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Non-enzymatic detection of urea using unmodified gold nanoparticles based aptasensor

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ABSTRACT

Biosensing nitrogenous compounds like urea is required to control the incidents of Economically Motivated Adulteration (EMA). In this study, we report the FluMag Systematic Evolution of Ligands by Exponential Enrichment (FluMag-SELEX) method to isolate a urea specific DNA aptamer with a dissociation constant (K_d) of 232 nM. The interaction of DNA aptamer with urea has been confirmed by affinity assay, CD analysis, melting curve analysis and truncation studies. Unlike other urea sensing methods reported so far, using this urea aptamer, we demonstrate a simple, 'non-enzymatic' easy-to-use, dual readout aptasensor that exploits unmodified gold nanoparticles (AuNPs) to transduce the signals of aptamer binding to urea in terms of intrinsic fluorescence differences and color changes simultaneously. This method is free from complicated sample processing and labeling steps. The urea aptasensor displays high selectivity for urea and is free from interference from common milk adulterants. The developed aptasensor reliably detects urea adulteration in milk. The response signals linearly correlate with the increasing concentrations of urea in milk ranging from 20 mM to 150 mM with detection limit of 20 mM. We also show that this aptasensor can also be used as a simple fluorescence based "turn-on" sensor. The results obtained in this study are comparable to the commercial urease based detection methods.

1. Introduction

Economically Motivated Adulteration (EMA) incidents in food and dairy products have reached alarming proportions globally. Food adulteration has caused several cases of serious illness amongst infants, adults and animals (Xin and Stone, 2008; Trivedi et al., 2009; Kobayashi et al., 2010; FSSAI, 2012). Even though, the exact proportions of such food fraud cannot be conclusively estimated, according to the Grocery Manufacturers Association (GMA) of the United States of America, the adulterated food products cost the industry 10–15 billion dollars per annum (GMA, 2010). Thus, it is of paramount importance to identify EMA events quickly to minimize the ill health effects and economic losses. Amongst the food commodities, milk is an important item in international commerce. An escalating population with increasing demand for milk invariably surpasses the available sources of supply which leads to milk adulteration mainly in developing countries. Food and Drug Administration (FDA) of United States of America has categorized urea amongst chemicals most likely be used in protein adulteration (Paradkar et al., 2000; Sadat et al., 2006; Sharma et al., 2008; FDA, 2011). In milk, urea is allegedly added with an aim of increasing solid not fat value (SNF) and nitrogen content to mislead conventional testing of protein content. However, adulteration of milk with cheaper and toxic chemicals poses health hazards. Beyond a certain limit (70 mg/dL in milk), urea has deleterious effects on human health as it can cause indigestion, renal failure, urinary tract obstruction, gastrointestinal bleeding and cancer (Nikoleli et al., 2010). Further, in bovines, high concentration of urea impairs the reproductive performance and leads to pathological problems (Butler, 1998). The importance of urea in biological fluids, the environment and EMA incidents have motivated researchers to develop biosensors for urea. The majority of the extant urea biosensors are based on urease enzyme. Urease catalyzes hydrolysis of urea to generate ammonia and carbon dioxide; which are measured directly or indirectly using conductimetric, amperometric, potentiometric, and cyclic voltameteric methods etc (Francis et al., 2002; Singh et al., 2008; Srivastava et al., 2012). However, these methods suffer from issues like complicated sample pre-treatment, poor urease stability, vulnerability to urease inhibitors, lack of reproducibility, complicated design and requirement of sophisticated instruments. A facile, reliable, quick and robust non-enzymatic aptasensor for urea

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detection can go a long way to address the problem of EMA of urea in milk.

AuNPs have been the choice of researchers for developing nanosensors. Literature survey reveals that most of the urea biosensors utilize urease enzymes in combination with different transducers. Recently, urease functionalized AuNPs based biosensors have been developed (Nair et al., 2013; Rajesh et al., 2014). However, to the best of our knowledge, there are limited methods for non-enzymatic urea detection which either require either hazardous chemicals or are time consuming. Moreover, these methods have not been developed for quantitative urea estimation.

Nucleic acid aptamers have gained acceptance as a category of functional nucleic acids that have shown promise as molecular recognition elements in biosensing applications. Aptamers rival antibodies in terms of affinity, specificity and stability (Navani and Li, 2006; Cho et al., 2009). Unlike antibodies, aptamers are selected in-vitro by a process known as SELEX (Ellington and Szostak, 1990; Tuerk and Gold, 1990; Robertson and Joyce, 1990), and thus, it is possible to exclusively tailor the selection strategies to generate aptamers targeting small molecules, non-antigenic targets and toxins (Xia et al., 2010; Wang et al., 2011; Kimoto et al., 2013; Alsager et al., 2014; Fan et al., 2015). Recently aptamers have also been proposed as replacement for antibodies in enzyme linked aptasorbent assays (Toh et al., 2015). However, no research has been carried out using aptasensor for detection of urea.

AuNPs have been exploited as transducers in developing colorimetric or fluorescence aptasensors due to their properties such as biocompatibility, chemical stability, strong distance dependent optical properties, fluorescence quenching ability and high extinction coefficient (Dubertret et al., 2001; Yeh et al., 2012). However, aptamer or AuNPs functionalization is costly, time consuming, laborious and may weaken the binding affinity between the target and aptamer and hence, influence the sensitivity for detection. Due to this, unmodified gold nanoparticles based methods have been preferred for developing optical and fluorescence aptasensors. Herein, we report the isolation of a urea specific DNA aptamer, characterization of its interaction with the target and its application for developing an easy-to-use, label-free, non-enzymatic urea aptasensor. The proposed aptasensing method obviates the tedious modification steps, relies on a simple sample processing and can be adapted for carrying out the 'in field' measurements.

2. Materials and methods

2.1. Chemicals and materials

Urea, L-glycine, L-alanine, L-tyrosine, L-phenyl alanine, L-glutamate, sodium chloride, glucose, sodium bicarbonate and 1-ethyl-3-3-dimethylaminopropyl carbodiimide (EDC) were purchased from Himedia (India). Gold (III) chloride ($HAuCl_4 \cdot H_2O$) was purchased from Sigma Aldrich, USA. Biomag®Plus carboxyl beads (20 mg/mL) was bought from PolySciences Inc. (USA). Polymerase Chain Reaction (PCR) reagents and InsTAclone™ PCR Cloning Kit were procured from Fermentas, Thermo Fisher Scientific (USA). Custom synthesis of oligonucletides was performed by Integrated DNA Technologies, Inc. (USA). The random DNA library (RDL) 5′-GTCTTGACTAGTTACGCC-N_{40}-TCATTCAGTTGGCGCCTC-3′, forward primer (DrF); 5′-FITC-GTCTTGACTAGT′TACGCC-3′, reverse primer (DrR); 5′-GAGGCGCCAACTGAATGrA-3 were obtained from Integrated DNA Technologies Inc. (USA). All other chemicals used were of analytical grade and were used without purification.

2.2. Immobilization of urea onto magnetic beads

Biomag®Plus carboxyl beads were used as immobilization support and handled according to manufacturer's instructions. Aliquots of 20 mg/mL carboxyl magnetic beads were activated using 1.6 mg/mL of EDC and incubated for 1 h with mild shaking at room temperature (RT). A final concentration of 100 mg/mL urea was mixed with carboxyl activated magnetic beads and incubated overnight at RT with mild shaking. After completing the reaction, the concentration of unbound urea in supernatant was estimated by plotting the standard curve using diacetyl-monoxime method (Ormsby, 1942). After immobilization, urea coupled magnetic beads (urea-CMB) were resuspended and stored in selection buffer. EDC activated magnetic beads without urea were used for negative selection.

2.3. In-vitro SELEX procedure

Aptamers for urea were selected from a random pool of 10^{15} single stranded DNA (ssDNA) molecules using FluMag-SELEX. For the first round, ssDNA library was denatured by heating for 10 min at 92 °C and allowed to cool down at RT for 15 min before mixing it with urea-CMB. The annealed ssDNA library was incubated with urea-CMB (2 mg) resuspended in binding buffer (250 mM NaCl, 100 mM Tris–HCl, 25 mM $MgCl_2$, 5 mM KCl, 0.02% Tween 20, 0.1 μg yeast t-RNA, 20 μg/mL BSA, pH 7.5) for 45 min with mild shaking. The incubated solution was washed twice with 500 μL of selection buffer and urea-CMB bound DNA sequences were eluted with 100 μL of elution buffer (selection buffer with 6 mM urea, pH 7.5). After elution, PCR was performed to enrich the population. The PCR product was precipitated using 2.5 volumes of ethanol (Merck, molecular grade, India) and kept at −20 °C for 2 h. After incubation, the samples were centrifuged at 13,000 rpm for 30 min at 4 °C. Ethanol was gently removed and the pellet was washed twice with 70% ethanol. The samples were centrifuged at 13,000 rpm for 30 min at 4 °C, subsequently ethanol was drained and the pellet was air dried. For every 100 μL of PCR product, the dried pellet was resuspended in 250 μL of 0.25 N NaOH and incubated for 10 min at 92 °C to cleave the ribo linkage thus facilitating easy separation of ssDNA. After heating, the samples were snap chilled on ice for 5 min, followed by addition of 25 μL of 3 M sodium acetate pH 5.2 and precipitated with 2.5 volume of absolute ethanol by placing the samples at -20 °C for 2 h. DNA was recovered by centrifugation at 13,000 rpm for 30 min at 4 °C, followed by two washes of 70% ethanol. The pellet was dried and resuspended in 20 μL of nuclease free water. The product was rendered single stranded using 10% denaturing urea Polyacrylamide Gel Electrophoresis (PAGE). These ssDNAs further act as the input sequences for next round of selection. Initial four rounds of SELEX were performed against urea-CMB and counter selection step using unmodified carboxyl magnetic beads was introduced to eliminate the nonspecific binding of ssDNAs. After 10 rounds of selection, each round of the selected population were enriched by PCR using FITC-labeled forward primer and appraised for their binding affinity towards urea by measuring the fluorescence intensities. Based on the preliminary binding study the best pool of DNA sequences were cloned in pTZ57R vector using InsT/A Cloning kit (Fermentas, Europe) as per manufecturer's instrutions and were sequenced. The sequences obtained were aligned based on homology by BioEdit software (Hall, 1999) and divided into different categories.

2.4. Screening of urea binding aptamer by fluorescence based assay

Binding affinity assay for different FITC-labeled putative aptamer was performed by fluorescence based assay. The assay was

performed by incubating each aptamer candidates (125 nM) with 2.0 mg of urea-CMB for 45 min in 750 μL of binding buffer under mild shaking conditions. Subsequently, the unbound candidates were removed by washing. The bound aptamer candidates were eluted with 500 μL of elution buffer. The amount of eluted aptamer was determined by measuring the fluorescence intensity at 525 nm with an excitation of 490 nm. A FITC-labeled naive library of ssDNAs (RDL) at the same concentration (125 nM) was taken as the control.

2.5. Determination of dissociation constant (K_d)

In order to determine the binding affinity of the selected aptamer (U38), a fixed concentration of urea-CMB (2.0 mg) was incubated with increasing concentration of annealed FITC-labeled U38 aptamer (0–350 nM) under mild shaking conditions for 30 min at RT. The unbound aptamer was removed by two gentle washes with selection buffer. The concentration of the eluted aptamer was estimated by measuring the fluorescence intensity and saturation curve was plotted. The dissociation constant (K_d) value was calculated by employing the nonlinear regression analysis using GraphPad Prism software (version 6.0, San Diego, CA, USA).

2.6. Circular dichroism (CD) measurements

The CD spectra of free U38 aptamer, U38 aptamer with 100 mM urea and U38 aptamer with a non-target molecule (100 mM glycine) at a final concentration of 5 μM in a selection buffer were recorded on Applied Photophysics spectropolarimeter interfaced with a computer and equipped with a Peltier temperature device. The spectra were recorded in wavelength range of 200–320 nm; the data gathered were the average of three scans. The scan of the selection buffer alone recorded at room temperature was subtracted from the spectra of above test samples. Melting curve was monitored by CD signals at 276 nm for U38 aptamer alone and in presence of urea from 20 to 100 °C and the graph was plotted for changes in ellipticity (θ) versus temperature.

2.7. Preparation of milk sample

Milk samples (200 μL) spiked with increasing concentrations of urea (0–250 mM) were precipitated using 4 volume of methanol followed by vortexing for 5 min to eliminate the interfering proteins. Subsequently, these samples were centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was collected in fresh tubes and used for detection of urea. The control sample was the same milk sample without any added urea. All the samples were freshly prepared prior to its use.

2.8. Synthesis and characterization of AuNPs

AuNPs were prepared by citrate reduction method as previously described in literature (Storhoff et al., 1998). Before starting with the preparation of AuNPs, glasswares required for preparations were treated with freshly prepared aqua regia (3HCl:1HNO_3) for 1 h. After aqua regia treatment, the glassware were thoroughly rinsed with milliQ water and then oven dried for 2–3 h to remove any traces of acids. For preparation of AuNPs, 50 mL of 1 mM gold (III) chloride was heated in a two neck flask under constant stirring till condensation appeared on the neck of the flask. To the boiling solution of gold salt, 5 mL of 38.8 mM of sodium tri-citrate was added drop wise. A gradual color change from yellow-colorless-purple-wine red was seen within 5 min which indicated the formation of AuNPs. After wine red color was attained, the flask was removed from the hot plate and kept on another magnetic stirrer maintained at RT and was stirred continuously in order to bring down the temperature of the solution to RT. The AuNPs thus prepared were characterized by UV–visible spectroscopy, dynamic light scattering (DLS), transmission electron microscopy (TEM) and atomic force microscopy (AFM).

2.9. Sensing of urea using unmodified AuNPs–aptamer approach

Three different aptamer candidates (U7, U34, U38) and a control random sequence at 250 nM concentration were incubated with AuNPs (2.75 nM) for 2 min at RT. Subsequently, 50 μL of processed milk samples spiked with 100 mM urea was added to each test sample and further incubated for 2 min at RT. After incubation, 1.5 μL of 5 M NaCl (250 mM) was added to observe the visible change in color. Processed milk without urea serve as a neagative control.

2.10. Secondary structural analysis and truncation studies of aptamer

Urea specific full length U38 (FL U38) aptamer (80 bp) was shortened by truncating either forward (FT U38) or reverse (RT U38) or both the primer (FRT U38) domains of the aptamer sequence. The truncated sequences were commercially synthesized, HPLC purified and assessed for their performance to detect urea in milk using same method as mentioned for full length U38 (FL U38) aptasensor, with appropriate control for each oligonucleotide. Secondary structural analysis of all U38 aptamer variants (FL U38, FT U38, RT U38 and FRT U38) was performed using M-fold software with default settings and predefined salt conditions as used in SELEX (Zuker, 2003).

2.11. Specificity and interference studies

In order to evaluate the specificity of the U38 aptamer towards urea, structurally related small molecules such as glycine, alanine, serine and tyrosine were individually examined for their ability to bind the U38 aptamer. Milk samples spiked with 100 mM of each of the above small molecule in the presence of urea were processed as aforementioned. From each sample, 50 μL volume of test sample was incubated with U38 aptamer (250 nM) with subsequent addition of salt and was observed for color change. Similarly, interfering components of synthetic milk such as sodium chloride (500 μM); glucose (600 μM); sodium bicarbonate (20 mM) and tween-20 (1%; v/v) were also tested. Normal milk and urea (100 mM) spiked milk were considered as negative and positive controls, respectively.

2.12. Limit of detection (LOD)

To evaluate the detection limit of aptamer–AuNPs based sensor, U38 aptamer (250 nM) was incubated with AuNPs (2.75 nM) for 2 min at RT. Subsequently, 50 μL of processed milk samples spiked with different concentrations of urea (0–250 mM) were added and further incubated for 2 min at RT. After incubation, addition of 1.5 μL of 5 M NaCl (250 mM) was followed by visual observation, UV–visible (400–750 nm) and fluorescence emission spectra of AuNPs were recorded immediately by exciting at 350 nm and scanned up to 700 nm. The limit of detection (LOD) was calculated by using the equation $LOD = LOB + 1.645 \times SD_{(low\ concentration\ sample)}$ (Armbruster and Pry, 2008). The limit of blank (LOB) was estimated by measuring replicates of blank sample and calculating the mean result and the standard deviation (SD) and computed using the equation $LOB = Mean + 1.645 \times SD$.

2.13. Fluorescence recovery assay

FITC labeled U38 aptamer (250 nM) was mixed with AuNPs (2.75 nM) and incubated for 2 min. Following this, 50 µL of the processed milk with urea (100 mM) was added to the above solution. A recovered fluorescence reading was measured at zero and 10 min time intervals, with excitation at 490 nm and emission at 525 nm. The fluorescence intensity of the FITC aptamer alone and FITC aptamer with AuNPs without any urea were used as the standard for establishing maximum and minimum fluorescence values. Milk spiked with 100 mM glycine instead of urea was used as a control. In another set of control experiment, 100 mM urea spiked milk was treated with 100 unit of Jack bean urease (Sigma Aldrich, USA) and its effect on fluorescence recovery was measured.

3. Results and discussion

3.1. Principles of the non-enzymatic urea aptasensor

A urea specific aptamer is prerequisite for developing a urea aptasensor. Since there was no urea specific aptamer, we carried out in-vitro selection and isolated a ssDNA aptamer against urea by affinity based FluMag-SELEX method (Stoltenburg et al., 2005). To adapt this aptamer into a urea aptasensor, we designed a simple "unmodified AuNPs" based strategy with dual readout signals; color and intrinsic fluorescence. In addition, we exploited the fluorescence quenching properties of AuNPs to develop a "turn-on" urea aptasensor. The working principles of aptamer based assays developed in this report are illustrated in Fig. 1. In case of aptamer–AuNPs assay, the aptamers get adsorbed on the AuNPs surface and protect the AuNPs from salt induced aggregation. Upon addition of urea spiked sample, aptamers, due to their affinity towards urea, leave AuNPs rendering AuNPs susceptible to salt induced aggregation. This aggregation is reflected in terms of change in color from wine red to purple (Fig. 1A). In case of fluorescence quenching assay, fluorescence of FITC-labeled aptamer is efficiently quenched in the vicinity of AuNPs through fluorescence resonance energy transfer (FRET). However, upon addition of urea spiked sample to FITC–aptamer–AuNPs complex, aptamer gets dissociated from the surface of AuNPs, restoring the fluorescence of FITC leading to a "turn-on" aptasensor (Fig. 1B).

3.2. Selection of aptamers against urea by SELEX

To start *in-vitro* selection process, urea was coupled to carboxyl modified magnetic beads using EDC chemistry according to manufacturer's instructions. Efficiency of urea coupling to carboxyl modified magnetic was calculated to be 61% (data not shown). Further, a FluMag-SELEX process was employed to generate urea specific aptamers from a random library of 10^{14}–10^{15} ssDNA molecules. A total of 10 rounds of selection were performed against urea-CMB. After 10th round, enriched population of each round were screened for their binding towards urea-CMB as compared to the naive ssDNA library (RDL) by performing fluorescence assay. The fluorescence data of round 4, 7 and 8 populations indicated that there was a monotonic increase in binding towards urea-CMB (Fig. S1). However, after the eighth round of SELEX, a decline in the fluorescence signal was observed, suggesting that no further enrichment of urea specific DNA sequences occurred. Following this, round eight enriched DNA pool was cloned and sequenced. The alignment of selected sequences yielded eight different classes based on the sequence similarity (Fig. S2). The affinity of one representative sequence from each category was further assessed for binding to urea-CMB using fluorescence assay. The amount of putative aptamer bound to urea-CMB was measured on the basis of the fluorescence signal. Out of eight putative aptamer sequences tested, sequence U38 displayed the best binding affinity to urea while naive DNA library (RDL) and other sequences showed low or negligible binding (Fig. S3). Based on the fluorometry data, U38 was selected for further study.

3.3. Dissociation constant (K_d) measurement

To evaluate the binding strength of U38 aptamer towards urea, dissociation constant (K_d) value was determined using the fluorescence based assay. The fluorescence values of the eluted aptamer DNA were fitted in the saturation curve using the nonlinear regression. The K_d of aptamer was calculated to be 238 nM demonstrating that the U38 aptamer exhibited high affinity comparable to other reported aptamers against small molecules (Win et al., 2006; Kim et al., 2010; Song et al., 2011; Mehta et al., 2012) (Fig. S4).

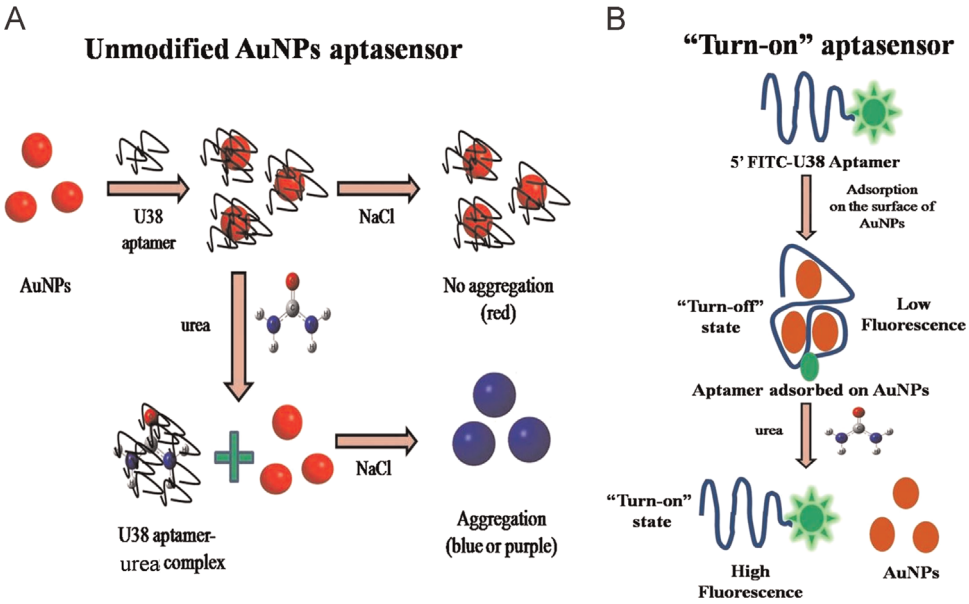


Fig. 1. Schematic representation of aptamer–AuNPs based methods for the detection of urea. (A) Unmodified AuNPs–aptasensor and (B) "turn-on" aptasensor.

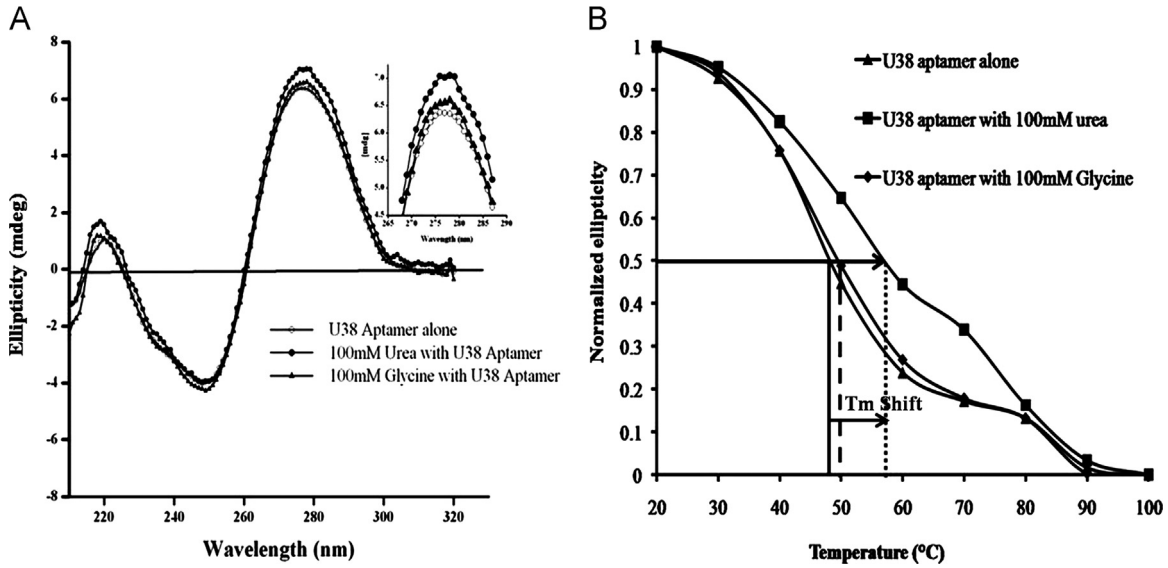


Fig. 2. Biophysical characterization of U38 aptamer interaction with urea. (A) Circular dichroism spectrum of U38 aptamer (5 μM) (o) alone, (•) in presence of 100 mM urea, (▲) in presence of 100 mM glycine (negative control); (B) The melting curves of (▲) U38 aptamer alone, (■) U38 aptamer with 100 mM urea and (♦) U38 aptamer with glycine followed at 276 nm using CD spectroscopy.

3.4. Structural analysis by circular dichroism (CD) spectroscopy

To confirm the interaction of U38 aptamer with urea, the changes in conformation of U38 aptamer were investigated before and after the addition of urea by monitoring the circular dichroism (CD) signals. The CD spectrum of the free U38 aptamer displayed positive maxima peak at 276 nm and a negative minima peak at 248 nm which are characteristic of DNA in B-form (Kypr et al., 2009). Upon addition of 100 mM urea to the U38 aptamer, we observed a significant increase in the ellipticity (θ) of the positive peak at around 276 nm (Fig. 2A). The increase in ellipticity attributes to a strong interaction of urea with U38 aptamer thereby stabilizing the secondary structure. This observation is also supported by the melting curve data which shows an increase of melting temperature (T_m) value of U38 aptamer by 8.5 °C in the presence of urea (Fig. 2B). The presence of glycine (a structural analog) did not alter the CD signal and T_m of U38 aptamer significantly. These spectral changes indicated that the conformational change in U38 aptamer was urea specific.

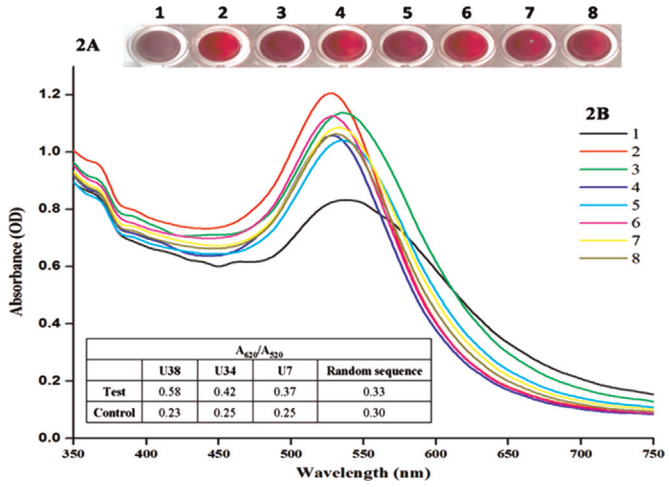
3.5. Sensing of urea using unmodified aptamer–AuNPs approach

To test if the aptasensor is workable in a realistic sample we optimized the detection method for milk. Milk is a complex biological fluid containing over 100,000 different molecular species (Wong and Nostrand, 1998). This complexity of milk makes the detection of individual species a difficult task. For the detection of urea in milk, a simple and rapid processing method was developed for eliminating interfering species like proteins and lipids. Urea spiked milk samples were precipitated by adding methanol, and clear supernatant was collected followed by centrifugation. This, supernatant was further used for detection purposes. This method of sample preparation offers advantages (simple; as it avoids multiple pre-treatment steps, requires less time for sample treatment, and does not rely on hazardous chemicals *viz.* chloroform, trichloroacetic acid and hydrochloric acid etc) over other methods used in AuNPs based approach for small molecule detection (Liang et al., 2011; Song et al., 2012).

In combination, AuNPs and aptamers have been favored to develop sensitive biosensors for detection of various analytes (Zhao et al., 2007; Zhang et al., 2010; Mei et al., 2013). Urease based urea detection methods are vulnerable due to urease inhibition by heavy metals (Zaborska et al., 2004). In fact, heavy metal based urease inhibition has specifically been used for detection of heavy metals (Hu and Jang, 2011; Syshchyk et al., 2015). To present an alternative, in the present study, we applied unmodified gold nanoparticles–aptamer based method to detect urea in processed milk. For the aptasensor development, AuNPs were prepared by citrate reduction method and characterized using biophysical techniques (Fig. S5). An aptamer–AuNPs based colorimetric sensor was developed using three best binding putative aptamer sequences (U7, U34, U38) and a control DNA (random DNA sequence). Each aptamer was incubated with unmodified AuNPs so as to adsorb them on the surface of AuNPs. Subsequently, the addition of processed urea spiked milk resulted in binding of the aptamer to urea by virtue of ligand induced conformational changes. This binding event leaves the AuNPs vulnerable to high salt induced aggregation, which is observed by a change in color from red to purple or blue. Fig. 3A shows the AuNPs color change in the presence of urea (test) for selected aptamers (U38, U34 and U7) along with their respective controls. The AuNPs adsorbed with a control random DNA oligonucleotide exhibited no such change on the addition of processed urea spiked milk. TEM images of aptamer–AuNPs also confirmed aggregation in the presence of urea (Fig. S6A and B). The absorption spectra of all the samples were taken and were converted into quantitative data. The absorption spectra showed a dip in the intensity at 520 nm and a clear red shift was observed for urea spiked samples. The intensity of the color change was also measured by calculating the absorbance ratio at 620/520 nm which corresponds to the degree of AuNPs aggregation (Fig. 3B). These results indicate that U38 aptamer displayed maximum binding affinity for urea. This observation was also supported by the fluorescence binding assay (Fig. S3).

3.6. M-fold analysis and truncation studies

In order to ascertain the involvement of primer binding regions of the U38 aptamer in ligand recognition, sequential truncation of these regions was done and the effect on performance of aptasensor was studied. The truncation of the reverse primer region in aptamer (RT-U38) marginally affected the performance of the



A_{620}/A_{520}

	U38	U34	U7	Random sequence
Test	0.58	0.42	0.37	0.33
Control	0.23	0.25	0.25	0.30

Fig. 3. Colorimetric detection to assess the performance of different aptamer candidates. (A) color images of (1) U38-T, (2) U38-C, (3) U34-T, (4) U34-C, (5) U7-T, (6) U7-C, (7) random sequence-T, (8) random sequence-C; (B) UV–visible spectra of all the samples tested. A620/A520 nm ratio gives a direct correlation of the color changes observed in the presence of urea. Test (T)=AuNPs+aptamer candidate+processed urea spiked milk+NaCl; Control (C)=processed normal milk without urea.

aptasensor. However, there was a dramatic decrease in the performance of the aptasensor with respect to the absorbance ratio at 620/520 nm when the forward primer region in aptamer was truncated (FT-U38). The M-fold analysis of truncated and full length aptamers reveals that the hairpins present in forward primer of U38 aptamer (extending from nucleotides 26-43; region 1 along with 11-23 nucleotides; region 2) are likely to be involved in interaction with urea (Fig. S7).

3.7. Specificity and interference studies

The specificity of the U38 aptamer was evaluated by performing cross reactivity studies using structurally related molecules to urea (*viz.* glycine, alanine, serine and tyrosine). AuNPs get aggregated only in the presence of urea spiked samples, while structural analogs failed to induce aggregation (Fig. 4A). These results demonstrated that U38 aptamer displays specific affinity towards urea.

One of the drawbacks of the available sensors based on AuNPs aggregation is the interference by other analytes which compromises with the usefulness of a sensor. The robustness of the aptasensor was tested by performing the assay in the presence of common milk adulterants (*viz.* sodium chloride, glucose, sodium bicarbonate and tween-20). No considerable color change was observed in the presence of different interfering agents in the processed milk. However, urea spiked milk induced aggregation with color change indicating high selectivity of U38 aptamer for urea (Fig. 4B). The above assay showed that the urea specific aptamer has insignificant interference from common milk adulterants.

3.8. Limit of detection (LOD)

Further, the LOD of the aptasensor was determined by spiking milk with increasing concentration of urea (0–250 mM). When urea spiked sample was added to aptamer–AuNPs complex, concentration dependent aggregation was observed (Fig. 5A). The aggregation was monitored by change in the absorbance (Fig. 5B) and intrinsic fluorescence values of the AuNPs (Fig. 5C). A linear correlation was obtained between A620/A520 nm and urea concentration ranging from 20 to 150 mM with regression value (R^2) of 0.995 (Fig. 5B; inset). From these comparisons, it can be inferred that the detection limit of the present method for urea is 20 mM. The LOD for urea in this study falls within the seasonal variation of urea which is naturally secreted in the milk of bovines (Carlsson et al., 1995). Further, we also confirmed the LOD using intrinsic fluorescence of AuNPs under the same experimental conditions and plotted it as a function of urea concentration. The results of both assays were in agreement showing linear correlation from 20 to 150 mM with R^2 value of 0.991 (Fig. 5C; inset). The urea aptasensor does not detect the natural levels of urea in milk, however, in cases of adulteration where the urea levels are spiked 10–20 times of the natural levels (upto 1400 mg/dL; Bansal and Bansal, 1997); the sensor works efficiently over a broad range so as to detect the levels of EMA of urea in milk.

3.9. Comparison of urea aptasensor with commercially available kits

The analytical features of the urea aptasensor were compared and validated with two urease based commercially available kits (ERBA Urea Enzymatic kit from Transasia Biomedicals, India and ROBOniK® prietest Urea Enzymatic colorimetric assay kit from ROBOniK, India). As shown in Fig. S8 the parameters like dynamic range, repeatability and analysis time of the method are comparable to the commercially available kits. The results suggest that the urea aptasensor is robust and may be suitable for on-site urea detection.

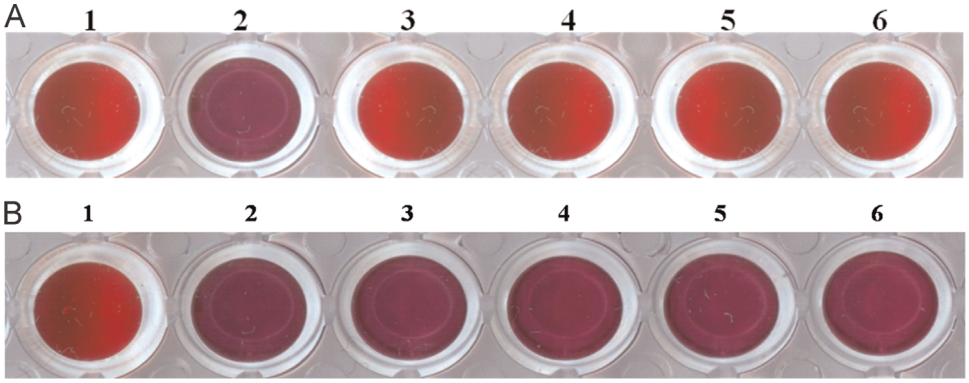


Fig. 4. Specificity and interference studies of urea aptasensor. (A) Specificity: Color changes in aptamer–AuNPs system in (1) absence of urea, (2) presence of 100 mM urea, presence of (3) 100 mM alanine, (4) 100 mM glycine, (5) 100 mM serine and (6) 100 mM tyrosine upon addition of NaCl; (B) Interference: Color changes in aptamer–AuNPs system in (1) absence of urea, (2) presence of 100 mM urea, (3) 100 mM urea+500 M NaCl, (4) 100 mM urea+600 μM glucose, (5) 100 mM urea+20 mM sodium bicarbonate, (6) 100 mM urea+1% tween-20 upon addition of NaCl.

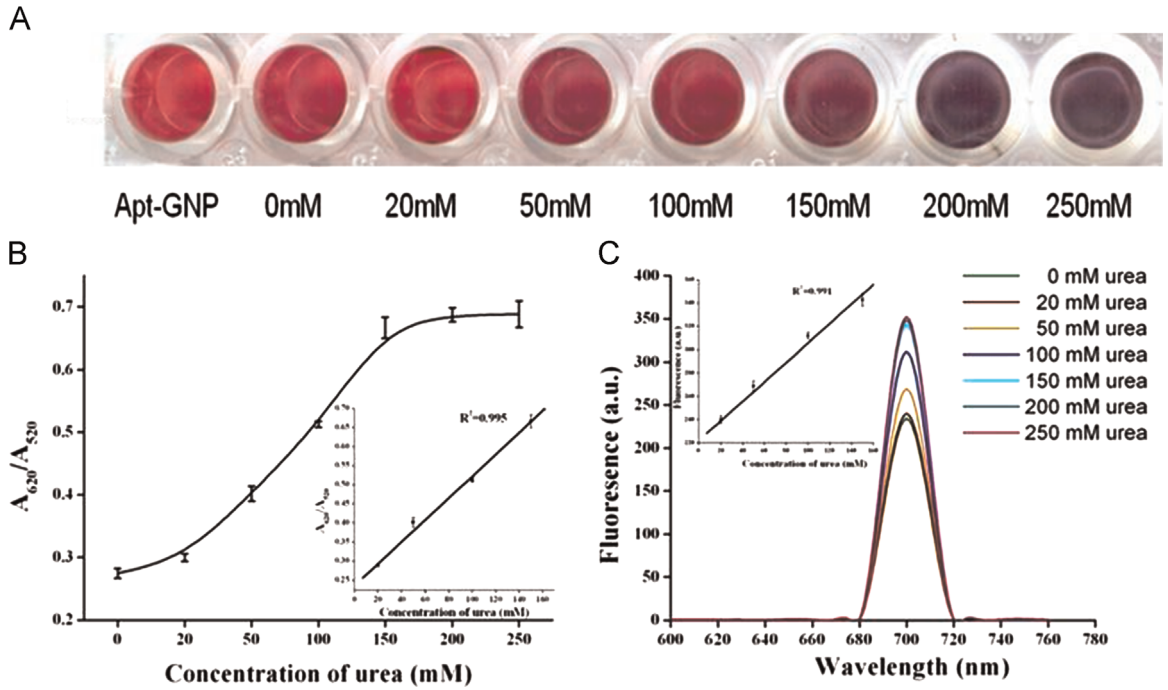


Fig. 5. (A) Incremental color change observed for aptasensor with increasing concentration of urea (0–250 mM); (B) plot of urea concentration vs absorbance ratio (620/520 nm) for quantification of urea in milk. The linearly fitted absorption spectra of the AuNPs solution containing U38 aptamer versus the concentration of urea (0–250 mM) (inset); (C) fluorescence spectra of aptasensor in the presence of U38 aptamer and increasing concentrations of urea (0–250 mM). The inset indicates dose dependent changes in fluorescence intensity of the AuNPs solution with respect to increasing concentration of urea.

3.10. Fluorescence recovery assay

Fluorescence based sensors have been developed in "turn-on" and "turn-off" modes (Wang et al., 2011). Sensors based on the "turn-off" mode are considered as less sensitive than those based on the "turn-on" mode (Xiao et al., 2006; Li and Ho, 2008). We exploited the specificity of the U38 aptamer and fluorescence quenching property of AuNPs to develop fluorescence based "turn-on" method for urea detection. For this, FITC-labeled U38 aptamer was adsorbed on AuNPs. The adsorption led to the quenching of fluorescence of FITC. Upon urea binding, a conformational change in FITC-U38 aptamer led to the displacement of the aptamer from the AuNPs surface. As a consequence, the distance between the fluorophore and the AuNPs increased, thereby enhancing the fluorescence signal of FITC-U38 aptamer (Fig. 6). The specificity of the aptamer was tested further by evaluating the fluorescence response in presence of glycine as negative control. The fluorescence recovery in presence of glycine was insignificant (15.65%) as compared to urea (85.8%). To further demonstrate the specificity of the aptamer, the milk spiked with urea was treated for 20 min with urease enzyme followed by the addition to FITC-U38 aptamer–AuNPs complex. This led to the restoration of 47% of fluorescence indicating that the fluorescence signal correlates to the amount of urea present in the sample after urease treatment (Fig. 6).

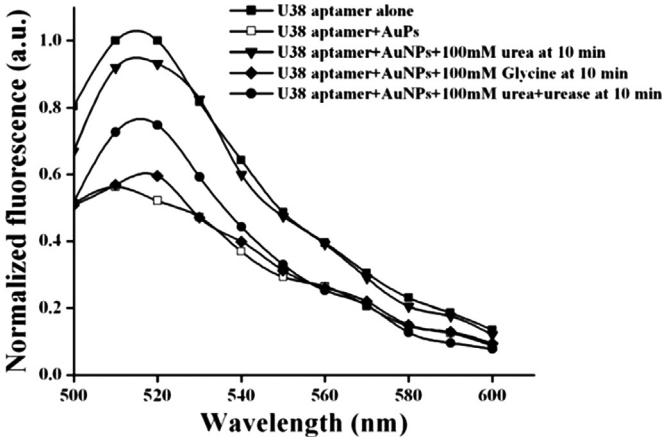


Fig. 6. Fluorescence recovery assay based on the fluorescence quenching of FITC-labeled U38 aptamer in presence of AuNPs. Fluorescence emission spectra of (■) FITC-labeled U38 aptamer alone, (□) FITC-labeled U38 aptamer with AuNPs, (▾) FITC-labeled U38 aptamer with AuNPs and 100 mM urea, (◆) FITC-labeled U38 aptamer with AuNPs and 100 mM glycine as a control, (●) FITC-labeled U38 aptamer with AuNPs and 100 mM urea incubated with 100 unit of Jack bean urease.

4. Conclusions

We have isolated a high affinity DNA aptamer for urea using FluMag-SELEX methodology. The interaction of selected aptamer with urea has been confirmed by affinity assay, CD analysis, melting curve analysis and truncation studies. This is the first report of urea specific DNA aptamer isolation and its development into a label-free, non-enzymatic colorimetric and intrinsic fluorescence aptasensor for urea, which is based on aggregation of AuNPs. The aptasensor displays high selectivity for urea and is not affected in the presence of interfering agents. This aptasensor shows linearity in the range of 20–150 mM of urea. Further, U38 aptamer can also be used for developing a "turn-on" fluorescence based urea sensor. Extant urease based AuNPs biosensors require functionalization of the nanoparticles which compromises the cost and reproducibility due to nonspecific enzyme functionalization. The 'non-enzymatic' aptasensor developed in the present report exploits the unmodified gold nanoparticles and aptamer for development of direct, simple and quick, label-free biosensing system which complements enzyme and other analytical urea sensing methods. In short, this study extends the usefulness of aptamers as an excellent alternative to urease based biosensing of urea which can lead to effective preventive measures of EMA of urea. Future

studies can be conducted to integrate these aptamers with Micro-Electro-Mechanical System (MEMS) to develop "point-of-care" devices for use in medical settings.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.05.029.

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