NUCLEIC ACID THERAPEUTICS
Volume 23, Number 6, 2013

DOI: 10.1089/nat.2013.0437

Sol-Gel SELEX Circumventing Chemical Conjugation of Low Molecular Weight Metabolites Discovers Aptamers Selective to Xanthine

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Sensitive detection of the metabolites indicative of a particular disease contributes to improved therapy outcomes. Developing binding reagents for detection of low molecular weight metabolites is hampered by the difficulty with immobilization of targets through appropriate covalent chemical linkage while ensuring that selected reagents retain specificity to unmodified metabolites. To circumvent chemical modification of targets, we employed sol-gel droplets deposited onto a porous silicon chip to entrap a purine metabolite, xanthine, which was found at lower levels in urine samples from patients with non-Hodgkin lymphoma. By sol-gel SELEX (systematic evolution of ligands by exponential enrichment) against xanthine, specific aptamers ($K_D \sim 10\,\mu M$) with sensitivity of detection at as low as $1\,\mu M$ were isolated, which bound to other purine metabolites at more than 100-fold lower affinity. In contrast, we failed to isolate xanthine-specific aptamers when SELEX was performed against xanthine covalently linked to polymer resin. This study demonstrates that the sol-gel platform for entrapping low molecular weight metabolites without chemical modifications can be utilized for SELEX to discover aptamers against clinical metabolite markers for diagnosis application.

Introduction

Recent advances in proteomics and biochemical profiling have validated numerous cell metabolite markers linked to disease progression, prognosis, and response to therapeutics (Christians et al., 2012). Quantitative detection of the metabolites obtainable from patients' urine and serum samples, therefore, can be developed into monitoring disease progression, therapy outcome, and drug toxicity. However, one of the challenges in applying the metabolite markers to disease diagnosis is finding suitable detection reagents possessing high sensitivity and specificity to targets. Although antibodies have been widely used as detection reagents for protein targets, few antibodies are available against low molecular weight metabolites (less than 1,000 Daltons) due to the difficulty with their low immunogenicity profile and generating antibodies against naturally abundant molecules in animals.

To circumvent the challenge with isolating antibodies against clinical metabolite markers, aptamers, single-stranded oligonucleotides that fold into a unique secondary and tertiary structure and are isolated *in vitro* through a process called SELEX (systematic evolution of ligands by exponential enrichment) (Tuerk and Gold, 1990), have shown a great potential as detection reagents. Studies have shown that aptamers are capable of recognizing diverse targets, including low molecular weight organic and inorganic compounds, proteins, nucleotides, and peptides (Zhu et al., 2012). However, *in vitro* selection of aptamers against small metabolites is challenging, as they may not possess appropriate chemical moieties for immobilization, normally required for conventional SELEX. Even with a successful chemical conjugation of metabolites, the possibility still remains that selected aptamers no longer retain full capacity binding to unmodified targets.

Recent studies have reported purine metabolites as a disease biomarker for cancers including non-Hodgkin lymphoma (NHL) (Linder et al., 2009; Yoo et al., 2010). It has been shown that the patients of breast, gastric, and colon cancer had lower or non-detectable xanthine oxidoreductase activity in their tissues (Linder et al., 2009). Moreover, xanthine and hypoxanthine levels in NHL patients' urine were significantly reduced (compared with a normal level of xanthine at $\sim 40\,\mu M$) when examined by mass spectrometry, suggesting purine metabolites as new potential diagnostic markers for NHL (Yoo et al., 2010). Lactate dehydrogenase levels in the

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serum have been used for prognosis and guiding treatment options for NHL, but a definite prognosis has been very difficult and the survival rates vary widely, prompting for the development of new diagnostic tools (Alexander et al., 2007).

In this study, we have chosen xanthine as a model metabolite and sought to discover aptamers using two different methods: (1) polymeric beads containing chemically coupled xanthine and (2) nanoporous sol-gel with xanthine entrapped without chemical modification. Sol-gel is a silicate material synthesized at room temperature that contains both nanoscale pores and microscale channels that are formed by hydrolysis and polycondensation chemical reactions (Kim et al., 2006; Ahn et al., 2008). Through micron-sized channels, aptamers can freely move but can be retained by their interaction with targets entrapped within nanoscale pores, providing an environment for keeping both aptamers and targets in their native states while permitting physical contacts between them (Kwon et al., 2008; Ahn et al., 2012). By the next generation sequencing of the selected aptamer pools generated by bead or sol-gel based SELEX, we isolated highly enriched sequences only in sol-gel SELEX pools (accounting for 11% of the total reads). Selected aptamers by sol-gel SELEX bound xanthine with exceptional specificity, exhibiting more than 100-fold lower affinity toward other structurally related purine metabolites. These aptamers were able to detect xanthine at as low as 1 μM, well suited for detecting the difference of xanthine levels in normal versus patients' urine samples. In contrast, we failed to isolate specific aptamers utilizing bead-based SELEX requiring chemical coupling of xanthine even with twice more rounds of bead-based SELEX than was with sol-gel SELEX. This study demonstrates that the sol-gel platform for entrapping low molecular weight metabolites without chemical modifications can be applied to the discovery of aptamers against a large set of clinical metabolite markers.

Materials and Methods

Materials

Epoxy-activated sepharose™ 6B (GE Healthcare) beads were used in bead-SELEX for immobilization of xanthine (Sigma-Aldrich). Poly-Prep® chromatography columns (Bio-Rad) were used as reservoirs for the coupled beads for SELEX procedure. Sol-gel was synthesized using SolB™ Kit (PCL Inc.) and spotted onto a silicon chip (PCL Inc.) using Sciflexarrayer DW (Scienion AG). A single-stranded DNA (ssDNA) library was chemically synthesized with the sequence composition of 5′-ATACCAGCTTATTCAATT-**N**40-AGATAGTAAGTGCAATCT-3′ (IDT, USA). Purine metabolites (hypoxanthine, adenine, guanine, caffeine, and uric acid) were obtained commercially (Sigma-Aldrich).

Bead-SELEX

Two-tenths of a gram of epoxy-activated sepharose-6B beads in 700 μL of distilled water were mixed with 120 μmol xanthine dissolved in water to couple secondary amine moieties in xanthine to the beads via stable ether linkages to hydroxyl groups. The amount of immobilized xanthine was calculated by spectrophotometer absorbance at 284 nm (PerkinElmer). The remaining active groups in sepharose were blocked by 1 M ethanolamine (pH 8.0). For depletion of aptamers nonspecific to the sepharose beads (negative selection), activated sepharose beads were blocked by 1 M ethanolamine without the conjugation of xanthine. Xanthine coupled and control beads were packed into chromatography columns, to which 10^{15} ssDNA library suspended in binding buffer (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM KCl, 10 mM $MgCl_2$) was applied. After loading, the column was washed to remove unbound DNAs, and was eluted by elution buffer (50 mM xanthine with 100 mM Tris-HCl, pH 8.0, 200 mM NaCl, 25 mM KCl) to collect reactive aptamers. Eluted DNAs were amplified by polymerase chain reaction (PCR) (performed with a pair of a forward primer with phosphothioate at the 5′ end and a reverse primer with OH at the 5′ end), and anti-sense strands were digested by T7 exonuclease [New England Biolabs (NEB)] to recover aptamer strands (Jo et al., 2011; Marimuthu et al., 2012). For every three positive selections, a negative selection was performed using the beads without xanthine to remove DNAs nonspecific to the beads. After the 7th and 14th SELEX rounds, enriched aptamer pools were subjected to next generation sequencing.

Sol-gel chip SELEX

To entrap xanthine within sol-gel, xanthine and the sol-gel were mixed, and this mixture was spotted onto porous silicon chip. At 12 hours post gelation, the sol-gel chip was equilibrated with binding buffer (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM KCl, 10 mM $MgCl_2$) and blocked for 1 hour with blocking buffer (binding buffer with 20 μg/mL tRNA). The 10^{15} ssDNA library in binding buffer was applied to the sol-gel droplets. After 2-hour incubation, sol-gel droplets were washed three times in every 5 minutes by wash buffer (binding buffer with 0.2% Tween 20). Bound ssDNAs from sol-gel were eluted three times by heat elution at 95°C. Negative selection with sol-gel without entrapped xanthine was performed every round after the third round SELEX. A total of seven rounds of SELEX were performed and enriched sequences were analyzed by next generation sequencing.

Next generation sequencing

Next generation sequencing was performed using Illumina's Genome Analyzer 2x (Gendocs, Inc.). The number of unique sequences in the randomized 40 nucleotides was computationally counted after removing the PCR primer sequences flanking the randomized nucleotide region. The secondary structures of selected aptamer candidates (the entire 76 nucleotides, including the randomized 40 nucleotides flanked by 36 nucleotides of primer sequences) were predicted by the *Mfold* program (http://mfold.rit.albany.edu) using the ionic condition of 100 mM NaCl, 10 mM $MgCl_2$ at 25°C (Zuker, 2003).

Real-time PCR to monitor progressive enrichment of reactive aptamers

Equal amounts of aptamer pools from an initial library, and rounds 3, 5, and 7 pools were applied to sol-gel containing xanthine. After washing out non-bound fractions, bound DNAs were heat eluted by incubation at 95°C. The relative amounts of bound aptamers were quantified by real-time PCR (StepOnePlus, Life Technologies).

Biolayer interferometry

The equilibrium dissociation constants (K_D) of aptamers against purine metabolites were measured by the label-free biolayer interferometry technique using Octet Red 384 (ForteBio, Pall Life Sciences). Biotinylated aptamers at 100 μg/mL were immobilized on the Octet Red's streptavidin-coated biosensor. Aptamer-conjugated biosensors were then exposed to the target chemicals suspended in a microplate well. Binding kinetics of the aptamers against the target chemicals were recorded as sensorgrams. The sensorgram signals by control biosensors, which were the sensors blocked with biocytin, served as a reference to correct for systematic artifacts and non-specific binding to the biosensor (Papalia et al., 2006; Rich and Myszka, 2007). Sensorgrams were collected and analyzed by Fortebio Data Analysis 7.0 software (ForteBio, Pall Life Sciences).

Fluorescent aptamer dot blot assay

Xanthine was mixed with sol-gel, and this mixture was spotted onto the porous silicon chip at various concentrations (1.5, 3, and 7.5 pmol of xanthine per spot). The chip was blocked with blocking buffers [20 μg/mL tRNA, 2% bovine serum albumin, 0.1% Novec^{TM} Fluorosurfactant (3M) in binding buffer] for 1 hour. One hundred picomoles of Cy3-labeled aptamers (PCL Inc.) in binding buffer were applied to the chip and incubated for 4 hours. After repeating three washing steps, the chip was dried in a dark chamber and scanned and analyzed with a fluorescence scanner and a Multi-Image Analyzer (Fujifilm).

Results and Discussion

Next generation sequencing reveals highly enriched sequences by sol-gel SELEX but not by bead-SELEX

We performed seven rounds of SELEX in parallel using sol-gel and sepharose beads and subjected aptamer pools to next generation sequencing to identify highly enriched DNA sequences (Fig. 1). The overall distribution of the multiplicity of top-ranked sequences differed greatly between the two methods (Table 1): the top sequence enriched by sol-gel SELEX reached a sequence multiplicity of 11% with the second ranked sequence fell below 1% of multiplicity. In contrast, there were no sequences enriched by bead SELEX reaching 0.1% of multiplicity. The top sequence after the seventh rounds of sol-gel SELEX also appeared after the fifth round (data not shown). Considering a sequence diversity of an initial library at 10^{15}, we estimated that our sol-gel SELEX achieved an enrichment factor of close to 1,000-fold per round. To test the possibility that if the lack of dominant sequences by the bead-SELEX was due to a small difference in enrichment efficiency, we performed additional seven rounds of bead-SELEX and the aptamers were subjected to next generation sequencing. Even after a total of 14 SELEX rounds, there were no aptamers with more than 1% sequence multiplicity by bead-SELEX (data not shown), clearly illustrating the difficulty with selecting reactive aptamers against small molecular compounds chemically conjugated to polymeric beads. Considering the fact that bead-based SELEX has been used successfully for discovering aptamers against xanthine (Kiga et al., 1998), our inability to isolate aptamers may be ascribed to differences in aptamer design and the method for chemical conjugation of xanthine to polymeric beads. The combination of next generation sequencing and sol-gel SELEX, overall, was effective in obtaining highly enriched sequences even with a low number of rounds of SELEX.

Affinity and specificity measurements of xanthine aptamers by label-free optical interferometry and sol-gel chip

We then used a real-time PCR to examine if the emergence of highly enriched sequences in later rounds of SELEX by sol-gel (Table 1) would coincide with the increase in the amount of aptamers reactive to xanthine in the later rounds of DNA pools. Using real-time PCR, we confirmed a gradual increase

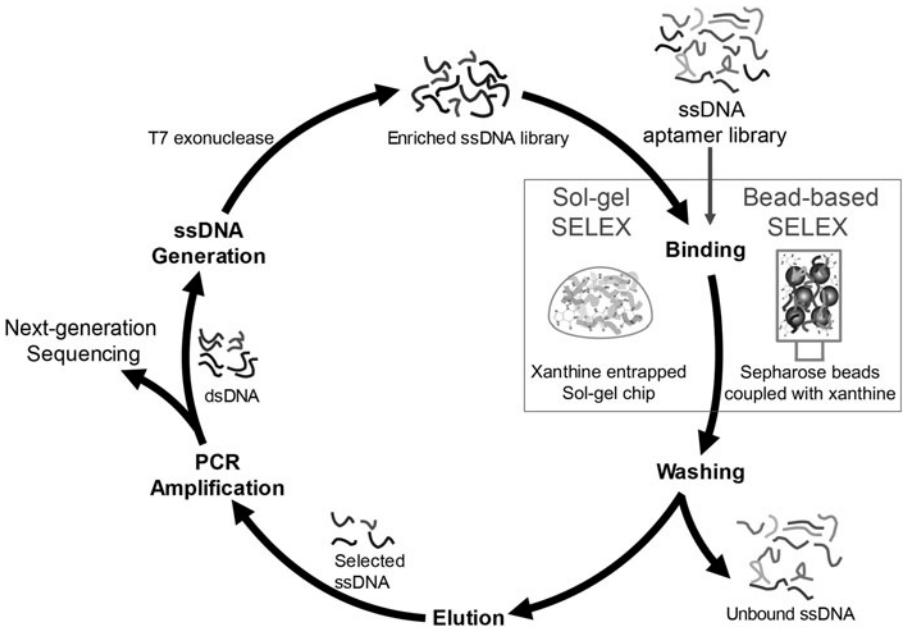

FIG. 1. Scheme for finding aptamers against xanthine chemically coupled to the beads versus entrapped within sol-gel. An initial library of 10^{15} single-stranded DNA sequences was used to isolate aptamers against xanthine via bead versus sol-gel SELEX. Enriched sequences were amplified by polymerase chain reaction (PCR), and the antisense strands were digested by T7 exonuclease to convert double-stranded DNA into single-stranded aptamer sequences. After designated SELEX rounds, amplified sequences were subjected to next generation sequencing to identify highly enriched sequences as potential aptamer candidates.

Table 1. Aptamer Sequences Enriched (Above 0.01% of Total Reads) by Sol-Gel-SELEX Entrapping Unmodified Xanthine Versus Bead-SELEX with Chemically Coupled Xanthine

Name	*Sequences enriched by sol-gel SELEX*	*% of total reads*
SG-1	ACACGACGCTCTTCCGATCTAGATTGCACTTACTATCTGT	11.14
SG-2	ACACGACGCTCTTCCGATCCAGATTGCACTTACTATCTGT	0.39
SG-3	ACACGACGCTCTTCCCGATCTAGATTGCACTTACTATCTGT	0.29
SG-4	GACGTGTGCTCTTCCGATCTAGATTGCACTTACTATCTCG	0.22
SG-5	ACACGACGCTCTTCCGATCTAGATTGCACTTACTATCCTGT	0.22
SG-6	ACACGACGCTCTTCCGATCCTAGATTGCACTTACTATCTGT	0.22
SG-7	ACACGACGCTCTTCCGATCTAGATTGCACTTACCTATCTGT	0.21
SG-8	ACACGACGCTCTTCCGATCTAGATTGCACCTTACTATCTGT	0.17
SG-9	ACACGACGCTCTTCCGATCTAGATTGCACTTACTATCTGTA	0.15
SG-10	ACACGACGCTCTTCCGATCTAGATTGCACTTACTATCTTGT	0.14
	Sequences enriched by bead SELEX	
B-1	CCGGGACATGTTCACTCTAAGGGACGTATATGTTGCTATG	0.07
B-2	CCCTTCCCAGATAGTAAGTGCAATCTAGATCGGAAGAGCA	0.04
B-3	CCCTTCCCAGATAGTAAGTGCAATCTAGATCGGAAGAGCG	0.04
B-4	GGACGAGATAGTAAGTGCAATCTAGATCGGAAGAGCACAC	0.03
B-5	GGACGAGATAGTAAGTGCAATCTAGATCGGAAGAGCGTCG	0.03
B-6	CACAATTATTGCGGGTGGGTGGGTGGTTGTATATTGATTA	0.01
B-7	AACGTGGGAGGGAGGGCGGGTAGTTAATTCTAGTTGGTAA	0.01
B-8	CCAGATAGTAAGTGCAATCTAGATCGGAAGAGCGTCGTGT	0.00
B-9	CCAGATAGTAAGTGCAATCTAGATCGGAAGAGCACACGTC	0.00
B-10	CCTGGGTGGGAGGGTGGGTATGGGAATTTATAGATGAGTG	0.00

The most enriched sequence by sol-gel systematic evolution of ligands by exponential enrichment (SELEX) occurred 643,657 times out of 5,776,221 total sequence reads, and by bead-SELEX 1,350 times out of 1,889,370.

of aptamers bound to xanthine, with the fraction of xanthine-reactive DNA in round 5 comparable with that in round 7 (Fig. 2A). This agrees well with our sequencing analysis, revealing the presence of the highest multiplicity sequence (named SG-1; Table 1) in both round 5 and round 7 pools. The predicted secondary structure of the top sequence by sol-gel SELEX showed that a part of the constant region at the 5′ end (1–18 bases) belongs to a stem-loop structure, while the constant region at the 3′ end (59–76 bases) fold into a stem-loop on its own (Fig. 2B). To test the importance of the first stem-loop in binding xanthine, SG-1 was trimmed to retain only the first stem-loop (named SG-1_trim1 containing DNA bases at 6–45), and the full-length and trimmed sequences were tested for binding to xanthine by biolayer interferometry. To our surprise, SG-1_trim1 bound xanthine with more than a four-fold increase in affinity over SG-1 (18.1 μM K_D for SG-1 and 4.2 μM K_D for SG-1_trim1) (Fig. 2C), indicating that the sequence following the first stem-loop, corresponding to bases 46–76, may interfere with the bases 6–45 from folding into the stem-loop structure required for xanthine recognition. Furthermore, another variant of SG-1 (named SG-1_trim2, containing DNA bases from 19 to 40) without the inclusion of the first stem-loop in SG-1_trim1 bound xanthine with ~15-fold reduction in affinity, confirming the first-stem loop as a key structure for binding to xanthine (Fig. 2C). In contrast, the top sequence from bead-SELEX (B-1) did not react with xanthine (Fig. 2C).

To test if sol-gel chip can be developed into a diagnostic tool for detection of clinical metabolite markers, we prepared a sol-gel chip array entrapping 0–300 μM of xanthine and measured the amount of bound aptamers by fluorescence (Fig. 2D, E). Despite the fact that concentrations as low as 1 μM xanthine could be measured by SG-1_trim1 using a label-free interferometry technique, a minimum of 125 μM of xanthine was needed for reliable detection by fluorescently labeled aptamers. However, sol-gel chip detection was specific, as the top sequences isolated by bead SELEX (e.g., B-1 and B-2) did not produce measureable signals above background levels (Fig. 2D). We anticipate that the sensitivity of sol-gel chip assay can be readily improved by several folds, for example, by adding multiple fluorescent dyes to one aptamer and enhancing a signal-to-noise ratio of the scanner and image analyzer. Sensitive and specific detection of xanthine in a simple assay format is clinically meaningful, as xanthine was found at a reduced level in urine samples from the patients with NHL (median levels are approximately 50 and 20 μM in control and NHL groups, respectively (Yoo et al., 2010). By taking advantage of the property of nucleic acids and versatility of the nanoporous sol-gel chip, a simple yet sensitive and specific diagnostic tool for purine metabolites can be developed employing sol-gel droplets containing urine samples and labeled-aptamers as probes.

Exceptional specificity of selected aptamers to xanthine

In order to confirm the specificity of selected aptamers, the trimmed aptamer, SG-1_trim1 was tested for detection of xanthine and other structurally related purine metabolites (Fig. 3). While as low as 1 μM of xanthine was detected with its binding affinity at 4.2 μM K_D, SG-1_trim1 aptamer bound other purine metabolites (hypoxanthine, adenine, caffeine, guanine, and uric acid) at more than 100-fold lower affinity (K_D ~ mM range) (Fig. 3B). This remarkable specificity toward xanthine may come from the fact that almost every single atom in xanthine is in close proximity to the molecules

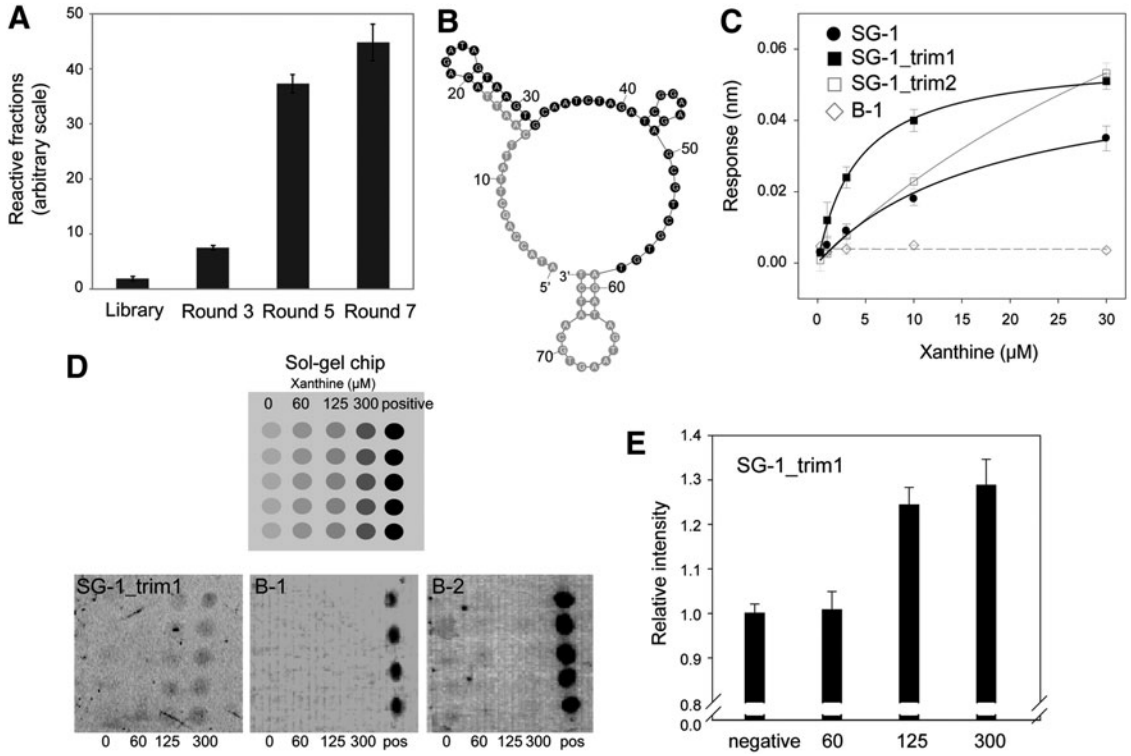

FIG. 2. Predicted secondary structure and affinity measurement of selected aptamer candidates to xanthine. **(A)** Reactive aptamers to xanthine from an initial library and rounds 3, 5, and 7 pools were quantified by real-time PCR. **(B)** The secondary structure of the highest multiplicity sequence from sol-gel SELEX was predicted by *Mfold* (Zuker, 2003). Constant regions at the 5′ and 3′ ends are indicated with nucleotides in gray circles. Nucleotides are numbered at every 10 bases, and 5′ and 3′ termini are labeled. **(C)** Biolayer interferometry was used to measure aptamer binding to xanthine ($n=3$). The K_Ds were estimated at 18.1 and 4.2 μM for the highest multiplicity sequence (SG-1) and its trimmed aptamer SG-1_trim1 (containing bases from 6 to 45), respectively. SG-1_trim2 (containing bases from 19 to 40) bound with more than 15-fold lower affinity (60 μM) to xanthine than did SG-1_trim1. B-1 (the top sequence from bead-SELEX) did not exhibit any measurable binding to xanthine. **(D)** The *top image* shows a layout of droplets containing xanthine entrapped within sol-gel. Cy3-labeled UTP (GE Healthcare) was used as a positive control for sol-gel droplet preparation and detection. The *three images below* show fluorescence detection of Cy3-labeled aptamers due to their affinity to xanthine. **(E)** The bar graph shows the fluorescence intensity of Cy3-labeled SG-1_trim1 against xanthine entrapped in sol-gel and control droplets (mean ± standard deviation, $n=5$).

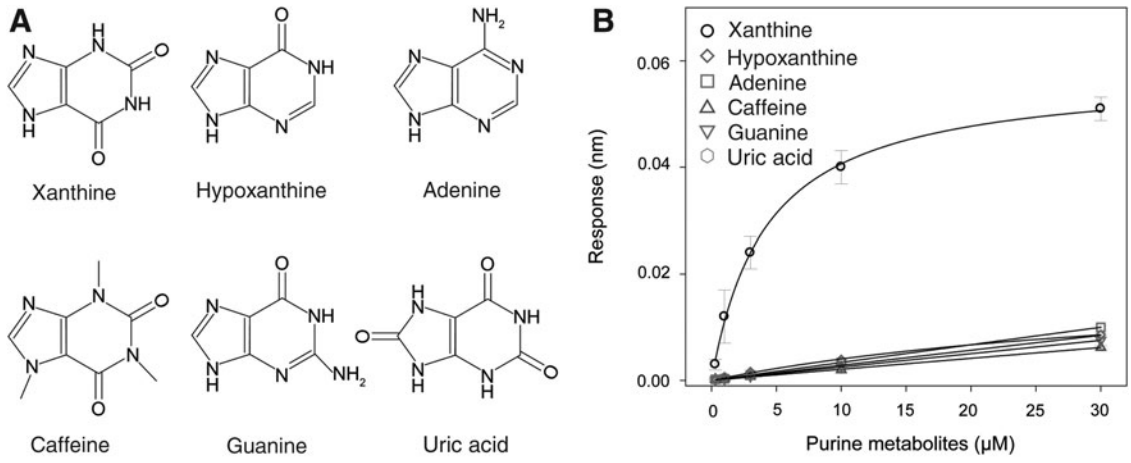

FIG. 3. The newly identified aptamer shows specificity to xanthine but not to other structurally related purine metabolites. **(A)** Drawn are the chemical structures of purine metabolites used in the biolayer interferometry analysis. **(B)** The binding responses of aptamer SG-1_trim1 against xanthine and other purine metabolites were measured by label-free biolayer interferometry (Octet Red 384) ($n=3$). The K_D of SG-1_trim1 for xanthine was 4.2 μM, and for other purine metabolites it was estimated to be in the mM range.

in aptamers. This is evidenced by the loss of binding to other structurally related compounds (i.e., hypoxanthine with a deletion of oxygen atom, guanine with a substitution of amine for oxygen, and caffeine with the addition of methyl groups). Compared to the aptamers selected in this study, other previously isolated RNA aptamers against xanthine were not specific and found to cross-react with other related molecules such as guanine (Kiga et al., 1998). Although many aptamers have been isolated against small molecules (e.g., caffeine, theophylline, ATP, or GTP) (Jenison et al., 1994; Huizenga and Szostak, 1995) employing chemical conjugation and conventional bead-based SELEX, the use of sol-gel should provide added advantage of isolating specific aptamers simply by avoiding chemical modification of the targets.

Conclusions

Low molecular weight metabolites present in serum and urine samples are increasingly being investigated as clinical biomarkers for early and sensitive diagnosis of many diseases. Unlike detection of proteins and peptides by antibodies, a majority of metabolites are not suitable for generation of antibodies due to their small size and the evolutionary conservation of the metabolic pathways and molecules across the animal kingdom. In contrast, as evidenced by the fact that low molecular weight dyes resembling metabolic cofactors were the target molecules of the first aptamer that was developed (Ellington and Szostak, 1990), aptamers are much more amenable for recognition of small molecules with specificity toward every chemical moiety. This is well illustrated in our study by the selected aptamer against xanthine, which bound much more weakly to other structurally related purine metabolites. However, not all low molecular weight molecules are suitable for aptamer discovery: conventional polymeric bead-based SELEX requires chemical conjugation for immobilization, and achieving conjugation to a specific moiety is often hard to achieve. In contrast, sol-gel chip, by providing a unique structure of a nanoscale compartment for retaining small molecules and microscale pores for macromolecules such as aptamers to freely move or remain trapped due to binding to entrapped target molecules, may provide a robust SELEX platform for selecting aptamers against clinical metabolites with a higher chance for selected aptamers to retain full capacity binding and specificity to the targets in clinical samples.

Acknowledgments

This work was supported by Korea Ministry of Environment as "EI project" (ERL E211-41003-0007-0), and Agency for Defense Development through Chemical and Biological Defense Research Center (2012-0126-005). This work was also supported by the Korea Ministry of Environment as "Converging Technology Project" (212-101-004) and R&BD program supported by the MOTIE (The Ministry of Trade, Industry, and Energy) and KIAT (Korea Institute for Advancement of Technology).

Disclosure Statement

No competing financial interests exist.

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Received for publication June 13, 2013; accepted after revision September 6, 2013.