique selection protocols. In addition, in complex mixtures with a large range in analyte abundance, such as plasma, the binding of highly abundant molecules may be the dominant signal even though they bind “nonspecifically” with a K_D as poor as 1 mM. To generate a large number of specific modified aptamers, a general methodology for specificity was devised based on a classic kinetic manipulation technique described in the 1970s [26, 27]; modified aptamers were selected for very slow off-rates in the presence of polyanionic competitors. No such universal competitor exists for proteinaceous affinity reagents. This new class of modified aptamers selected under these conditions is more specific in complex mixtures: low affinity, nonspecific interactions do not re-form after dissociation, while slow off-rate, specific interactions remain intact. [10, 28].

This new generation of reagents that contain modified nucleotides and are selected for slower off-rates are called “SOMAmer” (Slow Off-rate Modified Aptamer; Fig. 2A). SOMAmer reagents with subnanomolar affinities are routinely generated (Fig. 2B) to proteins that span the isoelectric spectrum of the predicted human proteome (Fig. 2C). The

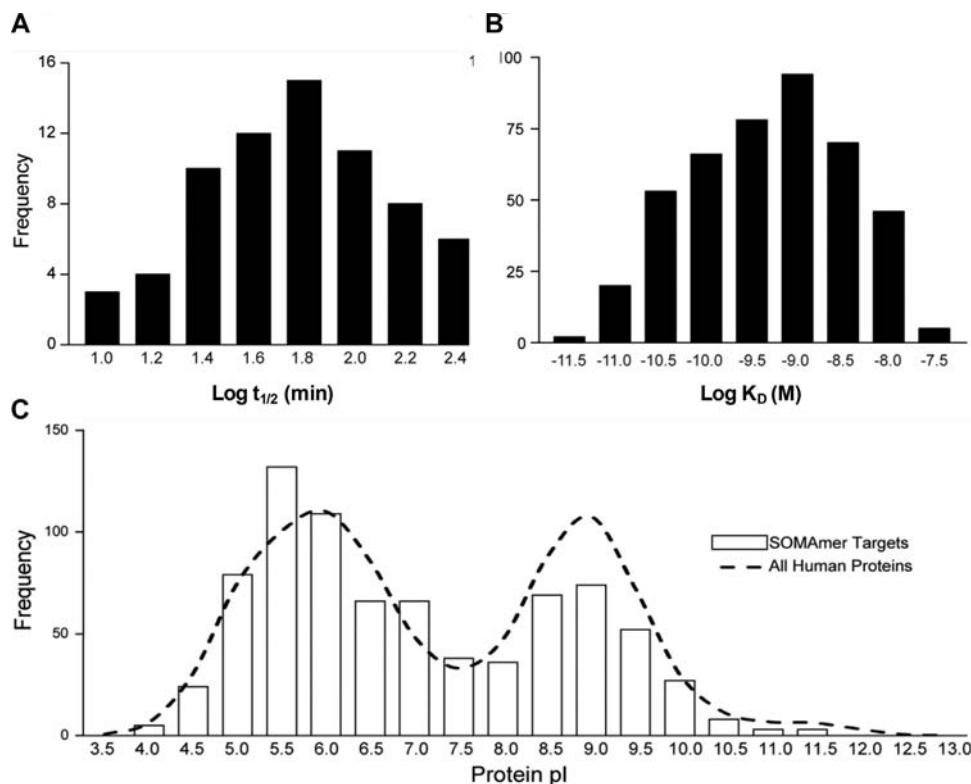


Figure 2. Off-rates and range of dissociation constants ($\log K_D$) of the SOMAmer:protein binding interactions and isoelectric points (pI) of target proteins. (A) Histogram of off-rates of 72 SOMAmer reagents dissociating from target proteins. (B) Histogram of 434 SOMAmer reagents delineated by $\log K_D$ for target protein. (C) Histogram of SOMAmer reagents delineated by pI of target protein. The dotted line is the pI of the predicted human proteome.

importance of the modified nucleotides in the binding interaction of SOMAmer reagents is exemplified by a recent X-ray crystal structure of a SOMAmer reagent interacting with its target, PDGF-BB: seven of the eight modified nucleotides interact with a hydrophobic groove on PDGF-BB [29], further underlining the novel chemical interactions that characterize SOMAmer reagents as compared to traditional aptamers. Other crystal structures have shown similar findings, as well as instances where the modified nucleotides interact with one another to create shapes that would not naturally occur with unmodified DNA.

4 Comparison of SOMAmer reagents to antibodies

The majority of the top SOMAmer reagents evaluated directly after selection have K_D between 10 nM and 1 pM (Fig. 2B) [10], and 22% have a K_D below 100 pM. While this range is comparable to antibodies, the ability to generate such a large number of affinity reagents with $K_D < 100$ pM without affinity optimization is remarkable: antibodies typically require affinity maturation to reach low picomolar affinities [3, 30]. It is likely that this result arises from the diversity of molecules in the library used for the selection of SOMAmer reagents, that is, $> 10^{14}$, which is much larger than typical in vitro phage display libraries for antibodies that are on the order of 10^7 – 10^{10} [30]. When desired, the affinity can be further improved, at least 100-fold, by postselection optimization strategies [29] simi-

lar to those used for traditional aptamers [17, 31]. SOMAmer reagents (like traditional aptamers) bind to native 3D proteins and therefore are similar to antibodies that recognize tertiary structures and, as such, most likely bind to a biologically competent molecule. That SOMAmer reagents (and aptamers) bind to native, 3D proteins is further reflected in the relative ease with which function-blocking aptamers and SOMAmer reagents can be selected [25, 29, 32, 33], as well as the successful applications of staining cell-surface receptors in flow cytometry [12, 32, 34] and histochemical staining of fresh frozen, but not formalin fixed, tissue [35, 36]. On the other hand, since SOMAmer reagents tend to bind globular structures, it is more difficult to generate SOMAmer reagents to flexible polypeptides smaller than 5 kDa, which is not a limitation for antibodies. Although typically smaller than antibody fragments (Fab; ~8–27 kDa vs. 50 kDa) and much smaller than intact antibodies (150 kDa), X-ray crystallography of a SOMAmer reagent binding to its target protein [29] demonstrates epitope dimensions remarkably similar to that reported for antibody–antigen interactions [37].

5 Advantages over antibodies

Although SOMAmer reagents are comparable to, and often better than, high-quality antibodies in terms of affinities, they also offer additional characteristics that make them more practical to use. These properties of SOMAmer reagents (and traditional aptamers), such as thermal stability, flexibility of

Table 1. Metrics for SOMAscan assay v3.0

Metric	Condition	Current version (3.0) of SOMAscan assay
Sensitivity	Median LLOQ	100 fM
	Median LOD	38 fM or 1.6 pg/mL
Dynamic range	Over all proteins in serum or plasma	10 ⁸
	Median range per SOMAmer reagent	4.2 logs
Precision	Median total %CV	5.1%
Sample volume	Human serum, plasma, or CSF (per sample)	65 µL
Multiplex size	Current # of proteins per sample	1129 human targets

selection conditions (over in vivo derived antibodies), etc., have been thoroughly described in previous reviews [12,15]. A notable advantage of SOMAmer reagents is that they are synthesized by standard nucleic acid synthesis techniques and therefore the same molecule can be reproducibly generated in large quantities, in contrast to the heterogeneity observed in monoclonal antibodies manufactured via cell culture or across batches of polyclonal antisera [38, 39]. In addition, this synthetic process enables the easy and consistent addition of functional moieties, such as biotin, fluorophores, etc., to defined positions as opposed to in vitro protein-labeling techniques that yield a distribution of modifications over the molecule that can compromise structure and may not be consistent from batch to batch.

6 SOMAscan, a highly multiplexed assay using SOMAmer protein binding reagents

But perhaps the most remarkable positive attributes of SOMAmer reagents are (i) their ability to be detected and quantified by standard, sensitive DNA detection methodologies, such as DNA microarrays or PCR, and (ii) their ability to be combined together in a very highly multiplexed protein detection assay (called SOMAscan). SOMAmer reagents do not cross-react with each other to the extent that antibodies in multiplexed immunoassays tend to do. Multiplexed ELISA limitations of 40–50 analytes are frequently quoted [40, 41], whereas the SOMAscan assay currently contains 1129 SOMAmer reagents without an apparent limit to multiplexing. Knowing the sequences of the SOMAmer reagents allows one to screen for potential interactions in silico and then make sequence modifications, such as truncations, to minimize such interactions. The lack of a secondary reagent in the SOMAscan assay likely further reduces nonspecific interactions. Furthermore, the series of chromatographic steps with unique washes and the slow off-rate combined with an anionic competitor lends specificity to the SOMAmer reagents during the SOMAscan assay; specific interactions are maintained in the presence of the anionic competitor while nonspecific interactions with faster off-rates dissociate and do not re-form in its presence [10, 42]. This combination of attributes of SOMAmer reagents yields the most highly multiplexed and most sensitive affinity reagent-based assay currently available.

Individual SOMAmer reagents operating in the context of the SOMAscan assay generally have a very large quantification range, with a median of 4.2 logs of protein concentration. However, an assay dynamic range of over eight logs is achieved by using three sample dilutions covering nearly four logs, so the detection and quantification of proteins is enabled over the range in abundance from micromolar to femtomolar. The assay has excellent reproducibility with the median total% CV (intra- and inter-run) of 5.1%. This performance (summarized in Table 1) consumes just 65 µL of human plasma or serum. In the current form of the SOMAscan assay, 168 samples can be profiled per day per instrument, and the assay is highly scalable. In addition, the assay format is amenable to many biological sample types, and equally small volumes of cerebrospinal fluid, tissue homogenates, synovial fluid, cell lysates, and cell supernatants have been successfully assayed [36, 43].

7 The SOMAscan assay as a biomarker discovery tool

The excellent reproducibility, throughput and small sample volume requirements (Table 1) make the SOMAscan assay well suited to overcome many of the issues that plague current proteomic biomarker discovery and subsequent validation efforts, namely profiling enough samples and proteins reproducibly to find key biological effects [44]. To date, the assay has been used to profile 49 000 samples for numerous clinical studies. One of the most fully developed projects is a signature of protein changes that can detect the presence of nonsmall cell lung cancer in long-term heavy smokers and those with indeterminate pulmonary nodules [45]. This signature was developed by profiling 1326 serum samples from four different clinical sites in less than 2 weeks. The signature panel was then used for blinded verification with 341 patients and controls, and results confirmed the initial discovery [42, 45]. Another notable example of a successful disease signature identification and clinical validation using the SOMAscan assay was for the early detection of mesothelioma, accomplished by profiling over 259 samples [46].

The ability to profile large numbers of analytes in large sample sets from multiple clinical sites has some important advantages, the most important of which is detecting variation in sample handling, which is all too common and

likely one of the top reasons so few biomarkers pass clinical validation [47]. Empirically derived signatures for complement or platelet activation or cell lysis to mimic those that arise from inconsistent blood processing such as described in Ostroff et al., [48] enabled unsupervised learning techniques such as PCA to find characteristic patterns of proteins which represent variation in sample handling that—when manifested differently in cases and controls—often masquerade as disease signatures [42]. Proprietary algorithms were developed alongside the SOMAscan assay to avoid false discoveries from this prevalent menace. Additionally, if a large number of candidate biomarkers (>30) are found to be differentially expressed, many are typically redundant with each other and can be interchanged in a diagnostic algorithm without loss of classifier performance [45, 46]. This permits easy attrition of candidate biomarkers downstream that is often necessary due to intellectual property constraints or potential difficulties in either analytical or clinical validation.

Because it profiles large numbers of analytes, the SOMAscan assay not only reconfirms some known biology, but it also often uncovers previously unknown mechanisms in the pathobiology of disease or, in studies with therapeutic agents, novel mechanisms of action and potential off-target effects. For example, when profiling lung tumor tissue and normal adjacent tissue, two thirds of the 36 analytes that were significantly differentially expressed were previously reported as associated with the disease and two were commercial drug targets; the remaining 13 analytes were novel discoveries [36].

A frequently asked question is how the results from the SOMAscan assay compare to antibody-based ELISAs, which are still the predominant method used in protein analyte detection. For the most part, they are very similar, providing the analyte measured is above the LOD in both methods. There are discrepancies in some cases, which are most likely because the SOMAmer reagents and antibodies are measuring different epitopes of the same protein. Indeed, similar observations have been made for two different antibodies [49, 50]. These differences could be the result of PTMs that affect epitope conformation or, in complex matrices such as plasma, the presence of a binding protein that can block accessibility, which may point to interesting biology. One such case led to the first commercially available SOMAmer-based product, developed and offered by Quest Diagnostics, that monitors Erbitux® and Vectibix®-unbound EGFR. When assaying patient samples, it was discovered that these therapeutic monoclonal antibodies blocked the SOMAmer reagent from binding to EGFR, whereas an ELISA was unaffected [51, 53], providing a potential surrogate indicator of drug efficacy. The inability to derive specific information on PTMs suggests a potential disadvantage of the current SOMAscan assay, though this kind of information can be obtained using a mass spectrometry-based approach or antibodies specifically generated to PTM [3].

Other assay formats such as qPCR, Luminex beads, and traditional sandwich assays have been developed for SOMAmer

reagents [52–54], and aptamers [15], that offer the ability to detect smaller numbers of analytes in a manner similar to the single analyte or small panel ELISAs. Finally, although SOMAmer reagents are created to bind to native human proteins, the SOMAscan assay has been evaluated for its ability to measure proteins from other species, such as mice [55], nonhuman primates, dogs and cats (unpublished data), with good success.

8 Conclusion

This article describes a new class of high-affinity protein-binding reagents and a robust assay that takes advantage of the unique chemical structure and characteristics of these reagents to measure more than 1100 proteins across eight logs of concentration in multiple biological sample types. Although antibodies and their associated detection technologies dominate the current affinity reagent landscape, SOMAmer reagents—which are essentially nucleic acid sequences—allow the use of current, sensitive DNA measurement technologies to convert a protein measurement into a nucleic acid measurement. To date, working alone and with partners from both academia and industry, 49 000 samples across numerous clinical and preclinical studies have been run in the SOMAscan assay. This new proteomics technology is now commercially available directly from SomaLogic, and efforts are underway to increase its accessibility by transferring it to additional academic and contract research centers. It is hoped that the SOMAscan assay will significantly expand the proteomics community's tool kit and help resolve the dearth of clinically approved protein biomarkers.

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