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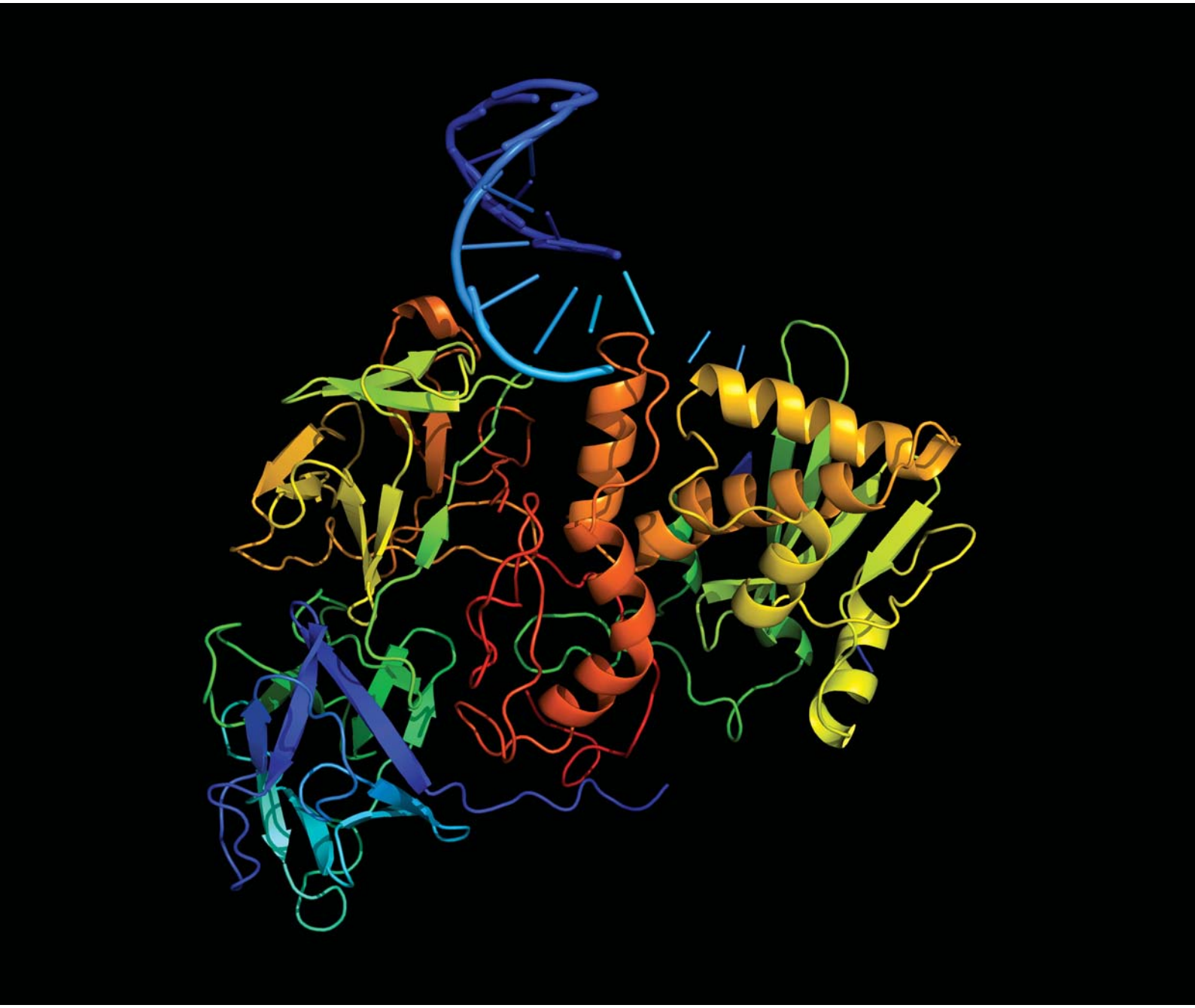

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PAPER

A single DNA aptamer functions as a biosensor for ricin†

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The use of microorganisms or toxins as weapons of death and fear is not a novel concept; however, the modes by which these agents of bioterrorism are deployed are increasingly clever and insidious. One mechanism by which biothreats are readily disseminated is through a nation's food supply. Ricin, a toxin derived from the castor bean plant, displays a strong thermostability and remains active at acidic and alkaline pHs. Therefore, the CDC has assigned ricin as a category B reagent since it may be easily amendable as a deliberate food biocontaminate. Current tools for ricin detection utilize enzymatic activity, immunointeractions and presence of castor bean DNA. Many of these tools are confounded by complex food matrices, display a limited dynamic range of detection and/or lack specificity. Aptamers, short RNA and single stranded DNA sequences, have increased affinity to their selected receptors, experience little cross-reactivity to other homologous compounds and are currently being sought after as biosensors for bacterial contaminants in food. This paper describes the selection and characterization of a single, dominant aptamer, designated as SSRA1, against the B-chain of ricin. SSRA1 displays one folding conformation that is stable across 4–63 °C ($\Delta G = -5.05$). SSRA1 is able to concentrate at least 30 ng mL^{-1} of ricin B chain from several liquid food matrices and outcompetes a currently available ELISA kit and ricin aptamer. Furthermore, we show detection of 25 ng mL^{-1} of intact ricin A–B complex using SSRA1 combined with surface enhanced Raman scattering technique. Thus, SSRA1 would serve well as pre-analytical tool for processing of ricin from liquid foods to aid current diagnostics as well as a sensor for direct ricin detection.

1.0. Introduction

The growing centralization of food production and processing and wide distribution of products causes a concomitant rise in concern over the potential introduction of a biocontaminate into the food network.[1] Ricin, a toxin derived from the castor bean plant (*Ricinus communis*), is considered to serve as a likely food biocontaminant because of its stability at a wide range of temperatures and pH.[2–4] The Centers for Disease Control and Prevention (CDC) has classified ricin as a category B reagent due to its relative abundance, ease of extraction from the castor bean without the need for specialized tools, readiness for dissemination, and the resultant requirement for enhanced diagnostics and surveillance.[5] Ricin is a glycoprotein lectin composed of two parts: an A chain and a B chain linked by a disulfide bond.[2,6–8] The B chain (32 kDa) functions as a lectin, which binds to galactose residues expressed on the cell surface and facilitates the translocation of the A chain to the cytosol.[2] The ricin A chain (32 kDa) is free to inactivate the ribosome by depurination of a single adenosine. The lethality elicited by ricin, as with most toxins, depends upon the route of exposure. For example, the lethal dose (LD_{50}) for direct injection or inhalation of ricin is estimated to be 5–10 μg kg^{-1} of body weight.[1,2,9] This is in stark contrast to exposure by ingestion, for which the LD_{50} is 1–20 mg kg^{-1} (an equivalent of 8 castor beans).[1,2,9] Although ricin has a high oral LD_{50}, intentional ricin poisoning is of interest due to public fear.[10] Particular concern over fruit juices contaminated with ricin creates anxiety due to the fact that some of the major consumers of these liquids are children.[1] Also, ricin contamination may serve as a prelude to future planned acts of bioterror on the food supply.

Current systems used to diagnose possible ricin contamination include methods that detect: (1) immunogenic interactions (*i.e.*

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† Electronic supplementary information (ESI) available: Fig. S1: Schematic of ricin B chain aptamer selection *via* systemic evolution of ligands by exponential enrichment. An aptamer library (~10^{14} sequences) was exposed twice to unconjugated tosylactivated beads to remove non-specific candidates. Aptamer candidates in the supernatant were subsequently incubated with ricin B chain bound tosylactivated beads for 8 rounds of SELEX. Upon the eighth round of SELEX, aptamers were cloned, sequenced, and examined for redundancy. See DOI: 10.1039/c1an15352h

enzyme-linked immunosorbent assay (ELISA) kits), (2) enzymatic activity of ricin, and (3) the presence of castor bean DNA.[1,11–15] Although the goal of any diagnostic test or system is to be sensitive and specific (little to no cross-reactivity to similar molecules) for the target agent and easily operated, none of the ricin diagnostic tools fit all of these needs.[11] Assays that solely rely on antibodies or enzymatic activity are often confounded by complex matrices, such as food.[1] Food components, such as lactose, may interfere with binding of ricin to a capture antibody and may result in a false negative result during surveillance testing.[4,16,17] As opposed to a false negative result, systems that rely on amplification of castor bean DNA may report false positive results since the underlying assumption is that castor bean DNA correlates with the presence of ricin.[18] Next generation diagnostics for biothreats include isotope labelling, tandem mass spectrometry and flow cytometry.[1,11] While these systems are highly sensitive and will also differentiate between active *versus* inactive ricin, they are labour intensive and are not feasible to be employed in a field study or in level A laboratories, which are often the first responders for a suspected biocontaminant.[11]

Aptamers, short RNA and single stranded DNA sequences, have increased affinity to their selected receptors and experience little cross-reactivity to other homologous compounds and are currently being sought after as biosensors for public health and food surveillance.[19–26] Published aptamers have shown increased binding affinities to target molecules when compared to available antibodies.[19] Furthermore, aptamers are relatively inexpensive to generate and produce and are easily modified to fit testing/diagnostic purposes.[19]

In this article we report the selection and characterization of a single DNA aptamer directed against the ricin B chain. The selected aptamer is able to concentrate dilute amounts of ricin B chain from complex liquid food matrices and is stable throughout a range of pHs. Moreover, in a previous study, we have shown that the anti-ricin B chain aptamer can be used with surface enhanced Raman scattering (SERS), an analytical tool for molecular fingerprinting, to create a ricin B chain sensor for liquid food matrices. However, ricin B chain detection may have limited utility for detection in field samples containing the intact ricin toxin. In order to address this issue, we have validated SERS aptamer–ricin capture using the entire ricin toxin. Aptamer capture of ricin *via* SERS spectra data displays greater sensitivity when compared to ricin B chain. Thus, the anti-ricin B chain aptamer can be readily modified to be used with current ricin diagnostics as a pre-analytical processing tool and sensor.

2.0. Experimental

2.1. Materials

Aptamers and primers. A randomized DNA aptamer library (SP40) was produced by IDT Technologies Inc. (Coralville, IA). Primers and aptamers were synthesized by the BioMedical Genomics Centre at the University of Minnesota (Saint Paul, MN).

Proteins and antibodies. Ricin B chain and ricin were acquired from Vector laboratories (Burlingame, CA). Mouse monoclonal ricin B (RB127), goat polyclonal ricin B (rcL-17) and donkey anti-goat horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-mouse IgG HRP was obtained from R&D systems (Minneapolis, MN).

Other laboratory reagents. Dynabeads® M-450 tosylactivated beads and streptavidin magnesphere paramagnetic particles were purchased from Invitrogen (Carlsbad, CA) and Promega (Madison, WI), respectively. Lightshift chemiluminescent Electromobility shift assay kit was acquired from Pierce (Rockford, IL). TA cloning kit containing TOP 10 chemically competent *E. coli* cells was acquired from Invitrogen (Carlsbad, CA). Well-coated™ amine binding plates were obtained from G-Biosciences (Maryland Heights, MO). Commercialized enzyme-linked immunosorbent assay (ELISA) kits for ricin B chain detection were bought from Tetracore, Inc. (Rockville, MD). Ultra-sensitive 3,3′,5,5′-tetramethylbenzidine (TMB-US) substrate used for ELISA development was obtained from Moss substrates, Inc. (Pasadena, MD).

Instruments and software. The MLR-350H environmental chamber was obtained from Sanyo Electric Co., Ltd. (Japan). An infusion pump (Model 780100) was purchased from Kd Scientific Inc. (Holliston, MA). All generated aptamer sequences were analysed using Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, MI). LabWorks 4.6 software for ethidium bromide and chemiluminescence imaging was obtained from LabWorks Inc. (Costa Mesa, CA). TQ Analyst software and DXR Raman microscope were obtained from Thermo Scientific (Madison, WI). The DXR Raman microscope facilitates a 780 nm excitation of SERS scattering through a 10× confocal microscope objective, resulting in a laser spot diameter of approximately 3 μm and 5 cm^{-1} spectral resolution. Graphpad Prism software was purchased from Graphpad Software Inc. (La Jolla, CA).

2.2. Ricin B chain conjugation to tosylactivated beads

Approximately 150 μL of tosylactivated beads were separated from solution with a magnetic apparatus, washed and resuspended in 150 μL of 0.1 M borate solution (pH = 9.5). Next, tosylactivated beads were combined with 450 μL of 0.1 M NaH_2PO_4 (pH = 7.4) and 100 μL of ricin B chain (1.0 μg μL^{-1}) and incubated overnight in a Labquake tube shaker at 37 °C. After overnight incubation, tosylactivated beads were washed thrice with cold (buffer kept at 4 °C until use) 1.0 mL of PBS containing 0.1% BSA (pH = 7.4), resuspended in 1.0 mL of 0.2 M Tris buffer containing 0.01% BSA (pH = 8.5), and incubated for 4 h at 37 °C. Beads were washed twice more and suspended in 150 μL of PBS containing 0.1% BSA. Successful binding of ricin B chain to tosylactivated beads was determined by Western blot analysis using a mouse monoclonal antibody against ricin B chain.

2.3. Selection of DNA aptamers against ricin B chain using Systemic Evolution of Ligands by Exponential Enrichment (SELEX)

Aptamer candidates were generated against ricin B chain using a modified SELEX protocol (Fig. S1†). Briefly, the SP40 aptamer library first underwent two counter-SELEX iterations by 30 min

incubations with 100 μL of unbound tosylactivated beads and 50 μL of 1× PBS containing 0.1% BSA (binding buffer) with gentle rotation (Labquake tube shaker). Aptamers that bound to tosylactivated beads were discarded and the supernatant was collected and used for positive rounds of SELEX against ricin B chain. Positive rounds of SELEX involved exposure of the remaining aptamers in the SP40 library to 100 μL of tosylactivated beads conjugated to ricin B chain (~67 μg) and 50 μL of binding buffer followed by six wash steps with 1× PBS containing 0.01% Tween 80 to remove non- or poor binders. Ricin B chain tosylactivatated beads bound to aptamers were subjected to polymerase chain reaction (PCR) after every round of SELEX to amplify potential candidates. The PCR reaction was performed using biotin labelled primer on the anti-sense strand (SK38) (Table 1) and the following program: 95 °C for 15 min, 94 °C for 15 s, 58 °C for 20 s, 72 °C for 20 s at 30 cycles and 72 °C for 7 min. Subsequent rounds of SELEX required only the aptamer sense strand; therefore, the antisense strand was removed by snap cool method with 50 μL of streptavidin magnesphere paramagnetic particles. This process was repeated for a total of 8 rounds of SELEX. PCR from the eight round of SELEX used a biotin label on the sense (SK39) primer to be used for electromobility shift assay (EMSA) analysis. Once binding of the enriched aptamer pool to ricin B chain was determined, aptamers were cloned into a TA vector (pCR2.1) and transformed by heat shock into chemically competent TOP 10 *E. coli* cells as per manufacturer's instructions. Transformed TOP 10 *E. coli* cells were plated at 100 μL on LB plates containing ampicillin (50 μg mL^{-1}) and X-gal. Successful transformants were selected by blue/white screening for disruption of *lacZ*. Colony PCR was performed on transformants using M13 primers and the following program: 94 °C for 15 min, 94 °C for 30 s, 56 °C for 30 s 72 °C for 1 min at 30 cycles and 72 °C for 5 s. 100 transformants showing the correct amplicon size were sequenced and analysed for redundancy using Squencher. Sequences of aptamer candidates selected for further investigation were entered into the m-fold program (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) available from the RNA Institute.

2.4. Electromobility shift assay (EMSA) and supershift assay

The following reagents were combined with binding buffer at room temperature for 20 min for EMSAs: (1) 20 attomole (amole) of biotin labelled (5′) aptamer pool or biotin labelled (5′) SSRA1 (5′bio/SSRA1), (2) 20 amole of 5′bio/aptamer pool or 5′bio/SSRA1 and 1.0, 2.0 or 3.0 μg of ricin B chain, 3) 20 amole of 5′bio/aptamer pool or 5′bio/SSRA1, 40 amole of unlabelled aptamer pool or SSRA1, and 1.0, 2.0, or 3.0 μg of ricin B chain. Strands from the enriched aptamer pool were separated by heating at 95 °C for 10 min before combining with ricin B chain. All reactions were mixed with 5× loading buffer prior to loading on a native 10 per cent polyacrylamide gel (prepared from a 30% acrylamide/bis solution and 0.5× TBE buffer). Samples were electrophoresed for 1 h at 100 V and later mechanically transferred overnight to a nylon membrane pre-soaked in 0.5× TBE. The nylon membrane was processed and developed using the Lightshift chemiluminescent EMSA kit according to manufacturer's instructions. Briefly, the membrane was blocked with 20 mL of pre-warmed blocking buffer for 15 min at room temperature with subtle shaking, which was followed by a 15 min incubation of a 1 : 300 dilution of streptavidin–HRP conjugate at room temperature. Next, the nylon membrane was washed four times in 20 mL of 1× washing solution in 5 min increments and combined with 30 mL of substrate equilibrium buffer for 5 min at room temperature. The nylon membrane was developed using a 1 : 1 mixture of luminal/enhancer and substrate and imaged as described in electroblot analysis. Supershift assays were conducted in a similar fashion as EMSAs with the addition of 1 : 250 (Hi) and 1 : 500 (Lo) dilutions of mouse monoclonal antibody against ricin B chain. All assays were conducted a total of three times.

2.5. Construction of gold electrospun fibers

Gold electrospun fibers were constructed using a described method.[27] Briefly, poly(lactic acid) (100 g l^{-1}) was added to *N*,*N*-dimethylformamide : chloroform (1 : 9 ratio) and sonicated VibraCell™ for 1 min to disperse the polymer. The poly(lactic acid) solution was transferred into a 10 mL glass syringe attached to a Teflon tube and a 16-gauge stainless steel blunt end tip needle spinnert. The spinnert was connected to the positive terminal of a DC power supply that was polarized at 10 kV. The apparatus was held within an environmental chamber maintained at 20 °C and relative humidity of 50%. The poly(lactide) fiber was formed using a polymer solution flow rate of 0.1–0.3 mL h^{-1} and a voltage between 10 and 12 kV for 10 min from a fixed distance of 16 cm. The polymer solution was spun

Table 1 Primers, aptamers and aptamer modifications used in this study

Name	Sequence and direction
BioSK38	bio-5′ ATAATCCACCTATCCCAGTAGGAGAAA T 3′
BioSK39	bio-5′ TTTGGTCCTTGTCTTATGTCCAGAATG C 3′
SK38	5′ ATAATCCACCTATCCCAGTAGGAGAAAT 3′
SK39	5′ TTTGGTCCTTGTCTTATGTCCAGAATG C 3′
SSRA1	5′ ACACCCACCGCAGGCAGACGCAACGCCTCGGAGACTAGCC 3′
BioSSRA1	bio-5′ ACACCCACCGCAGGCAGACGCAACGCCTCGGAGACTAGCC-3′
ThiolSSRA1	thiol-5′ ACACCCACCGCAGGCAGACGCAACGCCTCGGAGACTAGCC 3′
AmineSSRA1 amine	amine(C6)-5′ ACACCCACCGCAGGCAGACGCAACGCCTCGGAGACTAGCC 3′
AmineSSRA1 scramble	amine(C6)-5′ TAACGCAAACCGCGGGAACACGCGCGGATCCACAGCCCGA 3′
AmineC5	amine(C6)-5′ CCGTAGGTTCGGGGCGGAGTGGTCCGGAAGGTGGCGTGG 3′
AmineC5 scramble	amine(C6)-5′ CGGGTAGGCCCGGAGGTTGGGCACTTGGAGTGGGTGCGG 3′
M13 Forward	5′ GTTTTCCCAGTCACGAC 3′
M13 Reverse	5′ CAGGAAACAGCTATGAC 3′

towards vertical position onto a Low Density Polyethylene (LDPE) film attached to a circular steel plate of 25 cm diameter grounded to zero potential. Poly(lactic acid) fibers were cut into 1 cm^2 sections prior to sputter coating with a gold layer at 20 mV for 2 min.

2.6. Spike and recovery in multiple food matrices

Approximately 100 μL of streptavidin magnesphere paramagnetic particles were washed thrice using 100 μL of binding buffer with the aid of a magnetic particle separator in a 1.5 mL eppendorf tube. Streptavidin particles were combined with 0.25 μM of 5′bio/SSRA1 and 150 μL of binding buffer and incubated at room temperature for 2 h with rotation using a Labquake tube shaker. Ricin B chain was spiked in PBS, apple juice, orange juice, lemonade and 2% milk at 30 ng mL^{-1}, 50 ng mL^{-1}, 100 ng mL^{-1}, 250 ng mL^{-1} and 500 ng mL^{-1}. The pH of all ricin B chain spiked food matrices were recorded prior to exposure to 5′bio/SSRA1. The pH of each solution is as follows: PBS and 2% milk = 7, apple juice and orange juice = 4, and lemonade = 2. Ricin B chain spiked orange juice and lemonade contained a high percentage of pulp and were not amenable to incubation with paramagnetic particles in their current state; therefore, solutions were passed through a 0.45 μm syringe filter. Two per cent milk treatments were mixed with 0.01% Tween-80 to prevent clumping of paramagnetic particles. The 5′bio/SSRA1 coated streptavidin magnesphere paramagnetic particles or unconjugated streptavidin magnesphere paramagnetic particle controls were separately combined with ricin B chain spiked solutions in individual 1.5 mL DNase/RNase free eppendorf tubes and incubated for 30 min at room temperature with rotation using the Labquake tube shaker. Following incubation, 5′bio/SSRA1 coated streptavidin magnesphere paramagnetic particles were separated from solution using a magnetic apparatus, washed thrice in 500 μL of 1× PBS containing 0.01% BSA and 0.01% Tween-80 and resuspended in 20 μL of binding buffer. The ricin B chain spiked supernatants were saved in separate 1.5 mL DNase/RNase free eppendorf tubes. Ricin B chain spike and recovery experiments using gold electrospun fibers were performed in a similar fashion as streptavidin magensphere paramagnetic particles with the exception that 0.5 μM of 5′ thiolated SSRA1 (5′thiol/SSRA1) was conjugated to 3 cm × 3 cm gold ESF strips overnight and later washed three times with 1× PBS containing 0.01% BSA. In order to prevent non-specific binding, gold ESF strips were blocked with 1× PBS–Tween 20 containing 1% BSA for 1 h at room temperature followed by three washes with 1× PBS–Tween 20. 5′thiol/SSRA1 bound to gold ESF strips were exposed to 250 ng mL^{-1} of ricin B chain spiked solutions for 30 min at room temperature. All samples were stored at 4 °C until future electroblot analysis. Treatments were conducted in triplicate and spike and recovery assays were performed a total of three times.

2.7. Electroblot analysis

Ricin B chain spiked supernatants were concentrated to 100 μL using the Savant Automatic Environmental Speedvac System. 5′bio/SSRA1 bound streptavidin particles, unconjugated streptavidin particles, 5′thiol/SSRA1 bound gold ESF strips and unbound gold ESF strips from spike and recovery experiments were heated at 95 °C for 10 min to dissociate any ricin B chain from the substrates. Heated supernatants were removed and added to separate 0.2 mL thermo cycling tubes. Nitrocellulose membranes were assembled on the Minifold® I slot blot system and 1.0 μL of each sample was pipetted in duplicate into individual slot blot wells under vacuum pressure. Nitrocellulose membrane was dried and blocked for 2 h with 5% non-fat dried milk in 1× PBS–Tween 20 (blocking buffer). Primary, mouse monoclonal ricin B chain, and secondary, anti-mouse IgG_1, antibodies were applied separately to the nitrocellulose membrane in 1 : 1000 dilutions in blocking buffer for 1 h at room temperature with subtle shaking. Following all antibody incubations, the membrane was washed 5 times with 1× PBS–Tween 20 in 5 min increments. Nitrocellulose membrane was developed using the Western lightning ultra kit per manufacturer's instructions and imaged with LabWorks 4.6 software under simple biochem acquisition.

2.8. ELISA

Amine binding plates were washed three times using 300 μL of 1× femto PBS–Tween 20. Amine conjugated SSRA1 and C5 aptamers were diluted in 0.05 M carbonate–bicarbonate (pH = 9.6) and 100 μL of each solution was applied to individual amine binding wells in separate plates. Aptamer–amine binding plates were incubated for 18 h overnight at 4 °C and later washed three times with 1× femto PBS. Plates were blocked with 5% gelatin from cold water fish in 1× PBS containing 0.01% Tween-20 (pH = 7.0) for 1 h at 37 °C and washed three times with 1× femto PBS. A two-fold serial dilution of ricin B chain (1.0 μg mL^{-1}) in either tetracore blocking buffer (5% nonfat dried milk in PBS–Tween 20), apple juice, orange juice, lemonade or 2% milk was created in each plate to determine detection threshold in various liquid food matrices. The pH of all food solutions was adjusted to 7.0 in order to prevent cleavage of the aptamer–amine bond. Next, aptamer–amine binding plates were incubated with spiked ricin B chain solutions for 1 h at 37 °C, washed 3 times with 1× femto PBS–Tween 20 and incubated at 37 °C for 1 h with the detection antibody (rcL-17; 1 : 300 dilution). Upon completion of incubation, plates were washed in a similar fashion and incubated with donkey anti-goat HRP (1 : 10 000 dilution) for 1 h at 37 °C followed by three 1× femto PBS–Tween 20 washings. Approximately 100 μL of TMB-US substrate was added to each well and allowed to incubate for 5 min at room temperature. Fifty μL of a 2 M H_2SO_4 stop solution was pipetted into each well and the optical density of all plates was read at 450 nm using the SpectraMaxM2 microplate reader and software. Tetracore ricin B chain ELISAs were performed as per manufacturer's instructions with the exception of pH neutralization of food solutions and the replacement of 2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt (ABTS) with TMB-US. All treatments were conducted in triplicate with duplicate ricin B chain concentrations. Positive cut-off values were calculated from the negative control average and two standard deviations. Dissociation constants (K_d) were calculated for SSRA1 and C5 aptamers in each solution. K_d was calculated based on a nonlinear regression analysis standardized to the Hill equation using Graphpad software.

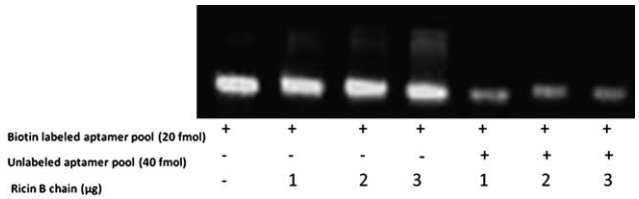

Fig. 1 EMSA of enriched aptamer pool from SELEX round 4. 20 fmole of the biotin labeled enriched aptamer pool was incubated with either increasing concentrations of ricin B chain (1–3 μg mL^{-1}) or in combination with 40 fmole of the unlabeled enriched aptamer pool. A noticeable band shift occurs with the addition of ricin B chain and is abrogated by the inclusion of the unlabeled apatmer pool.

$$OD = OD_{max} \frac{[ricin]}{[ricin] + K_d} \quad (1)$$

In eqn (1), OD = optical density, [Ricin] = the concentration of ricin B chain in nM, and K_d = the dissociation constant.

2.9. Ricin capture and analysis using SERS

Ag dendrites were prepared using a simple replace reaction using zinc and silver nitrate ($AgNO_3$) as stipulated.[28] Four hundred μg of Ag dendrites were conjugated to 4 μM of 5′thiol/SSRA1 overnight at room temperature under constant rotation. Upon completion of incubation, the Ag–SSRA1 complex was separated from solution by centrifugation at 6000 × *g* for 1 min and washed with 1.0 mL of double distilled water. Next, 20 μL aliquots of Ag dendrites alone or bound to SSRA1 were mixed individually with different ricin (whole toxin) concentrations (0, 25, 50 and 100 ng mL^{-1}) spiked in PBS and incubated at room temperature for 10 min under constant rotation. Ag dendrites/Ag–SSRA1 were collected by centrifugation at 6000 × *g* for 1 min, washed thrice in 1.0 mL of double distilled water, and adhered and air-dried on a glass slide prior to SERS analysis. Inactivated ricin (100 ng mL^{-1}) was prepared by boiling ricin for 30 min. Quadruplicate SERS measurements were analyzed using a DXR Raman microscope with 4 mW laser power and 25 μm slit width for a 15 s integration time. Spectra were collected using the Thermo Fisher Scientific OMNIC™ Software with array automation function. The array automation function automatically collects and processes data from groups of samples that are arranged in an ordered array. An array multiple (n = 25) was used to collect the data by randomly picking 25 spots on the Ag surface for each sample. SERS spectral data were analyzed by the TQ Analyst software. Variance of spectral data was examined using the principal component analysis (PCA) and the obtained standards were used to create a qualitative predictive model to view patterns and trends. Prior to PCA analysis, pre-data processing such as standard normal variant, second derivative transformation, and smoothing were applied as necessary to aid

Table 2 Selected aptamers against ricin B chain. Four repetitive aptamer sequences from one hundred transformants emerged from SELEX against ricin B chain. SSRA1, the dominant aptamer, showed a 93% redundancy

Aptamer	Sequence	Number of colonies[a]
SSRA1	ACACCCACCGCAGGCAGACGCAACGCCTCGGAGACTAGCC	93
SSRA2	ACACCCACCGCAGGCAGACGCAACGCCTCGAGACTAGCC	2
SSRA3	GCGAAGCGGGCAGAGGGGCAGGGGCGGGGTAGGTGGGCA	3
SSRA4	TGCTATAATGGAAGTAATGTTAATGATTGCGTCTCCTCCC	2

[a] Out of 100 transformants.

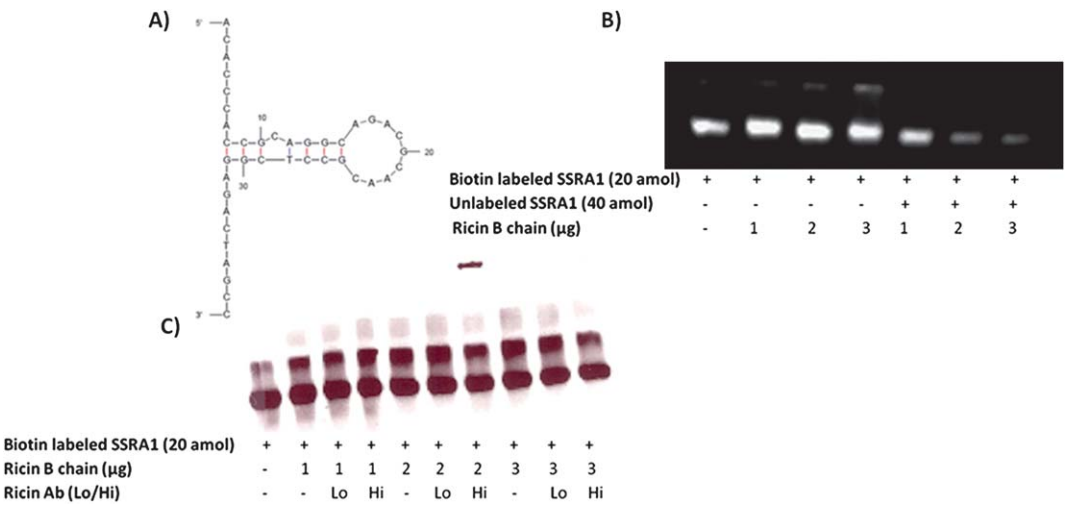

Fig. 2 SSRA1 forms a stable folding pattern and binds to ricin B chain. (A) Stem-loop structure of SSRA1. Structure remains stable at 4–63 °C. (B) EMSA of SSRA1 with increasing concentrations of ricin B chain protein. EMSA results are similar to those of enriched aptamer pool. (C) Supershift assay of SSRA1 in combination with ricin B chain and anti-ricin B chain antibody. Supershift occurs with 2 μg of ricin B chain and 1 : 250 anti-ricin B chain antibody.

PCA capture of significant variance.[29] The partial least square (PLS) model was constructed to predict the captured amount of ricin in comparison to the original (spiked) ricin concentration. The number of PLS latent variables was optimized based on the lowest root mean square error of prediction (RMSEP-see eqn (2)) values to avoid overfitting of spectral data.

$$\mathrm{RMSEP} = \sqrt{\frac{\sum_{\mathrm{i}}^{n}(c_{\mathrm{i}} - c_{\mathrm{i2}})^2}{n}} \quad (2)$$

In eqn (2), n = number of samples, c_{i2} = the amount of bound ricin (μg), and c_{i} = the amount of spiked ricin (μg). The correlation coefficient (R) and RMSEP were used to evaluate the model. The higher the R value or the lower the RMSEP value, the better the model predictions.

3.0. Results and discussion

3.1. SELEX based enrichment of DNA aptamers identified a dominant candidate against ricin B chain

An aptamer library (SP40) composed of 10^{14} sequences, each consisting of two 10-mer constant overhangs and a 40-mer random region, were exposed to ricin B chain using an iterative process called SELEX (Fig. S1†). After the fourth and eighth rounds of SELEX, the enriched aptamer pool was submitted to EMSA analysis to determine successful binding to ricin B chain (Fig. 1). Sequencing of 100 transformants containing apatmer inserts (SELEX round 8) revealed 4 sequences (Table 2). Aptamer library incubation with ricin B chain *via* SELEX selected for one dominant aptamer, herein referred to as SSRA1, with a 93 per cent redundancy (Table 2). It is also interesting to note that another aptamer sequence (SSRA2) is a truncation of SSRA1, which is missing a guanine at position 31 (Table 2). Single base pair variations of the dominant aptamer are also noted by Tang *et al.*, whom selected DNA aptamers against the total ricin toxin.[30] Tang *et al.* have suggested that these subtle changes, like the above, indicate saturation in the SELEX process. Due to the high per cent of redundancy, we selected SSRA1 for further characterization. The folding pattern for SSRA1 was determined by the m-fold program. SSRA1 has one configuration that contains a bulge loop (G_{10}–C_{29}) and a hairpin loop (C_{15}–G_{25}) (Fig. 2A). SSRA1 has an expected thermostability range of 4–63 °C ($\Delta G = -5.05$) and may successfully bind to ricin B chain under a number of temperature conditions. Next, we tested SSRA1 for binding to different concentrations of ricin B chain using EMSA (Fig. 2B). As little as 20 amol of SSRA1 shows binding to ricin B chain (Fig. 2B). SSRA1 displays enhanced binding with a concomitant increase in ricin B chain concentration. SSRA1 binding to ricin B chain appears to be a "real" event as cold competition with a 2× concentration of unlabelled SSRA1 abolishes visualization of binding as well as decreases the aptamer band (Fig. 2B). In order to validate this assertion as well as determine specificity to ricin B chain, SSRA1 was tested in a supershift assay with a commercially available antibody to ricin B chain (Fig. 2C). SSRA1 with the less diluted ricin B chain antibody showed the greatest retardation in migration when combined with 2.0 μg of ricin B chain (Fig. 2C). Surprisingly, the highest ricin B chain concentration (3.0 μg) did not show a supershift (Fig. 2C). This is likely due to the aptamer/antibody/protein complex forming in the well and not migrating in the gel. Together these data suggest that SELEX for aptamers against ricin B chain has reached saturation, which has resulted in selection of a single, dominant aptamer (SSRA1). SSRA1 is highly stable at a wide range of temperatures and successfully binds to ricin B chain.

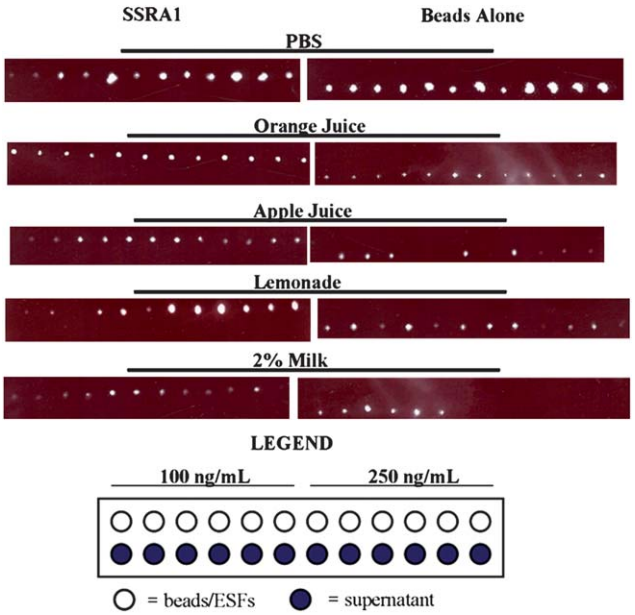

Fig. 3 SSRA1 pulldown assay captures dilute concentrations of ricin B chain spiked in multiple liquid food matrices. Ricin B chain spiked liquid foods were incubated with either streptavidin paramagnetic particles coated with biotin–SSRA1 (left) or unconjugated. Ricin B chain was dissociated from the paramagnetic particles and the samples were analyzed *via* dot blot. Only paramagnetic particles conjugated to SSRA1 are able to concentrate ricin B chain from all liquid samples.

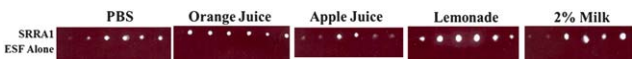

Fig. 4 Validation of SSRA1 pulldown assay. 5′thiol-SSRA1 was conjugated to gold electrospun fiber strips and later incubated with ricin B spiked liquid foods. SSRA1 easily captures 250 ng mL^{-1} of ricin B chain. Similar to the SSRA1–streptavidin paramagnetic particle assay, only ESF strips bound with SSRA1 were capable of concentrating ricin B chain from liquid matrices.

Table 3 Binding affinities of SSRA1 and C5 in multiple liquid samples

	K_d/nM	
Food matrix	SSRA1	C5
PBS	5	70
Blocking buffer	8	160
Orange juice	15	150
Apple juice	32	120
Lemonade	12	NA
2% Milk	22	157

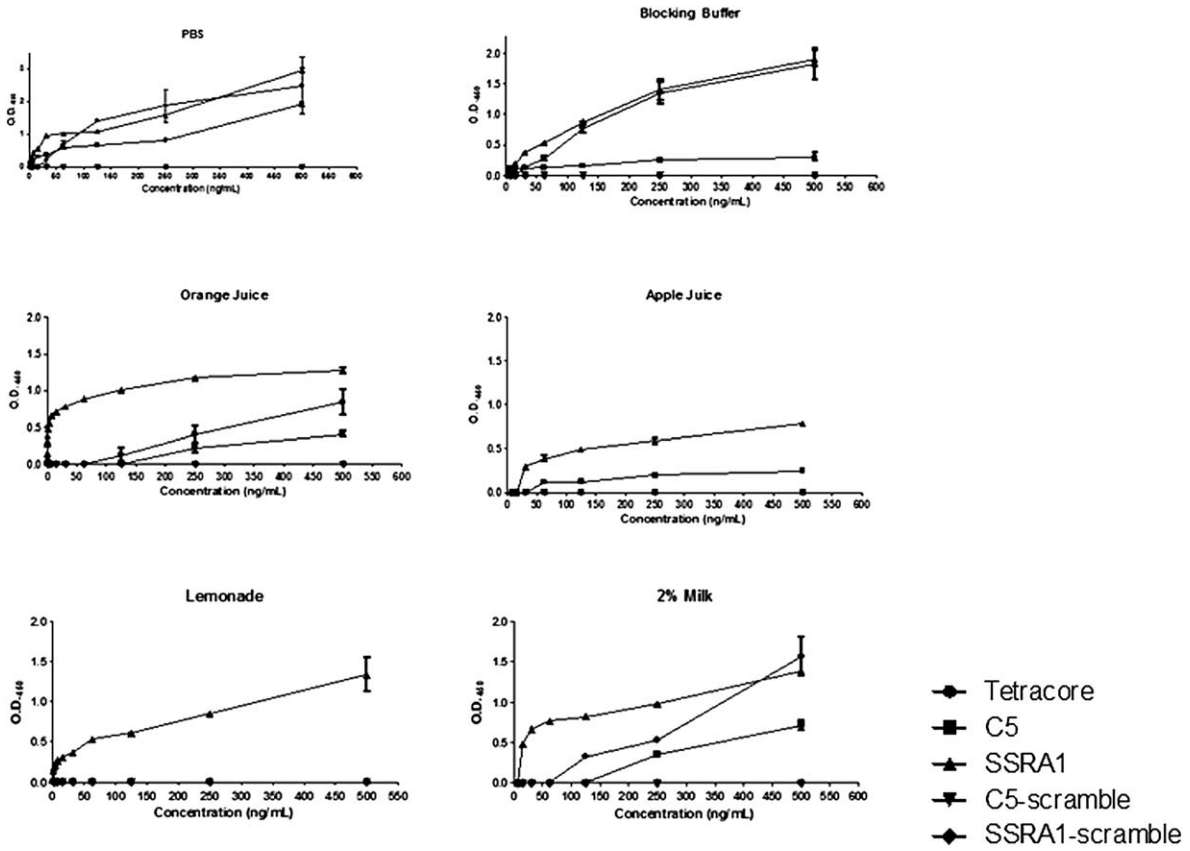

Fig. 5 SSRA1 has a greater range of detection compared to a commercialized ricin B chain ELISA and another published aptamer against ricin. Amine modified SSRA1 and C5 aptamers were bound separately to individual amine coated ELISA plates. SSRA1, C5 and the commercial ELISA were incubated with ricin B spiked neutral (pH = 7) liquid food samples. While SSRA1 and the commercial kit performed similarly at ricin B chain detection in blocking buffer, SSRA1 has an enhanced detection limit in orange juice, apple juice, lemonade and 2 per cent milk. The C5 aptamer displayed the least dynamic range of detection, which may be due to the method used to develop the aptamer.

3.2. SSRA1 concentrates dilute amounts of ricin B chain from liquid food matrices

A confounder in ricin B chain detection or concentration for pre-analytical purposes is complex food matrices.[1,3,4] Many liquid foods, such as milk, contain high amounts of lactic acid, which may bind to ricin and inhibit diagnostic tools from functioning properly.[16,31] In fact, a closely related compound, *Ricinus communis* Agglutinin 120 (RCA120) (also found in the castor bean

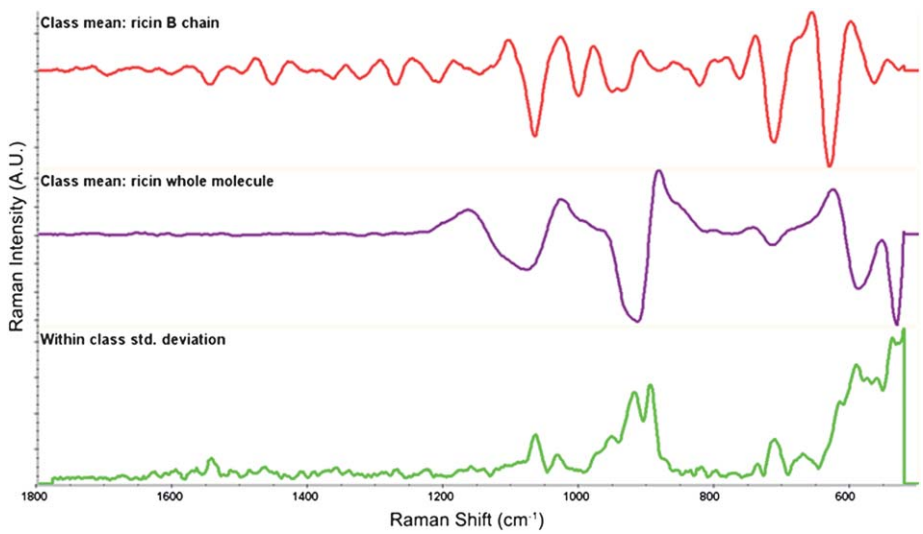

Fig. 6 SERS technique distinguishes ricin B chain from whole ricin toxin. Average spectra (second derivative transformation) of ricin B chain and ricin whole toxin.

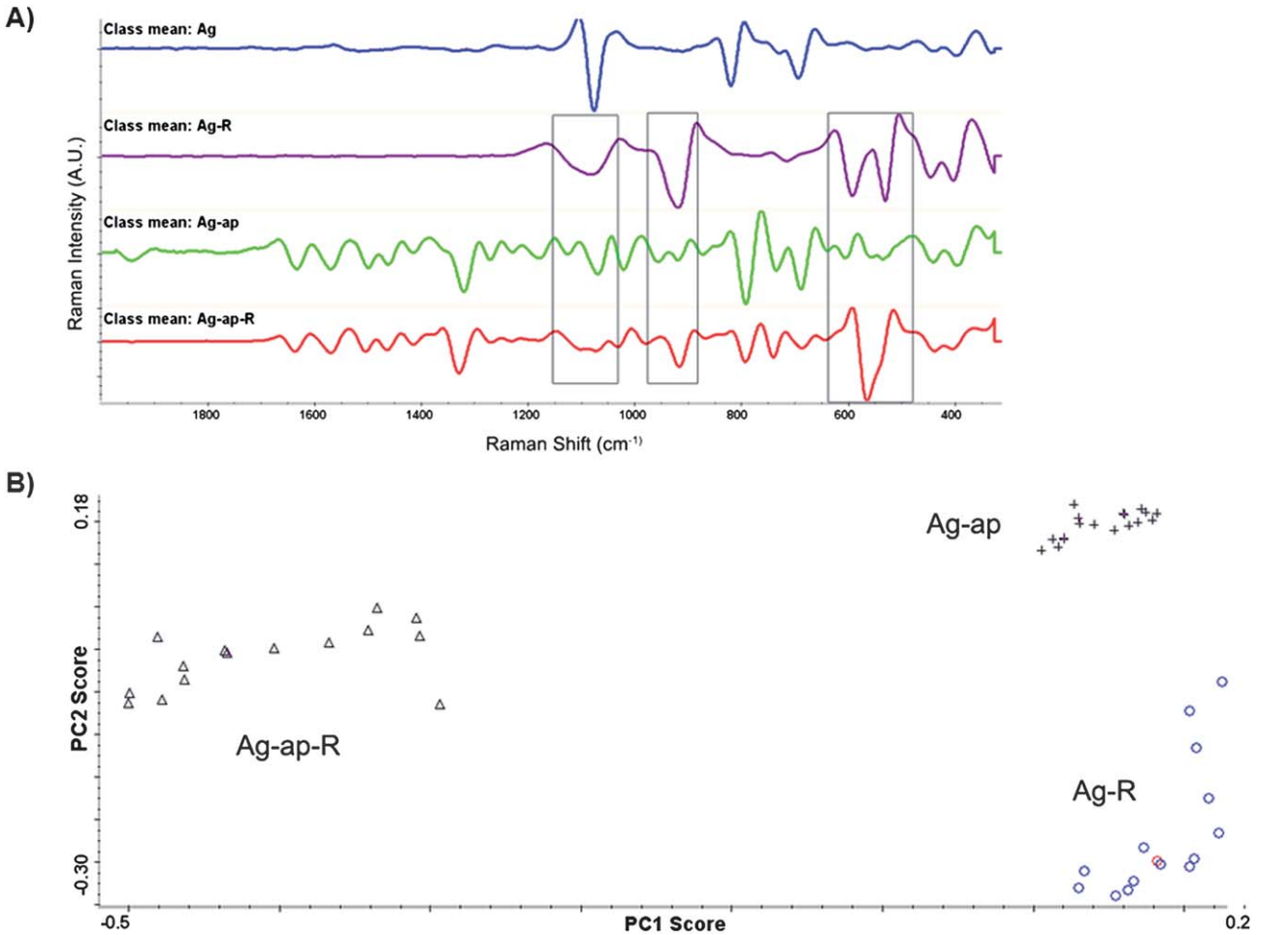

Fig. 7 SSRA1 captures the complete ricin toxin and is easily distinguished by changing spectra. (A) Average spectra (second derivative transformation) of Ag–Ap, Ag–Ap–R, and Ag–R. Ag. (B) PCA plot from SERS spectra of Ag–Ap (cross), Ag–Ap–R (open triangle), and Ag–R (open circles). Ag: silver, Ap: aptamer, R: ricin.

plant), binds to lactose and prevents sufficient binding to capture antibodies used in ELISAs.[31] Furthermore, ricin binding may also be impacted by pH.[17,32] For example, ricin binding decreases with pHs lower than 6.[17] This poses a significant problem for ricin detection in many juices that have a pH of 4 or lower. In order to determine if SSRA1 still performs in liquid food matrices, several pulldown assays using ricin B chain SSRA1 coated streptavidin magnesphere paramagnetic particles and gold ESF strips were conducted (Fig. 3 and Fig. 4). Supernatants from pulldown assays were saved to assess efficiency of SSRA1 concentration of ricin B chain. SSRA1 is capable of concentrating at least 100 ng mL^{-1} of ricin B chain from PBS, apple juice, orange juice, lemonade and 2 per cent milk *via* pulldown with streptavidin particles (Fig. 3). It is important to note that the pHs of these solutions range from 2 (lemonade) to 7 (2 per cent milk). SSRA1 is also capable of binding ricin B chain concentrations as dilute as 30 and 50 ng mL^{-1} (*data not shown*). Ricin B chain concentration from liquid foods *via* SSRA1 pulldown is extremely efficient as the B chain could not be detected in any of the supernatants (Fig. 3). Ricin B chain binding to streptavidin particles is dependent upon SSRA1 as unbound streptavidin particles were incapable of ricin B chain concentration from any solution (Fig. 3). Gold ESF are currently sought after as a new biosensor platform for food matrices due to its stability and high surface area, which increase binding to the target of interest.[33] Therefore, gold ESF strips were used in combination with thiol modified SSRA1 as a pre-analytical tool for ricin B chain detection. Gold ESFs strips conjugated to thiol modified SSRA1 concentrated 250 ng mL^{-1} of ricin B chain spiked in liquid food matrices (Fig. 4). Gold ESF strips alone did not bind to ricin B chain (Fig. 4). Thus, SSRA1 performance is not hindered by complex liquid food matrices and is efficient at concentrating dilute amounts of ricin B chain.

3.3. SSRA1 displays a wider range of detection for ricin B chain compared to a current commercial ELISA and previously published ricin aptamer

For purposes of pre-analytical processing, SSRA1 must have increased sensitivity (*i.e.* dynamic range) to ricin B chain binding compared to commercially available ELISA kits and ricin aptamers. Therefore, we developed a ricin B chain ELISA using SSRA1 as the "capture antibody" and compared the dynamic range of ricin B chain capture to a commercial ricin ELISA kit and a previously published ricin aptamer (C5).[30] PBS was used as a liquid control to calculate binding affinities for aptamer

comparison. The SSRA1 aptamer showed a K_d of 5 nM in contrast to C5, which was calculated to be 70 nM (Table 3). Both SSRA1 and the commercial ricin ELISA showed a similar detection range (31.2 ng mL^{-1}–500 ng mL^{-1} of ricin B chain) in blocking buffer spiked with ricin B chain (commercial kit blocking buffer) (Fig. 5). However, the other aptamer directed against ricin (referred to as C5) showed a limited detection range (125 ng mL^{-1}–500 ng mL^{-1}) (Fig. 5). This is also reflected by the binding affinities for SSRA1 and C5, K_d = 5 and 165, respectively. Like the blocking buffer, ricin B chain spiked in 2 per cent milk was easily detected by the commercial kit and SSRA1 aptamer (Fig. 5). The C5 aptamer was also able to detect ricin B chain in 2 per cent milk but at a limited dynamic range (250 ng mL^{-1}–500 ng mL^{-1}). Other liquid foods tested, particularly fruit juices, showed superior binding when SSRA1 was used as the capture antibody (Fig. 5). In both apple juice and lemonade, the commercial kit was not able to detect ricin B chain as opposed to SSRA1 that detected 7.8–500 ng mL^{-1} and 62.5 ng mL^{-1}–500 ng mL^{-1} in lemonade and apple juice, respectively (Fig. 3). Although the changes in optical density were not as great as in SSRA1 (K_d = 32 nM), the C5 aptamer (K_d = 120 nM) showed a similar detection range in ricin B chain spiked in apple juice (Fig. 5). Like the other juices, SSRA1 is more efficient at concentrating ricin B chain from orange juice in contrast to the commercial ELISA kit and C5 aptamer (Fig. 5). None of the aptamer scramble sequences (weighted for A, C, T, G content)

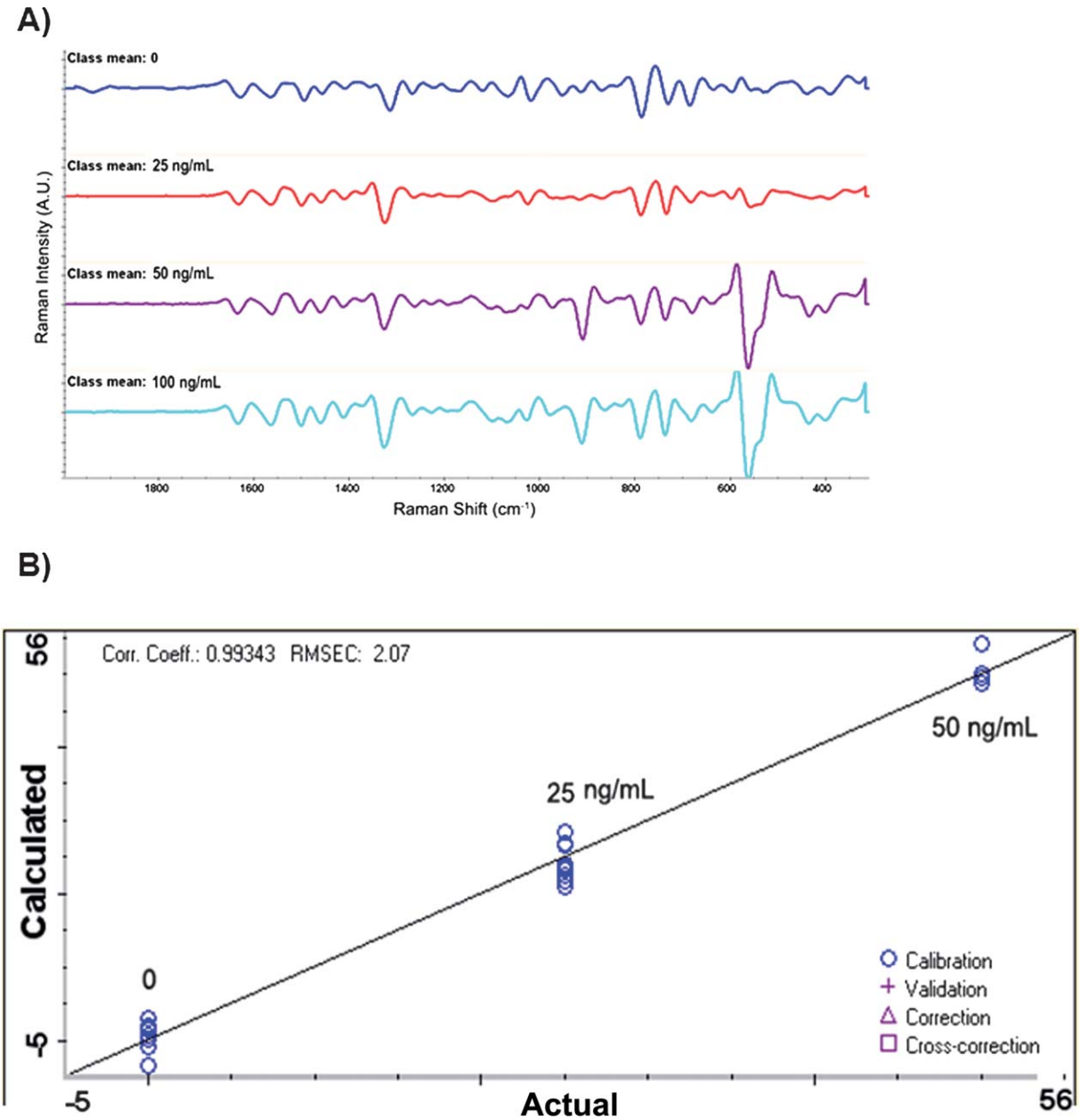

Fig. 8 SSRA1-SERS assay shows a dynamic range of 0–50 μg mL^{-1} of ricin. (A) Average spectra (second derivative transformation) of ricin at 0, 25, 50, and 100 μg mL^{-1} captured by aptamer modified silver dendrites. (B) PLS plot of ricin at 0, 25,and 50 μg mL^{-1} captured by aptamer modified silver dendrites.

reacted with ricin B chain for any of the spiked liquid samples (Fig. 5). Since all liquids were adjusted to a pH of 7 (to prevent breakage of the amine–amine bond), acidity was not a factor in inhibition of binding in the commercial kit and C5 aptamer. However, the C5 aptamer was generated against the whole ricin toxin and may specifically bind to the A chain or need the entire molecule to interact. It is also possible that the complex sugars present in juices may inhibit binding and further concentration by C5 and the commercial kit. These data suggest that SSRA1 may be used as a pre-analytical tool used to concentrate dilute amounts of ricin B chain from complex food matrices, which can be combined with other ricin detection systems, to be used in food surveillance.

3.4. SSRA1 detects complete ricin toxin using SERS technology

SERS techniques have shown great promise as rapid and sensitive analytical tools for molecular fingerprinting and detection based on scattering amplification in the near vicinity of nanoscale-roughened noble metal substrates.[34] In a previous study, SSRA1 combined with SERS was shown to function as a sensor for ricin B chain detection in liquid foods.[35] In order for ricin to be cytotoxic and consequently a viable bioterror agent, it must be intact (A chain and B chain linked by a disulfide bond). The possibility remained that SSRA1 may fail to bind to ricin B chain that is complexed to the A chain and would have limited utility for ricin detection in field samples. Therefore, the ricin B chain aptamer was tested to determine if: (1) SSRA1 was capable of binding the complete ricin toxin and (2) SSRA1–ricin binding performed with equal sensitivity as SSRA1–ricin B chain using SERS. SERS spectra data differed between ricin and ricin B chain (Fig. 6). This is advantageous as spectral changes due to aptamer capture of ricin is expected to have limited overlap in comparison to previously reported data using ricin B chain. In other words, resultant spectra from aptamer binding to ricin will be apparent from pre-capture spectra. 5′thiol/SSRA1 (ap) was conjugated to Ag dendrites prior to sensor use. After ricin (R) capture, changes in SERS spectra were used as the base of sensing. The spectrum of Ag–ap–R exhibited a clear spectra

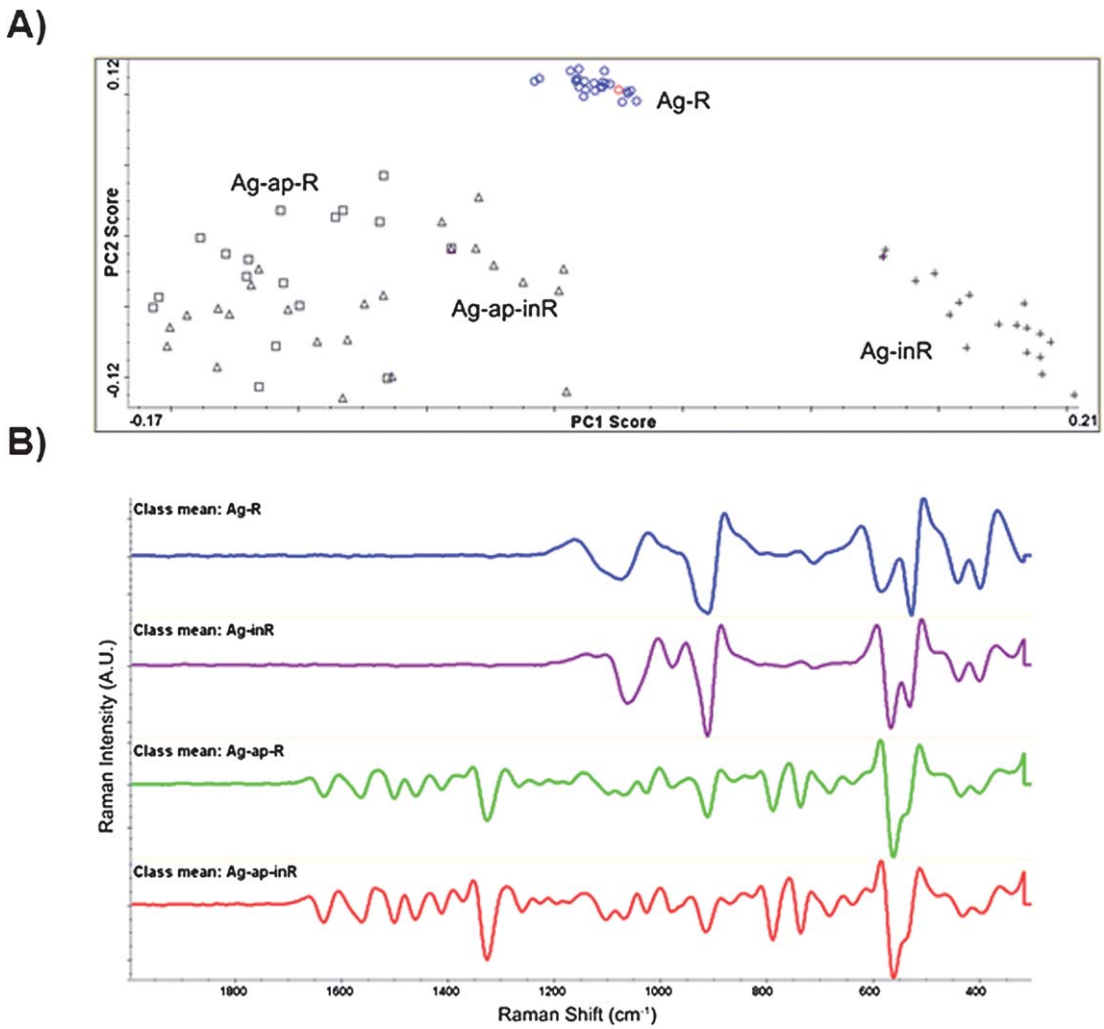

Fig. 9 A two-step SSRA1-SERS assay can distinguish native from inactive ricin. (A) PCA plot of native and inactivated ricin on Ag and captured by aptamer modified Ag dendrites. (B) Average spectra (second derivative transformation) of native and inactivated ricin on Ag and captured by aptamer modified Ag dendrites. Ag: silver, Ap: aptamer, R: native ricin, inR: inactivated ricin.

change at the 567, 916, 1079 cm^{-1} bands (Fig. 7A). PCA showed a clear separation of the data cluster between Ag–R, Ag–ap, and Ag–ap–R, which indicate the success of capture and detection of ricin using this technique (Fig. 7B). A dynamic range for the aptamer-SERS assay was established by using different concentrations of ricin. Spectra data show a clear increase of 567 and 916 cm^{-1} bands using 0 to 50 ng mL^{-1} of ricin; however, no significant difference was observed for ricin concentrations ranging from 50–100 ng mL^{-1} (Fig. 8A). No perceptible changes in spectra for greater concentrations of ricin may be due the Hook effect, which could indicate that aptamer capture of ricin has reached saturation.[36] These data indicate the potential quantitative ability at the range of 0 to 50 ng mL^{-1}, as shown in the PLS plot (Fig. 8B). The limit of detection and quantification of ricin in PBS was estimated to be 25 ng mL^{-1}. Compared with the previous SERS study on ricin B chain,[35] SSRA1–ricin binding performed with similar sensitivity as SSRA1–ricin B chain using SERS, but better quantification ability. However, one major concern of current hand-held-assays for ricin detection is the inability to differentiate between native and inactive ricin.[1] SSRA1 capture of denatured ricin (100 ng mL^{-1}) (inR) compared with native ricin was tested to determine differentiation potential. Inactivation of ricin is expected to expose more of its hydrophobic compounds. Post aptamer capture of ricin revealed similar spectra for both native and denatured ricin, which indicate that SSRA1 cannot differentiate between the two ricin forms (Fig. 9A). Direct conjugation of native and inactivated ricin on Ag dendrites showed major spectral differences, specifically at the 931–1160 cm^{-1} band assigned to the ricin three dimensional structure and hydrophobic compounds (Fig. 9B). The PCA plot showed separation when native/denatured ricin was bound to Ag dendrites in comparison to cluster overlap for aptamer capture (Fig. 9A). However, native/inactive ricin differentiation can be achieved using a two-step SSRA1-SERS assay by (1) aptamer capture of ricin and (2) breakage of the aptamer–ricin bond and direct conjugation to Ag dendrites. To summarize, SSRA1 combined with SERS may be used to detect the intact ricin toxin. The aptamer-SERS assay is easily amenable for native and inactive ricin differentiation.

Conclusions

This paper has described the characterization and selection of a single, dominant aptamer against ricin B chain using SELEX. SSRA1 binding of ricin B chain is not hindered by complex liquid food matrices and easily detects concentrations as low as 30 ng mL^{-1}. Additionally, the SSRA1 aptamer outcompeted a commercially available ELISA kit and published aptamer used to detect ricin. SSRA1 is readily modified to work with SERS technology to act as a sensor for ricin detection, which can also be used in a two-step system to distinguish native and inactive ricin. Thus, SSRA1 may be used as pre-analytical tool for ricin concentration or as a sensor for ricin detection.

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