Development of a DNA aptamer for direct and selective homocysteine detection in human serum

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PAPER

1

Development of nucleic acid probes capable of direct and selective homocysteine detection in human serum

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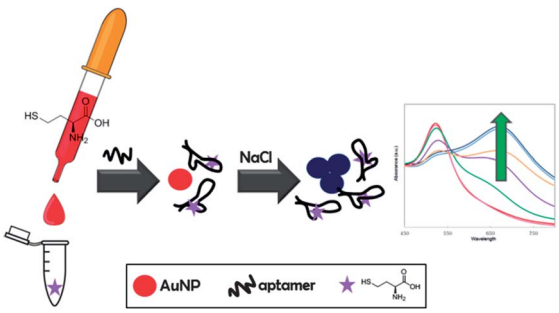
A simple, sensitive, and selective gold nanoparticle-based colorimetric assay for L-homocysteine in serum was developed using a newly-selected high affinity DNA aptamer. The assay is selective for homocysteine over other thiols, such as cysteine, and can detect homocysteine directly in filtered, diluted serum within the clinically relevant range.



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Development of nucleic acid probes capable of direct and selective homocysteine detection in human serum†

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L-Homocysteine has been an amino acid intermediate of interest for over 20 years due to its implication in various adverse health conditions, including cardiovascular disease. Here, we report the first *in vitro* selection and application of high affinity aptamers for the target L-homocysteine. Two novel aptamer sequences were selected following 8 rounds of selection that displayed high affinity binding and selectivity to homocysteine compared to other amino acids. One of the selected aptamers, Hcy 8 (K_D = 600 ± 300 nM), was used to develop a gold-nanoparticle biosensor capable of sensitive and selective homocysteine detection in human serum, with a limit of detection of 0.5 μM and a linear range of 0.5–3.0 μM. This biosensor allows rapid detection of free homocysteine in human serum samples at low cost, with little preparation time and could be adapted to be part of a point-of-care screening method.

Introduction

L-Homocysteine (Hcy) is an amino acid intermediate produced at the intersection of two major biochemical pathways, where it is irreversibly transformed *via* the transsulfuration pathway into cysteine or is remethylated back to methionine.[1] The proposed upper reference limit for plasma homocysteine levels in adults leading a healthy lifestyle or taking adequate vitamins is 12 μM.[2] Increased homocysteine levels have been shown to be associated with several diseases including cardiovascular disease,[3] Alzheimer's disease,[4] dementia,[5] pregnancy complications and birth defects,[6] osteoporosis,[7] and cancer.[1] Additionally, homocystinuria is a rare autosomal recessive condition where homocysteine plasma concentrations are severe (>100 μM). Homocystinuria results in a high risk of vascular disease if left untreated. Individuals that are homozygous for homocystinuria also experience ocular, skeletal, and neurologic complications.[8] As a result, rapid, sensitive, and selective detection of homocysteine is of great interest for point-of-care screening and diagnosis.

Several analytical techniques have been developed for the detection of homocysteine. These include high performance liquid chromatography (HPLC) separation-based methods, gas chromatography/mass spectrometry (GC/MS), capillary electrophoresis (CE), electrochemical detection, and capillary zone electrophoresis (CZE). Reviews on these methods have been previously published.[9] These methods are typically sensitive but time-consuming and costly, thus necessitating a simple, inexpensive screen that could be used at the point-of-care and could serve as a tool for decision-making about further testing. Some methods have been developed that utilize antibodies as effective molecular recognition agents. Examples include chemiluminescence immunoassays, fluorescence polarization immunoassays and ELISA.[10] While useful, these assays are costly and can be affected by antibody cross-reactivity.[11]

Due to their optical properties, ease of use, and simple synthesis, gold nanoparticles have been incorporated into a number of novel biosensor strategies. Aggregation of the particles leads to an easily detectable color change of the colloidal solution from red to blue. Gold nanoparticles have been used to detect all thiol-containing amino acids such as cysteine and homocysteine.[12] DNA-based gold nanoparticle sensor strategies have also emerged[13] since single-stranded DNA (ssDNA) adsorbs onto the surface of gold nanoparticles and can protect them from salt-induced aggregation.[14] Based on this principle, recent research has explored the detection of cysteine using AuNPs and random ssDNA.[15,16] The strong interaction between the cysteine thiol and the gold surface displaces the ssDNA molecules adsorbed on the AuNPs, making them once again susceptible to salt-induced aggregation (facilitating a red-to-blue color change). Unfortunately, these sensors lack specificity, and detect all thiol-containing compounds.[17,18] Thus, in order to exploit the simplicity of gold nanoparticle assays for the rapid, and importantly, selective detection of homocysteine, the incorporation of a specific recognition element into this assay was required.

Aptamers are single-stranded nucleic acid ligands that are discovered using an *in vitro* selection procedure called Systematic Evolution of Ligand by EXponential enrichment

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† Electronic supplementary information (ESI) available: Extended experimental section, aptamer secondary structure prediction, and sample binding isotherm. See DOI: 10.1039/c3ra43893g

(SELEX).[19–21] Like antibodies, these synthetically derived molecular recognition probes are selective and able to bind to their target with high affinity. However, aptamers offer several advantages over their antibody counter-parts including improved stability, lower cost and the ability to be modified at precise locations with reporter molecules often without affecting binding activity. As a result, the use of aptamers as recognition probes has emerged as a viable approach in biosensing and therapeutics. However, the conceptual and technical challenges involved in small-molecule aptamer development have resulted in relatively few new aptamers for these targets.[22] An RNA aptamer for S-adenosyl homocysteine (SAH) with an affinity of approximately 0.1 μM has been reported.[23] Despite the high affinity, competition studies of this aptamer revealed that adenine is the main contributor to binding affinity, with only a minor aptamer–target interaction at the thioether of homocysteine. As a result, novel, stable DNA aptamers selective for homocysteine that can be directly integrated into useful detection systems are still required.

In this study, we report the first isolation of aptamers that recognize and bind to homocysteine with good affinity and selectivity, particularly notable based on the small size of the target and the close structural resemblance to other amino acids. The most promising of these aptamers was incorporated into an unmodified AuNP-based colorimetric aptasensor enabling selective detection of homocysteine over other amino acids, including cysteine. Furthermore, the utility of this assay for the rapid and direct detection of homocysteine in diluted human serum samples is demonstrated.

Experimental

Reagents and materials

Phosphoramidites and reagents for DNA synthesis were obtained from Glen Research (Sterling, VA, USA). Standard support columns and acetonitrile were purchased from BioAutomation (Plano, TX, USA). Ultra High Purity 5.0 argon was purchased from Praxair. All PCR and electrophoresis components were purchased from BioShop Canada (Burlington, ON, Canada). L-Homocysteine thiolactone hydrochloride (L-HcyT), DL-homocysteine, L-homocysteine (L-Hcy), L-cysteine, L-methionine, L-serine, NHS Sepharose, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), human serum (from human male AB plasma) and chemicals for buffering agents, nanoparticle synthesis and all other purposes were purchased from Sigma-Aldrich and prepared with Millipore Milli-Q deionized water at 18 MΩ cm. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) was received from Pierce (Rockford, Illinois, USA). Spin-X cellulose acetate microcentrifuge filter tubes, Amicon-Ultra 0.5 mL 3 kDa centrifuge units and sulfo-NHS acetate were purchased from Fisher Scientific Canada. ProMag 3 Series carboxylic acid surfactant free magnetic beads were purchased from Bangs Laboratories Inc (Fishers, IN, USA).

Homocysteine-binding aptamers

L-Homocysteine-derivatized sepharose was prepared (see ESI S1.1†) and used to select homocysteine-binding aptamers using a modified SELEX process. The starting DNA library was prepared using standard phosphoramidite chemistry on a Bio-Automation Mermade 6 (Plano, Texas) using 200 nmol controlled pore glass (CPG) columns with a 1000 Å pore size. The sequence is 96 nucleotides in length and contains a central region of 60 random nucleotides flanked by two primer binding sites necessary for PCR and cloning: 5′-ATACCAGCTTATTCAATT-N_{60}-AGATAGTAAGTGCAATCT-3′. The following primers used for amplification and cloning of the selected oligonucleotides were synthesized on a 1 μmol scale: Primer1: 5′-ATACCAGCTTATTCAATT-3′ and Primer2: 5′-AGATTGCACTTACTATCT-3′. Modified primers were also synthesized to allow for fluorescent labeling of the binding DNA and separation of strands on a denaturing polyacrylamide gel: ModPrimer1: 5′-6-FAM-ATACCAGCTTATTCAATT-3′ and ModPrimer2: 5′-poly-dA_{20}-HEG-AGATTGCACTTACTATCT-3′. HEG represents the hexaethylene glycol Spacer Phosphoramide 18 (Glen Research). DNA was purified using denaturing polyacrylamide gel electrophoresis (12%) followed by extraction and clean-up with Amicon YM-3 Centrifugal Filter Devices.

In vitro selection SELEX was performed as previously described[19–21] with several modifications. Fresh aliquots of L-Hcy-immobilized sepharose, unmodified sepharose or sepharose modified with a counter-selection target (such as L-cysteine and L-methionine) were pre-equilibrated by washing 10 times with 0.5 mL of homocysteine binding (HB) buffer, 50 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 M NaCl (pH 7.4), before each SELEX round. Prior to the first selection, the library (5 nmol) was suspended in 0.5 mL of HB buffer and heated for 5 minutes at 95 °C then cooled to room temperature. In subsequent rounds, 200 pmol of library was used.

First, a negative selection was performed by incubating the library with 250 μL of either unmodified column material (rounds 1–5) or sepharose derivatized with counter-selection target molecules (rounds 6–8) with mild shaking for 30 minutes. Only DNA lacking affinity for the unmodified or counter-selection targets was collected by removal of supernatant following a 1 minute centrifugation at 5000 g. This library was subjected to the same heating and cooling process described above, followed by incubation with 100 μL of L-Hcy derivatized column for 30 minutes. Following incubation, sequences with little to no affinity for L-Hcy were removed with 10 washing steps using 200 μL of HB buffer. DNA bound to L-Hcy was then recovered with several washes of 0.1 M Hcy (rounds 1 and 2), 3.5 M urea at 90 °C (rounds 3–5) or 7 M urea at 90 °C (rounds 6–8). These conditions were chosen as the selection proceeded to accommodate the enrichment of the library. Recovery of putative aptamers was performed until the fluorescence of the elution fractions was negligible. The recovery of aptamers, representing the enrichment of the DNA library was assessed throughout the rounds of selection by monitoring the percent of DNA library binding to the L-Hcy column each round by measuring DNA absorbance at 260 nm using a Cary 300 BioUV-visible spectrophotometer and measuring fluorescence (excitation 490 nm, emission 520 nm) with a Fluorolog Fluorescence Spectrophotometer (Horiba Jobin Yvon, USA) with a SpectrAcq controller. The elution fractions were collected, purified using Amicon

Table 1 Putative aptamer sequences obtained after 8 rounds of selection against L-homocysteine

Sequence	5′-ATACCAGCTTATTCAATT-N_{60}-AGATAGTAAGTGCAATCT-3′
Hcy 8	ACCAGCACATTCGATTATACCAGCTTATTCAATTCACA
	GCTATGTCCTATACCAGCTTATTCAATT
Hcy 10	GTGGAAAGCCGAATGTGATTAGGGACCAGTGGAGAAG
	TAGTACGGACTGACCTCGCGTGTA

Ultra 3 kDa 0.5 mL centrifugal filters, and quantified using UV-visible spectroscopy at 260 nm and/or using fluorescence (ex: 490 nm, em: 520 nm). A total of 8 rounds were performed.

The entire selected oligonucleotide library was amplified in 30 parallel PCR reactions for the first round, in subsequent rounds only 90% of the selected library was amplified with the remaining 10% set aside for analysis and in case of PCR failure. Each reaction consisted of 0.1 M Tris HCl pH 9, 50 mM KCl, 1% Triton X-100, 1.9 mM $MgCl_2$, 0.3 mM dNTP mix, 1 μM each modified primer and 5 units of Taq DNA polymerase. The DNA was initially melted for 10 minutes at 94.0 °C, followed by 25 cycles of 94.0 °C (1 minute), 47.0 °C (1 minute) and 72.0 °C (1 minute). The final extension occurred at 72.0 °C for 10 minutes after the last cycle. PCR products were dried down, heated at 55 °C for 5 minutes in the presence of formamide and run on a 12% denaturing PAGE to separate the double stranded product. The 5′ fluorescein-labeled DNA strands (the selected sequences) could be identified using an Alpha Imager UV-illuminator. The corresponding DNA bands were cut from the gel and extracted in 10 mM Tris HCl buffer, pH 7.44. The DNA was then purified using the Amicon centrifugal filters, quantified and re-suspended in HB buffer and could be used in the next selection round.

The enriched DNA library obtained from SELEX round 8 was PCR amplified using the unmodified primers (Primer1 and Primer2) and cloned using the StrataClone PCR Cloning Kit according to the manufacturer's protocol. Positive colonies (white) were picked. DNA was prepared for sequencing by rolling-circle amplification with the TempliPhi Amplification kit using the protocol provided. The samples were sent for full service sequencing at the University of Calgary Core DNA Services (Calgary, AB, Canada). Sequences Hcy 8 and Hcy 10 (see Table 1) were present multiple times in the sequencing pool.

Selected sequences Hcy 8 and Hcy 10 were then synthesized with a 5′ fluorescein modifier, 6-FAM, followed by PAGE purification. Affinity chromatography assays were performed using magnetic beads covalently modified with either ≥98% L-homocysteine, L-methionine, L-cysteine and L-serine according to the instructions from the beads manufacturer (see ESI S1.2†). Varying concentrations of each Hcy 8 or Hcy 10 aptamer (1 nM–50 μM) were prepared in HB buffer and heated to 95 °C for 5 minutes and cooled to room temperature.

The magnetic beads assay was performed by incubating 100 μL of each aptamer sample with 2×10^8 L-Hcy-derivatized beads in HB buffer for 30 minutes with mild shaking. Non-binding DNA was removed with two 100 μL washes of HB buffer. Following the removal of non-binding DNA, the receiving tube was changed and the bound aptamers were eluted from the column by washing twice with 90 °C HB buffer. The DNA elution fractions were pooled and the fluorescence was measured (excitation wavelength 490 nm; emission wavelength 520 nm). The fluorescence intensity (Fig. S1†) was plotted *versus* the concentration of aptamer. The K_D was determined by minimizing the residual values between calculated and observed experimental fluorescence using the solver feature of Microsoft Excel.[24,25] The assay was performed in triplicate. For selectivity tests, the aptamer sequences were incubated with the L-cysteine, L-methionine or L-serine modified beads in the same way. One replicate experiment for each aptamer and counter target was performed.

Development of an aptamer–AuNP sensor

Gold nanoparticles were prepared using the Turkevich method[26] and characterized by UV-Visible spectrometry, displaying a $\lambda_{max} = 522$ nm (see ESI S1.3†). The assay was performed as previously described with modifications.[27] Nine samples of L-Hcy or L-cysteine in water were prepared at various concentrations ranging from 500 nM to 6 μM. 243 μL of each dilution of L-Hcy, L-cysteine or deionized water was added into microcentrifuge tubes containing 6 μL of Hcy 8 (10 μM in water). After a 30 minutes incubation period, 135 μL of the AuNP solution (11 nM) was added, vortexed briefly and allowed to react for 5 minutes. 72 μL of 0.25 M NaCl was then added to each microcentrifuge tube and allowed to incubate for a further 5 minutes. Each sample was then analyzed using UV-Visible spectrometry. The relative absorption ratio between 695 nm and 522 nm (A_{695}/A_{522}) was plotted against L-Hcy concentration. This experiment was performed in triplicate.

Human serum tests

The assay was performed as described in Section 2.4 using samples prepared in human serum as an alternative to deionized water. 10% human serum (from human male AB plasma) was prepared by diluting 1 : 10 in deionized water. The amino acid samples were dissolved in the 10% human serum samples. 500 μL of each concentration was filtered through Amicon-Ultra 0.5 mL 3 kDa centrifuge units. To determine the sensitivity of the test, 500 nM to 6 μM of L-Hcy was prepared in 10% human serum and tested with the AuNP assay. To assess the selectivity of the assay, 1.5 μM of L-cysteine, L-methionine and L-serine were prepared in 10% human serum and tested with the AuNP assay. All tests were performed in triplicate.

Results and discussion

Selection of homocysteine aptamers

The goals of this work were twofold: firstly to generate an aptamer for homocysteine, and secondly to take a previously non-selective gold nanoparticle detection platform and impart selectivity by including this aptamer as a recognition element. The first goal of this project was particularly challenging given

the small size of the target and its structural similarities with other amino acids.

As selection of aptamers towards small molecule targets often results in failure, careful consideration was made when choosing the selection conditions and materials. The primers chosen for this selection had been previously optimized for SELEX experiments to minimize 3′-mediated mis-priming and primer dimers.[28] A random region length of 60 nucleotides (nt) was chosen to strike a balance between two competing factors: shorter sequences are easier to synthesize and handle, while longer sequences allow for a greater structural complexity and possibly new functional motifs.[29] Small molecules must be tethered to a solid support matrix for selection; therefore, care was taken to confirm sufficient immobilization of homocysteine using the Ellman's test for free thiol groups. Negative selection steps with unmodified column material were performed with each SELEX round to ensure that sequences that displayed even weak binding or partial recognition to the matrix were removed from the selection library.[30] While a negative selection is typically only performed at the beginning of SELEX, integrating this additional step each round may reduce aptamer affinity derived from partial binding to the matrix and improve the likelihood that the final aptamers can effectively measure free target in solution. Additionally, counter-selections were performed to remove any sequences from the library that could bind to structurally similar molecules and therefore further enhance the selectivity of the aptamers. Counter-selections using L-cysteine and L-methionine were performed simultaneously in rounds 6 through 8 once significant enrichment of the library was observed. These amino acids were chosen as they are the only other amino acids with side chains containing a sulfur atom; methionine consisting of a thioether and cysteine containing a free sulfhydryl group. There are several other forms of homocysteine found in the body. For example, in its most common form, homocysteine is bound to the protein albumin by a disulfide linkage. Additionally, homocystine, which consists of two homocysteine molecules bound by a disulfide bridge and the homocysteine–cysteine disulfide (Hcy–Cys) can occur. The extra counter-selection steps to gain selectivity against these additional targets were not performed since total homocysteine is typically measured, which requires a general reduction step to convert all these forms to free homocysteine.[2]

To assess the enrichment of the library for target binding, as well as to determine when increased stringency could be applied to the selection, the percent binding of the DNA library was carefully monitored after each round using fluorescence and absorbance measurements (see Fig. 1). Beginning with round 6, the counter-selections with L-cysteine and L-methionine resulted in a reduction in the percentage of DNA eluted from the L-Hcy immobilized column. This was not surprising as L-cysteine, in particular, is structurally very similar to L-Hcy. With the drastic reduction in the percent recovery of the library (to <1%), it was expected that the resulting aptamers would have a significant preference in binding L-Hcy over the counter targets L-cysteine and L-methionine.

Elution of target-binding sequences by affinity with free ligand was employed in the first two rounds of selection, using

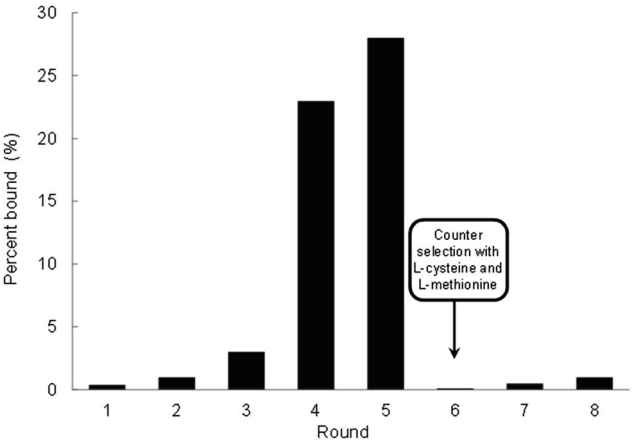

Fig. 1 Percent recovery of binding ssDNA to the L-Hcy immobilized sepharose after each selection round. Beginning at round 6, counter-selections with L-cysteine and L-methionine were performed prior to positive selection.

0.1 M L-Hcy. To ensure recovery of the highest affinity candidates, several other elution methods were employed in later rounds. In response to the subsequent library enrichment and improvement in binding affinity observed with each round, elution with high concentrations of urea and heating were tested. The most effective conditions for elution were found to be with 7 M urea at 90 °C over several minutes, allowing full recovery of putative aptamers (data not shown).

Measuring aptamer affinity and selectivity

After 8 rounds of selection, the pool was cloned and sequenced in order to assess the presence of any high affinity sequences. Two unique sequences, Hcy 8 and Hcy 10 (Table 1) emerged with high incidence in the sequencing results from the round 8 pool. Secondary structures of these sequences were predicted using RNAstructure[31] (see ESI Fig. S1†). Both structures appear to be simple stem loops with bulges. Work is ongoing to determine the putatitve binding pockets. Many K_D determination methods are ineffective for measuring aptamer binding to small molecules,[22] and as L-homocysteine is one of the smallest targets[32] ever used in an aptamer selection (M_W = 135.18 g mol^{-1}), determining the affinity of the selected aptamers was difficult. Affinity chromatography proved to be the most effective method to measure the affinity for the selected aptamers.

Aptamers selected using a solid support matrix may display affinity towards this matrix and/or the linker arm. Despite the efforts to eliminate any of this non-specific interaction by performing multiple negative selections, it is not always possible to avoid.[33] Therefore, binding affinity was tested using L-Hcy derivatized magnetic beads instead of the sepharose used in the selections. While there is a possibility for some non-specific interaction with the beads, it is far less likely since these beads were not used in the SELEX experiments. The measured K_D values of sequence Hcy 8 and Hcy 10 compared to counter targets are listed in Table 2 (a sample binding isotherm can be found in the ESI, see Fig. S2†). Notably, sequence Hcy 8 bound

Table 2 Dissociation constants (K_D) for sequences Hcy 8 and Hcy 10 obtained using affinity chromatography on target-immobilized magnetic beads ($n = 3$). Selectivity of Hcy 8 and 10 against similar amino acids is also noted (a sample binding isotherm can be found in the ESI†)

	Dissociation constant K_D (μM)	
Target	Hcy 8	Hcy 10
L-Hcy	0.6 ± 0.3	1.2 ± 0.7
L-Cysteine	22	91
L-Methionine	44	79

to the L-Hcy beads with a K_D in the low micromolar range (approx. 600 nM). With the exception of one L-arginine aptamer, most aptamers that bind to amino acids have K_D values in the high micromolar to millimolar range.[22] Additionally, homocysteine is one of the smallest DNA aptamer targets reported. Given the challenge of finding aptamers for such a small molecule, this is considered an excellent binding affinity. While both sequences bind to homocysteine beads with good affinity, Hcy 8 consistently resulted in the best K_D value, in the low micromolar range. However, the measured affinities of both sequences are suitable for detecting homocysteine within clinically relevant ranges (high micromolar).

Both aptamer sequences were further tested for selectivity against similar amino acid targets. Hcy 8 and Hcy 10 were found to bind selectively to L-Hcy, displaying K_D values that were orders of magnitude higher for L-cysteine and L-methionine (see Table 2). Even for small molecules, aptamers with K_D values in this range are considered very weak (>20 μM). The binding curves produced for these compounds did not reach a plateau for binding saturation, therefore it is likely that both sequences bind to cysteine or methionine with even lower affinity. This selectivity indicates that the Hcy aptamer is not merely interacting with the amino acid backbone, characteristic of all amino acids. The thiol group on the amino acid side chain is important in binding recognition. The observed selectivity emphasizes the power of *in vitro* selection, in particular counter selection techniques. For example, the selected RNA aptamer for theophylline was counter-selected against caffeine and as a result displayed a 10 000 times weaker binding affinity.[34] In our case, counter selections against methionine and cysteine proved successful.

Principle of the AuNP sensor

It is well known that ssDNA can be used to stabilize AuNPs against salt-induced aggregation by coiling around the AuNP (Fig. 2(i)). Several studies have shown that due to the strong gold–thiol interaction, cysteine and other thiol-containing molecules are able to displace ssDNA molecules adsorbed on AuNPs.[16] This leaves the AuNPs once again susceptible to salt-induced aggregation. Using these principles, several groups have developed AuNP biosensors capable of detecting thiol-based compounds at high concentrations (Fig. 2(ii)A).

Well-characterized aptamers for targets such as thrombin,[35] adenosine,[36] ochratoxin A,[37] and dopamine[27] have also been

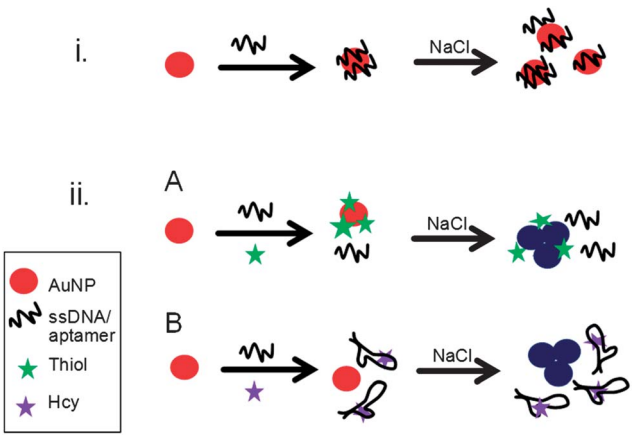

Fig. 2 Gold nanoparticle assay for target detection. (i) Single-stranded DNA (such as an aptamer) coils around the sodium citrate stabilized AuNPs surface, preventing salt-induced aggregation, therefore the colloid solution remains red after salt addition. (ii) Analyte detection strategies (A) upon addition of high concentrations of thiol-containing compounds, the gold–thiol interaction dominates, releasing the ssDNA from the AuNPs. Therefore, salt-induced aggregation returns and a blue-purple color is observed. (B) Upon addition of the aptamer's target, the aptamer folds into a rigid structure around the target, preventing the adsorption of the ssDNA on the AuNP surface. Therefore, salt-induced aggregation is possible and a blue-purple color is observed. While strategy A is effective for all thiols, strategy B should be selective for the aptamer's target, in this case, homocysteine.

used to protect AuNPs from aggregation. In the absence of target, the aptamer remains unstructured and behaves like ssDNA, coiling around the AuNPs. Upon the addition of target, the tested aptamers preferentially bind to the target, folding into a specific rigid tertiary structure.[27] This limits the ability of the aptamer to coil around the AuNPs; therefore, salt-induced aggregation occurs (Fig. 2 B). The ability of these aptamer–nanoparticle sensing schemes to detect low concentrations of their target provide precedent that a similar platform may be applied to newly developed aptamers. We therefore tested whether using the aptamers selected for L-Hcy, rather than random ssDNA, would result in the aptamer–target interaction dominating over the gold–thiol interaction, thus imparting homocysteine selectivity to the typical thiol AuNP biosensor (interaction B of Fig. 2).

Biosensing of homocysteine

Figure 3A shows the UV-visible results of the developed AuNP sensor under various conditions. The dispersed AuNPs have a characteristic absorbance at 522 nm, corresponding to a red color. Upon addition of salt, the nanoparticles aggregate and a shift in absorbance to 695 nm (blue/violet) is seen. In the absence of the aptamer, the addition of homocysteine (low or high concentrations) alone do not affect salt-induced aggregation. When the aptamer is added to the AuNPs, salt-induced aggregation is prevented, as indicated by the presence of the original 522 nm peak (and red color). With increasing concentrations of homocysteine, the gradual appearance of the peak at 695 nm occurs and a simultaneous decrease in the absorbance at 522 nm, results in a color change from red to blue (Fig. 3A

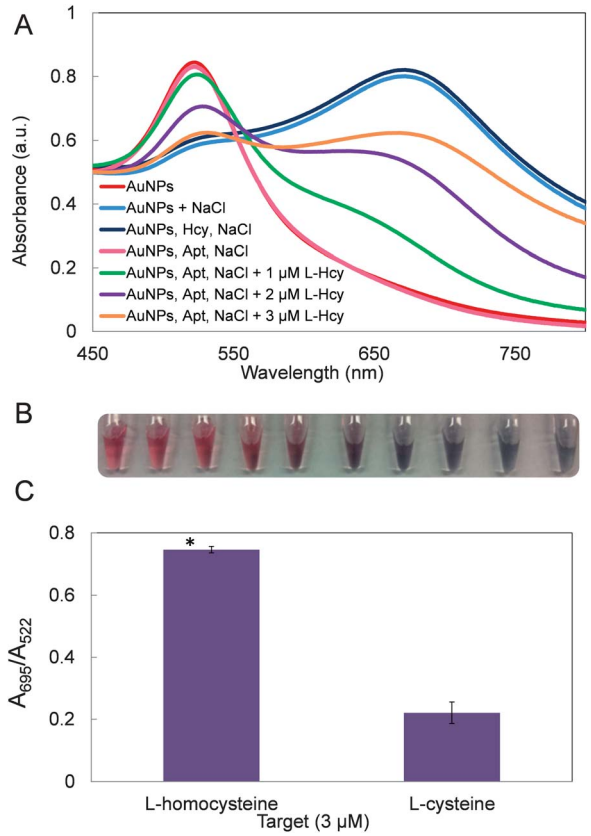

Fig. 3 AuNP–aptamer biosensor (A) UV-visible results under various conditions. The AuNPs have the characteristic absorbance at 522 nm, corresponding to a red AuNP color (red trace). Upon addition of NaCl, the nanoparticles aggregate and a shift in absorbance to 695 nm is seen (light blue trace). Homocysteine (Hcy) does not affect the salt-induced aggregation (dark blue trace) whereas the aptamer (Apt) prevents aggregation (pink trace). Upon addition of Hcy with the aptamer, aggregation is restored (green, purple and orange traces). (B) Using this biosensor, an increase in Hcy concentration (L to R: 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6 μM), results in aggregation and a change of color from red to blue. (C) Selectivity of our aptamer–AuNP biosensor comparing L-homocysteine and L-cysteine at 3 μM, nearly a 4 fold signal enhancement is observed ($p = 0.0006$).

and B). The response of the sensor was measured using the relative absorption ratio between 695 nm and 522 nm (A_{695}/A_{522}) plotted against L-Hcy concentration (Fig. 3C). We compared the response of our aptamer–AuNP biosensor to homocysteine and cysteine. At 3 μM target, a significant (4 fold) signal enhancement is observed for detection of homocysteine over cysteine (Fig. 3C). It is likely that within this region the aptamer–target interaction dominates, resulting in the selective detection of homocysteine compared to cysteine. At higher concentrations, greater than 5 μM, the signal enhancement is lost. At these concentrations, the gold–thiol interaction likely begins to dominate and the sensor loses its selectivity for homocysteine.

Homocysteine detection in human serum

A drawback of many rapid, selective and sensitive biosensors reported in the literature is that they are not conducive to direct use for measuring an analyte within a real-world sample.[38] Indeed, there is only one published report to date of an aptamer-based gold nanoparticle sensor that has been tested in a complex matrix such as serum.[39] While our sensor provides a rapid and selective means for homocysteine detection at clinically relevant concentrations, its utility in measuring homocysteine in human serum warranted investigation. High concentrations of protein and other macromolecules found in the serum are likely to interfere with detection. In particular, we found that even small concentrations of human serum (1 : 10 000 dilution) prevented the necessary salt-induced aggregation. It is likely that the large proteins, lipids and carbohydrates present in human serum[40] interact with and coat the nanoparticle surface, preventing aggregation.[41] Even high concentrations of NaCl (up to 10 μM) could not restore aggregation (data not shown). However, a rapid filtration step allowed removal of these large interfering macromolecules, without affecting homocysteine concentration. Following this step, a linear correlation between aggregation, measured as (A_{695}/A_{522}) and homocysteine concentration was found from 0.5 μM to 3 μM with a limit of detection (LOD) of 0.5 μM (3σ), see Fig. 4A, indicating that the biosensor is useful in direct analysis of homocysteine. The linear dynamic range of the sensor is comparable to that reported in immunoassay and HPLC formats; however, detection with our sensor is complete within

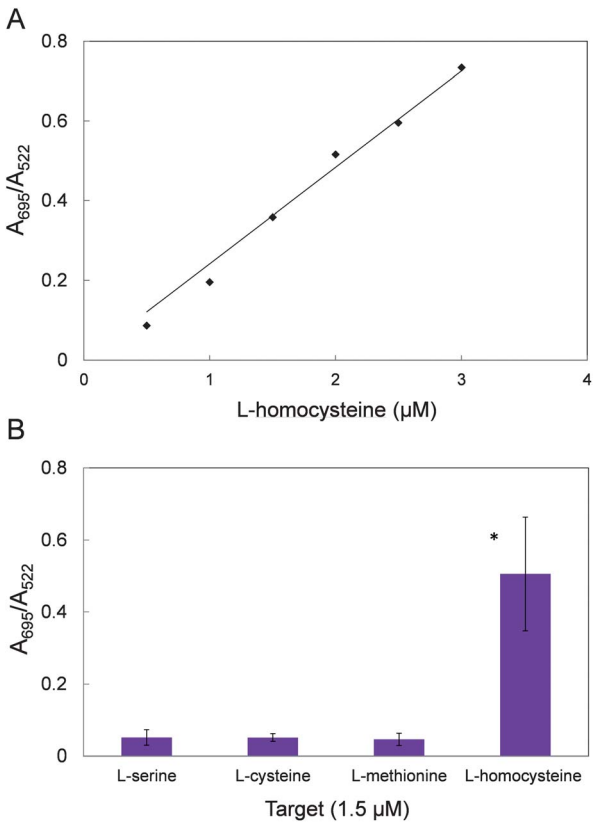

Fig. 4 AuNP–aptamer biosensor in 10% human serum (A) aptamer–AuNP biosensor response to L-homocysteine in 10% human serum. The sensor has a linear response between 0.5 μM and 3 μM ($y = 241\,741x$; $R^2 = 0.985$). (B) Selectivity of the aptamer-AuNP biosensor at 1.5 μM target concentration. The sensor is significantly more sensitive to L-homocysteine compared to the other amino acids ($p = 0.04$).

20 minutes whereas most immunoassay formats require over an hour.[10] This assay also modestly improves on sample preparation time compared to typical HPLC detection methods, where sample derivitization, clean-up and chromatography require 30–45 minutes.

Moreover, the use of an aptamer rather than an antibody reduces the costs and improves the shelf-life for the assay compared to ELISA methods. The cost of the materials for this AuNP test, for example the AuNPs and DNA, from common vendors such as Sigma Aldrich and Integrated DNA Technologies is approximately $150 for 100 samples. In contrast, ELISA kits for homocysteine detection are typically around $1000 for 100 assays. While the cost of consumables for HPLC detection-based methods are relatively low, the need for sophisticated equipment and highly trained personnel drastically increases the cost of these methods. While some other aptamer–AuNP sensors report improved LODs over what is seen here (0.5 μM *vs.* high nanomolar range), the sensitivity of this sensor is suitable for detecting homocysteine within clinically relevant ranges, *i.e.* after the filtration and 1 : 10 dilution, values above the upper reference limit of 12 μM would fall within the assay's linear range. This assay could therefore serve as a low cost, point of care screen that could inform whether further testing is warranted.

Furthermore, the majority of the aptamers used in these sensors use minimal aptamers of less than 58 nucleotides in length. Our aptamer, Hcy 8 is 102 nt in length. Chen *et al.*[16] found that longer ssDNA, even 30 nt in length compared to 24 nt in length, resulted in significant reduction in sensitivity of these biosensors. By performing truncation experiments, the minimal aptamer sequence of Hcy 8 may be identified. Incorporating the minimal aptamer into this assay may further improve the sensitivity of homocysteine detection.

Human serum also contains several other similar amino acids that may interfere with analysis, resulting in falsely high estimates of homocysteine concentration. We tested our sensor against several similar amino acids, namely cysteine, methionine and serine (see Fig. 4B). The concentration of target was chosen to reflect the expected concentration of homocysteine in samples, after the dilution step, at just above the upper reference limit. Not surprising, at 1.5 μM concentrations, minimal response was observed with methionine and serine. However, unlike previously developed AuNP biosensors for cysteine, our sensor also showed little response to cysteine compared to homocysteine (10 times signal enhancement with homocysteine). At higher concentrations (greater than 3 μM), the sensor remained selective against methionine and serine; however, the cysteine response was more similar to homocysteine (Fig. S3†). This further demonstrates that the aptamer–target interaction dominates at concentrations closer to the aptamer K_D (~1 μM) and at much higher concentrations the gold–thiol interaction out-competes the sensitive aptamer–target interaction.

Conclusions

While aptamer technology has existed for over two decades, challenges in aptamer development for small molecules have resulted in very few aptamers for these compounds.[42] As a result, developing new aptamers for medically-relevant small molecules is critical. We endeavoured to produce a viable aptamer biosensor for L-homocysteine. Eight rounds of SELEX were performed to obtain DNA sequences displaying high binding affinity to the target, L-homocysteine. Sequence Hcy 8 bound to L-Hcy with a KD in the low micromolar range, excellent affinity considering the small size and simple structure of the target. This sequence was also found to be selective, binding to the similar target L-methionine and L-cysteine with 200 and 100 times less affinity, respectively. With this full length sequence, we have developed a AuNP-based biosensor able to selectively detect low concentrations of L-homocysteine compared to other similar amino acids. The stability and selectivity of this sensor in serum was also confirmed, allowing for the rapid and direct detection of homocysteine in human serum samples. Further analysis will attempt to identify the minimal target-binding sequence in order to shorten the aptamer and improve binding while lowering its production cost. Adaptation of this sensor to a lateral flow assay and other practical colorimetric assays is ongoing.

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