nature chemistry

ARTICLES

PUBLISHED ONLINE: 28 SEPTEMBER 2014 | DOI: 10.1038/NCHEM.2058

Recognition and sensing of low-epitope targets via ternary complexes with oligonucleotides and synthetic receptors

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Oligonucleotide-based receptors or aptamers can interact with small molecules, but the ability to achieve high-affinity and specificity of these interactions depends strongly on functional groups or epitopes displayed by the binding targets. Some classes of targets are particularly challenging: for example, monosaccharides have scarce functionalities and no aptamers have been reported to recognize, let alone distinguish from each other, glucose and other hexoses. Here we report aptamers that differentiate low-epitope targets such as glucose, fructose or galactose by forming ternary complexes with high-epitope organic receptors for monosaccharides. In a follow-up example, we expand this method to isolate high-affinity oligonucleotides against aromatic amino acids complexed *in situ* with a nonspecific organometallic receptor. The method is general and enables broad clinical use of aptamers for the detection of small molecules in mix-and-measure assays, as demonstrated by monitoring postprandial waves of phenylalanine in human subjects.

Aptamers are often pursued as recognition elements for biosensors[1,2]. Although the development of new protocols[3,4] has enabled the isolation of high-affinity protein-binding aptamers in general, similar advances in the recognition of small molecules have not been forthcoming. This is because many small molecules of analytical interest lack epitopes that would elicit strong interactions with nucleic acids[5] or some other biomolecular receptors, such as antibodies. The introduction of modified oligonucleotides[6] and organic receptors as cofactors[7] has been suggested as helpful to isolate aptamers for challenging small molecules, but with no practical impact as yet. Although the approaches that use modified oligonucleotides might, indeed, improve the affinity for many targets, these may still fail if targets inherently lack epitopes. Furthermore, such modifications increase the production costs of sensors. The problem with previous attempts[7] to incorporate organic receptors for small molecules within aptamers was that the binding of receptors to oligonucleotides seemed to compete with binding to targets, which led to drastic drops in affinity. We now propose to avoid all these issues with targets that display no suitable epitopes by pursuing unmodified oligonucleotides that are selected to interact specifically with the *in situ* complex that low-epitope targets form with organic or organometallic receptors. This would lead to the formation of ternary complexes of oligonucleotides, organic receptors and targets, whereas oligonucleotides would avoid productive interactions with organic receptors on their own. Therefore, if isolated successfully, sensors formed from these oligonucleotides would respond with high sensitivity and selectivity to the presence of small molecules in the presence of an excess of organic/organometallic receptors, acting as *in situ* derivatization agents. A successful approach such as this could change clinical chemistry and enable rapid and sensitive mix-and-measure assays for almost any class of small-molecule guest that can interact with organic/organometallic hosts.

Results

Differentiation of monosaccharaides. Glucose might be the most prominent example of a small molecule that has up to now resisted attempts to isolate aptameric reagents that bind to it, despite a universally recognized need for improved sensors in the context of real-time glucose monitoring in patients with diabetes[8]. Our approach to sensing glucose or other monosaccharides with aptamers is based on the idea that we can improve the epitope presentation in glucose during the selection of oligonucleotides by targeting the complexes formed *in situ* with an organic receptor, as an example of which we chose Shinkai's receptor[9,10] (**1**) (Fig. 1a). Shinkai's receptor is a successful fluorescent glucose sensor based on bisboronic acid and designed to overcome the natural preference of monoboronic acids for fructose over glucose. Based on its mechanism of action, we expected Shinkai's receptor to change its conformation drastically depending on the monosaccharide target, which presents a spatially different arrangement of epitopes to aptamers, and thus enables their selectivity (Supplementary Fig. 1).

Our selection of oligonucleotides that bind to monosaccharide–receptor complexes started with a recently reported[11] variant of early solution-phase selection and amplification protocols (Fig. 1b)[12,13]. This protocol was chosen for two reasons: first, Shinkai's receptor, or any other organic host molecules, can be used without modification to attach it to a solid-state matrix, as done previously in reports using organic receptors as cofactors for aptamers[7]; second, at the end of the procedure isolated oligonucleotides that bind complexes (aptamers) are already in their sensor forms (Fig. 1c), which thereby simplifies the post-selection analysis. We added **1** to the elution buffer in the presence of an excess of a targeted monosaccharide (for example, glucose **2**, fructose **3** or galactose **4** in Fig. 2; excess sugar makes the receptor fully soluble) to ensure that the target for the aptamers is a

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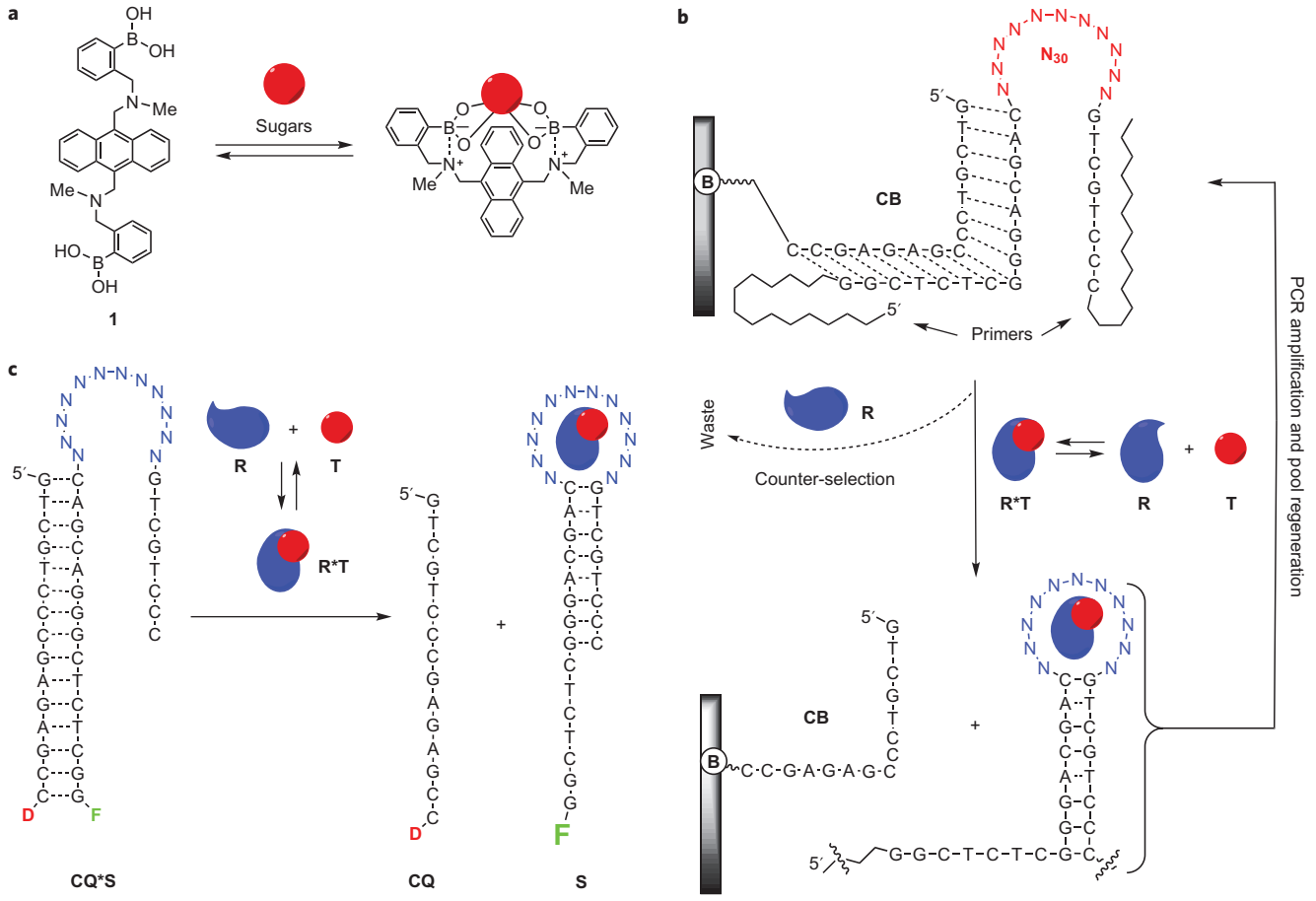

Figure 1 | Basic principles of glucose recognition with boronic acid receptors and of the isolation of aptamers against complexes with receptors. **a**, A schematic representation of the complexation of Shinkai's receptor **1** with glucose (presented as a sphere). **b**, A schematic representation of selections used in this work: a library (for example a randomized region N_{30} flanked with primers) is attached to an agarose–streptavidin column via a biotinylated complementary oligonucleotide (**CB**). On binding to a complex between a receptor (**R**, for example Shinkai's sensor) and a target molecule (**T**, for example glucose or other monosaccharides), aptameric structures are released preferentially from the column as a result of stabilization of the stem formation, PCR amplified and, therefore, evolutionarily favoured to survive through multiple selection cycles; counter-selection against the receptor itself is introduced to minimize competition between the aptamer and target for the receptor. **c**, An example of a structure-switching aptameric sensor that comes from the selection procedure. The aptameric structure selected in **b** is labelled with fluorescein (F), and the capture strand is labelled with a quencher **Q** (D in **CQ** is the quencher dabcyl (4-((4-(dimethylamino)phenyl)azo)benzoic acid) to yield a sensor **S**; on binding to a complex, **CQ** is displaced, which leads to an increase in the fluorescence signal.

complex of monosaccharide with **1**. We also introduced counter-selections against:

(1) Shinkai's receptor itself, to the extent that it is soluble on its own (Supplementary Fig. 5) in the presence of oligonucleotides, to focus selection on a receptor that serves as an *in situ* derivatization agent.
(2) A mixture of steroids to eliminate hydrophobic three-way junctions that would otherwise overwhelm the selection and provide no useful selectivity for a complex.
(3) In the case of galactose and fructose, two non-targeted sugars added in excess to the Shinkai's receptor (for glucose, the third type of counter-selection was not performed); this counter-selection reduces the affinity of the organic receptor for non-targets in ternary complexes or, in other words, eliminates the natural crossreactivity of **1**.

As a result of our selections, we isolated oligonucleotides in the form of aptameric structure-switching[11,12] sensors that respond with high specificity to each of the three targeted monosaccharides (for example, **I**–**III**). The evolutionary pressure in these selections is based on the selective ability of receptor × target complexes to enhance closures of the stems that displace the complementary oligonucleotide (**C**) from aptamers; thus, the mechanism of reporting glucose concentrations shown in Fig. 1c and Fig. 2a directly results from the selection[11]. The combination of positive and multiple negative (counter) selections was even able to completely overcome the initial preferences of **1** (for glucose) or monoboronic acids (for fructose) (Fig. 2). For example, although the original Shinkai's sensor is more responsive to both glucose and fructose over galactose (Supplementary Fig. 3), the aptameric sensor **III** against the galactose complex specifically responds to the addition of galactose. Mid-points of sensitivity of sensors **I**–**III** to monosaccharides were similar to the dissociation constants of Shinkai's receptor, corrected for the competition with complementary oligonucleotide **C**, which indicates that the aptamer and target did not compete for the receptor. Additionally, in this example sensor **I** was adjusted, by choosing the length of **C**, to respond to a concentration below 3 mM glucose, which is important for hypoglycaemia and is a region in which traditional glucometers do not perform well[8]. The aptamer on which sensor **I** is based will actually interact with a glucose complex in the range 1–10 μM (this is a competitive assay; solubility issues

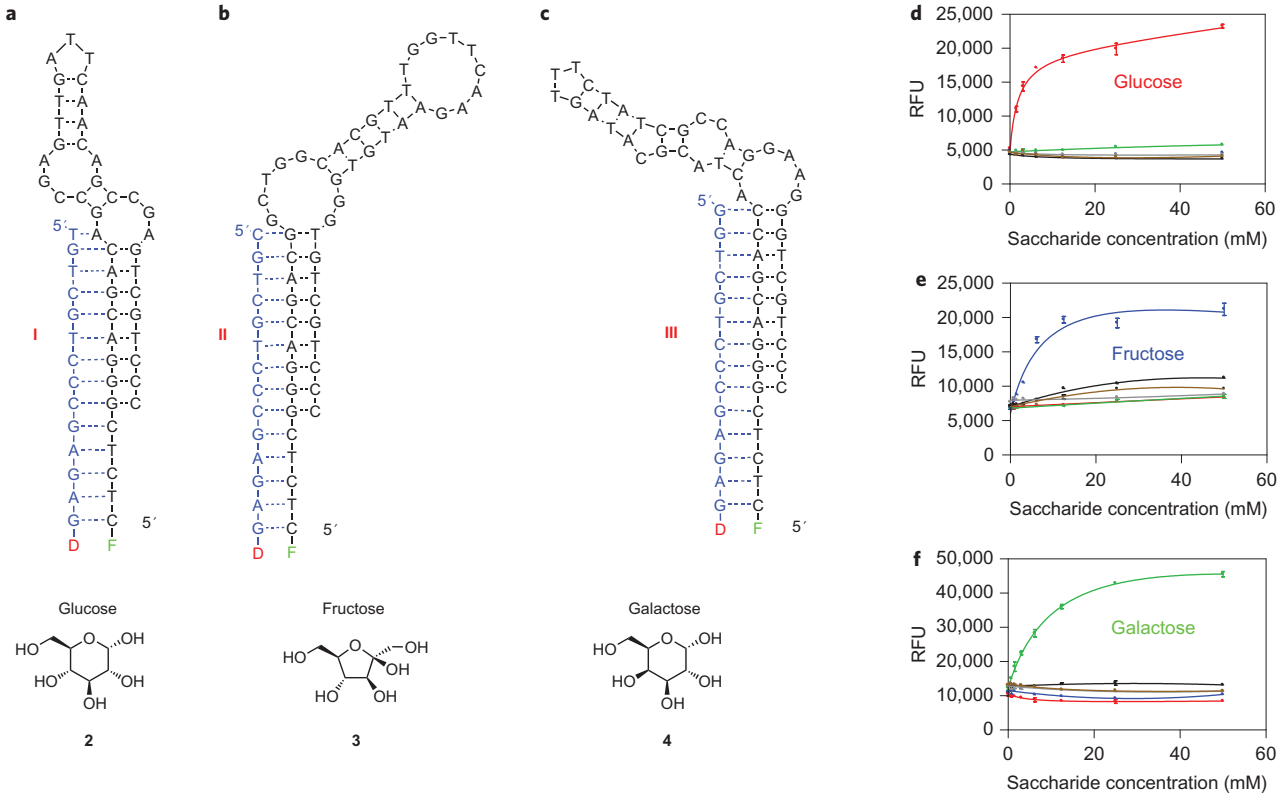

Figure 2 | Results of three selection procedures against monosaccharides. a–c, **I**, **II** and **III** are structure-switching aptameric sensors that respond to glucose (**2**), fructose (**3**) and galactose (**4**) (all monosaccharides are shown as a single, dominant form, which does not necessarily represent the form that is bound by Shinkai's receptor when recognized by the aptamers). **d–f**, The response of sensors **I–III**, respectively (relative fluorescence units (RFUs), as read from the plate reader, versus the concentration of monosaccharides; the initial concentration of Shinkai's receptor was 50 μM with 2% methanol used). The tested monosaccharides are glucose (red), fructose (blue), galactose (green), mannose (black), allose (grey) and altrose (brown). Each aptameric sensor is primarily responsive to a single monosaccharide, that used in the selection. All measurements were performed in triplicate; s.d. values are shown.

and multiple binding sites of the receptor to oligonucleotides interfere with a more precise characterization (Supplementary Figs 3 and 4)). One more benefit of using aptameric sensors is that they shift the fluorescence response of Shinkai's receptor to the fluorophore we choose for them (that is, fluorescein can be substituted with other fluorophores), and thereby allow multicolour detection.

Aptamers for aromatic amino acids. Our work on monosaccharides was designed to demonstrate the ability of aptamers to recognize analytes that have no epitopes suitable for tight interactions with nucleic acids and at the same time impose selectivity on organic receptors for analytes that are challenging because of their structural similarity. We next decided to address the issue as to whether this approach could lead to significant improvements to affinities for less-challenging targets that have, for example, aromatic epitopes, and at the same time address unmet needs, such as the rapid measurement of amino acids in body fluids. Elevated blood amino acids in newborns are indicative for inborn errors of amino-acid metabolism that require immediate and often lifelong treatment with a diet restricted in the specific amino acid to prevent disability and serious motor and mental sequelae. Inborn errors of amino-acid metabolism are detected in newborn screenings by the analysis of whole blood obtained from a heel prick about 24 hours after birth using mass spectrometry. Consecutive follow-up and monitoring of blood levels is carried out in specialized laboratories with a usual turnaround time between two and 21 days. There are currently no methods to determine amino acids rapidly and accurately other than in specialized laboratories. A simple test based on a measurement in a highly diluted (to avoid interferences) droplet of fasting blood or serum could be used to detect excessive amino acid and allow the adjustment of therapy in real time.

As our primary targets we chose three aromatic amino acids (Fig. 3): (1) phenylalanine (Phe, **5**), the measurement of which is important in the detection, confirmation and therapy monitoring for the most common inborn metabolic disease, phenylketonuria (PKU)[14]; (2) tyrosine (Tyr, **6**), the measurement of which is indicative of various types of tyrosinaemia[15] and (3) tryptophan (Trp, **7**), a physiological precursor of serotonin that is increased in familial tryptophanaemia and tryptophanuria[16]. Numerous RNA aptamers that interact with amino acids have been reported in the context of studies of the origins of life and translational machinery[17], but these would be challenging to adapt in fluorescent assays for clinical applications because their affinity is mismatched with the actual needs (assuming that the intrinsic instability of ribonucleic acid (RNA) can be addressed). Specifically, RNA aptamers with mid-micromolar affinities for Phe[18] and Tyr[19], and a DNA aptamer with low-micromolar affinity for Trp[20], were reported before, and these could serve as benchmarks for our method as well.

For an *in situ* derivatization of amino acids we chose a pentamethylcyclopentadienyl rhodium(III) (Cp*Rh(III)) complex (**8**)[21]. The complex interacts preferentially with bidentate and tridentate ligands, usually with micromolar affinities, but with no useful selectivity; it was introduced by Severin and colleagues[22] as the amino-acid complexation reagent in crossreactive arrays of indicator-displacement sensors for the identification of amino acids. Our goal was to isolate aptamers that tightly bind to complexes of individual

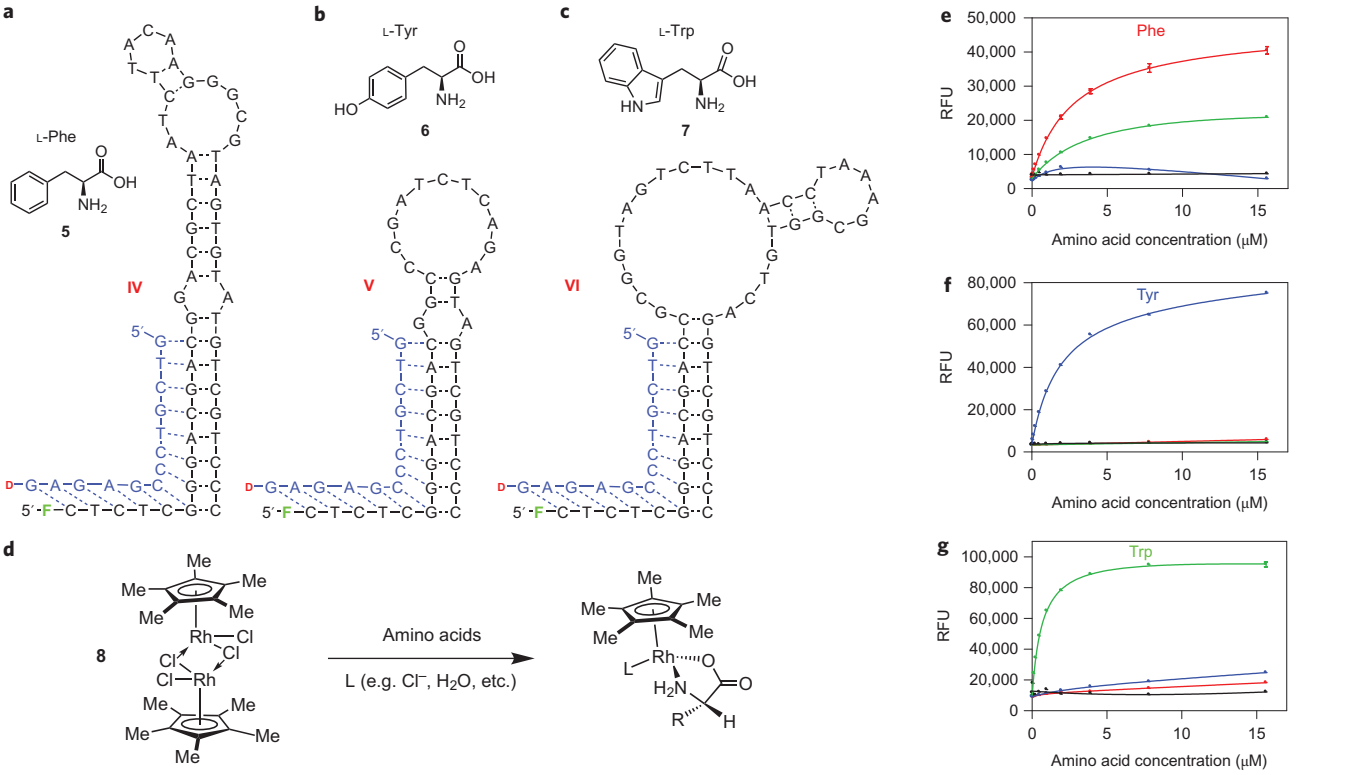

Figure 3 | Results of three selection procedures against amino acids. a–c, **IV**, **V** and **VI** are structure-switching aptameric sensors against L-Phe (**5**), L-Tyr (**6**) and L-Trp (**7**), respectively**. d**, Structure of $[(Cp^*RhCl_2)_2]$ complex **8**, which in solution provides the Cp*Rh(III) moiety that complexes amino acids (shown as a generic amino acid) and other nucleophilic ligands present in solution (L). **e–g**, The response of sensors **IV**–**VI**, respectively, at low concentrations of amino acids, representative of measurements in diluted blood or serum (RFUs, as read from the plate reader, versus the concentration of amino acids, with the complex added at 100 μM). The tested amino acids are: Phe (red), Tyr (blue), Trp (green) and a mixture of eight amino acids (black). All measurements were performed in triplicate; s.d. values are shown.

amino acids to be able to dilute body fluid in an excess of Cp*Rh(III) (globally derivatizing all the ligands) and directly measure the concentration of an amino acid with an aptamer. Our idea was that this complex would be suitable because it could effectively crosslink nucleic acids with amino acids by providing additional electrophilic and aromatic epitopes for binding to nucleic acids and simultaneously complexing amino acids.

We followed an aptamer selection procedure similar to that used for receptor **1** and isolated a number of sensors for targeted amino acids. In the case of Phe, counter-selections individually with both Cp*Rh(III) and Cp*Rh(III)–Tyr were performed; in the case of Tyr, only Cp*Rh(III) was used in counter-selection; in the case of Trp, both Cp*Rh(III)–Tyr and Cp*Rh(III)–Phe were used. The aptameric sensors for Tyr (**V**) showed selectivity over complexes with other amino acids at lower concentrations, with no observed response to Cp*Rh(III) in the absence of other tested potential ligands; at higher concentrations it showed some response to Phe/Trp (Supplementary Fig. 7). Targeted counter-selection was successful in eliminating the crossreactivity of Phe sensor **IV** with Tyr, but this sensor showed a response at low concentrations with Trp, against which no counter-selection was performed (Supplementary Fig. 7); subsequent counter-selection against Trp eliminated the response to this amino acid at low concentrations (see Supplementary Fig. 13). Aptamer **VI** against the Trp complex had exquisite selectivity against other tested amino acids. The dissociation constants of adducts of aptamers with Phe, Tyr and Trp complexes were 180, 60 and 120 nM, respectively (Supplementary Fig. 8), in each case an affinity between one and two orders of magnitude higher than those reported for the best aptamers without the addition of the Cp*Rh(III) complex.

Next, sensors for Phe and Tyr (**IV** and **V**) were tested for their ability (Supplementary Fig. 9) to differentiate healthy sera (fasting) diluted up to 1:512 in a solution that contained 100 μM Cp*Rh(III) from the same sera spiked (prior to dilution) with 0.25, 0.5 and 1 mM amino acids; the tests indicated that these sensors could be used in actual clinical assays. We performed additional confirmatory assays with the Phe sensor; clinically, in monitoring dietary intervention, it is important for a Phe sensor to distinguish at least three critical cutoff concentrations: (1) in the adult PKU patient population, the goal is to keep the Phe concentration in blood below 600 μM (ref. 14); (2) in children with PKU, the goal is to keep the Phe concentration below 360 μM (ref. 14); (3) the targeted concentration in patients who adhere to diet should also be kept above 120 μM to ensure the sufficient biosynthesis of neurotransmitters[14]. When we took five sera from healthy humans, spiked them with 100, 300 and 600 μM and 1 mM Phe (Fig. 4a), and diluted them 1:200 in a buffer with reagents, sensor **IV** was clearly able to stratify samples spiked at all these concentrations and the unspiked samples, which indicates a potential for clinical and at-home use. Second, in an IRB-approved self-experiment, two senior investigators (T.S.W. and M.N.S.) ingested 100 mg kg^{-1} Phe and observed transient postprandial waves of Phe in serum obtained from capillary blood (Fig. 4b). Although full clinical validation with a large number of samples, comparison with the standard clinical methods, studies of interferences, matrix effects and longitudinal monitoring of an actual patient population are necessary to establish firmly the suitability of these sensors to address patients' needs, the postprandial waves we observed in two human subjects correspond to those expected in a real-life situation[22], except that in PKU patients these would

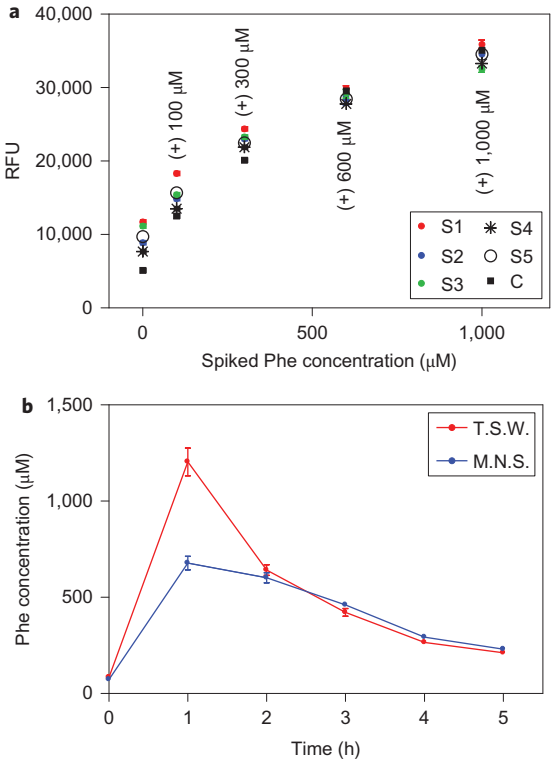

Figure 4 | Measurement in human serum and real-time monitoring of postprandial waves. a, Five samples of healthy human sera (that is, in the range 30–100 µM) were diluted 1:200 in buffer that contained 100 µM Cp*Rh(III) and the fluorescence intensity measured (RFUs as read directly from the plate reader). Samples were also spiked with clinically relevant concentrations of Phe, thus demonstrating the clear stratification based on concentrations. C (black squares) is a standard curve of the response of the sensor to these concentrations of Phe, but without sera present. **b**, Observation of a postprandial wave in the serum of the two senior investigators (T.S.W. and M.N.S.) after a Phe oral load (100 mg kg^{-1}); the concentration was read at a 1:400 dilution of a serum from a calibration curve without serum and with no further corrections. All measurements were performed in triplicate; s.d. values are shown.

persist longer because of the defective Phe catabolism that defines disease.

Discussion

It is instructive to compare the high affinities observed after our selection to the early work of Anslyn, Ellington and co-workers in which they used a bisorganoboronic receptor as a cofactor and isolated aptamers selective for tartarate over citrate[7]. Their characterized aptamer responded to tartarate, but not to citrate, in the presence of the bisorganoboronic receptor; however, the affinity of tartarate for the receptor within the aptamer dropped significantly in comparison to that of the free receptor (the authors estimated >30-fold, K_d changed from ~7 µM to >200 µM); that is, the aptamer binding to the receptor seems to have interfered with the receptor binding to targets. This observation is in sharp contrast to our results, in which oligonucleotides with closed stems are stabilized in comparison to their complexes with competitor oligonucleotides by binding to receptor × target complexes at low concentrations (Supplementary Fig. 4), but not by the receptors themselves. This leads to a system that is more, not less, sensitive to an analyte under conditions in which there is an excess of the derivatization agent, so it enables quantification of targeted analytes beyond that possible with a receptor or just an aptamer against a target alone, and enhances the potential for clinical applications.

We attribute our success in generating useful analytical reagents primarily to two factors that were different from previous attempts to isolate similar aptamers:

(1) Our selection was performed in a solution phase, with a receptor as an *in situ* derivatization agent, coupled with a successful counter-selection against the receptor itself, instead of against a dissimilar analogue; these select against the incorporation of a receptor as a cofactor.
(2) Our elution step was not based on the displacement of aptamer candidates from their complexes with targets by a competitor binding to a free receptor; such a procedure disfavours off-rate constant (k_{off}) values, and thereby further reduces the impact of affinity as the criteria in selection pressure.

Another perspective to analyse this difference is to look at the order of events in the formation of a ternary complex: in our case, small-molecule receptors have to bind their ligands (sugars or amino acids) and only thereafter will the complex favour closing of the stem of the aptamer in the ternary complex.

The importance of our results goes beyond a purely commercial potential in monitoring sugar and amino-acid concentrations using newly generated sensors and their non-fluorescent analogues. First, we offer a general solution to a long-standing problem that prevented aptameric sensors for small molecules being generally applicable by demonstrating that, with the judicious selection of *in situ* derivatization agents and tailored counter-selection conditions, even traditionally difficult small-molecule targets are not off-limits for sensors based on oligonucleotides. Second, our work supports earlier proposals[7] that oligonucleotide-based receptors can be used to tailor the specificity of synthetic receptors for small molecules in a way that is not fully accessible through traditional synthetic organic (or organometallic) chemistry and, vice versa, that an organic receptor endows oligonucleotides with binding and sensing properties that are not readily accessible otherwise. In contrast to this pioneering work[7], we show that under the right conditions we do not have to lose affinity of the organic receptor in the trade-off for enabling oligonucleotide binding (we also lose the affinity of receptors for non-targets through counter-selection). Third, for those small molecules for which aptamers have already been isolated, we demonstrate that the affinity of oligonucleotide-based sensors to small molecules can be increased by approximately two orders of magnitude, and thus adjusted to concentrations that are closer to being useful for addressing actual patient needs. Finally, the isolation of sensors for glucose based on nucleic acids opens up possibilities to integrate devices based on nucleic acids into molecular-level closed loop systems for the delivery of insulin[23] when this advance is combined with the progress in DNA nanotechnology[24].

Methods

All the technical details, procedures and sequences are provided in the Supplementary Information.

Received 23 April 2014; accepted 5 August 2014; published online 28 September 2014

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Acknowledgements

We are grateful to the National Science Foundation (CBET-1033288 and CBET-1026592), National Institutes of Health (RGM104960) and the Juvenile Diabetes Research Foundation (Innovative Program) for funding this research. We thank J. Loeb for help in correcting initial drafts of our manuscript.

Author contributions

M.N.S. proposed and led the project. K-A.Y. isolated the aptamers listed in this report. S.P. and S.T. performed the synthetic and characterization work on the organic receptors. T.S.W. provided the clinical context and organized self-experiments, with B.K. providing technical support in these. M.B., M.H., P.P. and D.M.K. performed earlier experiments on glucose aptamers that eventually led to the development of the method described here. All co-authors participated in the design of their own experiments and analysis of data. M.N.S., T.S.W., K-A.Y. and S.T. wrote the manuscript, with the exception of synthetic procedures written by S.P.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to T.S.W. and M.N.S.

Competing financial interests

The authors declare competing financial interests in the form of patent application(s).