UNIVERSAL FIELDABLE ASSAY WITH UNASSISTED VISUAL DETECTION

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References Cited


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ABSTRACT
A universal detection system based on allosteric aptamers, signal amplification cascade, and eye-detectable phase transition. A broadly applicable homogeneous detection system is provided. It utilizes components of the blood coagulation cascade in the presence of polystyrene microspheres (MS) as a signal amplifier. Russell’s viper venom factor X activator (RVV-X) triggers the cascade, which results in an eye-visible phase transition—precipitation of MS bound to clotted fibrin. An allosteric RNA aptamer, RNA132, with affinity for RVV-X and human vascular endothelial growth factor (VEGF 165) was created. RNA132 inhibits enzymatic activity of RVV-X. The effector molecule, VEGF 165, reverses the inhibitory activity of RNA132 on RVV-X and restores its enzymatic activity, thus triggering the cascade and enabling the phase transition. Similar results were obtained for another allosteric aptamer modulated by a protein tyrosine phosphatase. The assay is instrumentation-free for both processing and readout.

16 Claims, 11 Drawing Sheets
FIGURE 1.

A

100 amole RVV-X

10 amole RVV-X

1 amole RVV-X

100 zmole RVV-X

10 zmole RVV-X

100 amole BSA (control)

B

0.9

0.6

0.3

0

0

60

120

180

240

Time (minutes)

OD 450nm
FIGURE 2.

A  SEQ ID NO:1

B  SEQ ID NO:2

C  SEQ ID NO:3

D  SEQ ID NO:4
FIGURE 3.

A

B

\[ t_{1/2} \] (minutes)

RNA37s (fmole)

Relative Effect

Effectors (fmole)

- O VEGF_{165}
- △ PTPase
FIGURE 4
Phase transition (precipitate formation) caused by RVV-X in BCC-MS detection system

FIGURE 6
(A) Visual presentation of the 96-well plate obtained on a flatbed scanner at the time of 50% reduction in absorbance (T50) for a given concentration of RVV-X.

(B) Optical density of the same wells at 405 nm measured on a microplate reader at 5-minute intervals.

FIGURE 7
Schematic Representation of the Detection System based on Blood Coagulation Cascade with Microspheres (BCC-MS)

FIGURE 8
Inhibitory effect of RNA37s on the RVV-X-induced phase transition in the BCC-MS detection system
The effect of VEGF$^{165}_{\mathrm{f mole}}$ on the inhibitory action of RNA132 on RVV-X in RNA132-controlled BCC-MS detection system

FIGURE 10
Visual presentation of the kinetics of phase transition in the RNA 132-controlled BCC-MS detection system in the presence of different proteins

Buffer
Human VEGF_{165}
Murine VEGF_{165}
Zebrafish VEGF_{165}
Human VEGF_{121}
Endocrine Gland VEGF
Human VEGF/P/IGF
BSA
RNase
No RNA132
No RVV-X

0 15 25 30 35 40 MINUTES

FIGURE 11
UNIVERSAL FIELDABLE ASSAY WITH UNASSISTED VISUAL DETECTION

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. Provisional Patent Application Ser. No. 60/779,994, filed on Mar. 6, 2006.

FUNDING

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FIELD OF THE INVENTION

The present invention relates in general to aptamers. More specifically, the invention provides a universal detection system based on allosteric aptamers, signal amplification cascade, and eye-detectable phase transition.

BACKGROUND OF THE INVENTION

Detecting specific molecules is a crucial task for medicine, biotechnology, chemical and biodefense, and environmental protection. Many new detection systems are developed every year with increasing specificity and sensitivity. These systems include the latest developments in biotechnology and nanotechnology. All of them have one common feature: they employ sophisticated and expensive equipment for the processing and/or the readout of the results.

SUMMARY OF THE INVENTION

A broadly applicable homogeneous detection system has been developed. It utilizes components of the blood coagulation cascade in the presence of polystyrene microspheres (MS) as a signal amplifier. Russell’s viper venom factor X activator (RVV-X) triggers the cascade, which results in an eye-visible phase transition—precipitation of MS bound to clotted fibrin. An allosteric RNA aptamer, RNA132, with affinity for RVV-X and human vascular endothelial growth factor (VEGF_{165}) was created. RNA132 inhibits enzymatic activity of RVV-X. The effector molecule, VEGF_{165} reverses the inhibitory activity of RNA132 on RVV-X and restores its enzymatic activity, thus triggering the cascade and enabling the phase transition. As few as five femtomoles of VEGF_{165} could be detected by the naked eye within an hour. Similar results were obtained for another allosteric aptamer modulated by a protein tyrosine phosphatase. The assay is instrumentation-free for both processing and readout and can be modified to detect molecules to which aptamers can be obtained.

The above-mentioned and other features of this invention and the manner of obtaining and using them will become more apparent, and will be best understood, by reference to the following description, taken in conjunction with the accompanying drawings. The drawings depict only typical embodiments of the invention and do not therefore limit its scope.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Kinetics of the phase transition (precipitation of MS bound to the clotted fibrin) in the BCC-MS amplification cascade triggered by the different concentrations of RVV-X. (A) Visual presentation of the 96-well plate obtained on a flatbed scanner at the time of 50% reduction in absorbance (t(50)) for a given concentration of RVV-X. (B) Optical density at 405 nm measured on a microplate reader in the same well at 5-minute intervals.

FIG. 2. Schematic representation of the secondary structure folding for the RNA aptamers. (A) RNA37s (SEQ ID NO:1), (B) VTr44(-2)(SEQ ID NO:2), (C) RNA132 without pseudoknots (SEQ ID NO:3), (D) RNA132 with pseudoknots (SEQ ID NO:4).

FIG. 3. Kinetics of the phase transition in the BCC-MS detection system. (A) In the presence of different concentrations of RNA37s. (B) The reversal of the inhibitory effect of RNA132 on the phase transition by different concentrations of Human VEGF_{165} and PTPase. The relative effect was calculated according to the formula in the Experimental Procedures section.

FIG. 4. Gel-shift analysis of RVV-X and Human VEGF_{165} binding to RNA37s and RNA132.

FIG. 5. Visual presentation of the kinetics of clot formation in the RNA132-controlled BCC-MS detection system in the presence of 10 fmole of different proteins, except for 100 amole of RNase. Proteins were incubated with a mix of RVV-X and RNA132 for 15 minutes at room temperature in 5-10 µL of IC buffer, followed by the addition of the components of the BCC-MS detection system. At the indicated time points, wells of the 96-well plate were scanned on a flatbed scanner. The results are presented in duplicates.

FIG. 6. Phase transition (precipitate formation) caused by RVV-X in BCC-MS detection system.

FIG. 7. Kinetics of phase transition (precipitate formation) caused by RVV-X in BCC-MS detection system.

FIG. 8. Schematic representation of the detection system based on blood coagulation cascade with microspheres (BCC-MS).

FIG. 9. Inhibitory effect of RNA37s on the RW-X-induced phase transition in the BCC-MS detection system. RNA37s was incubated with RVV-X for 15 minutes at room temperature. One hundred microliters of the BCC-MS reaction mix were dispensed into wells of a 96-well plate to which 5-10 mL of a test solution were added. After that the plate was immediately placed into a microplate reader (Genios, Tecan) and shaken for 10-30 seconds prior to OD_{405} measurements at 5-10 minute intervals. T(50) is the time for a 50% decrease in OD_{405}.

FIG. 10. The effect of VEGF_{165} on the inhibitory action of RNA132 on RVV-X in RNA132-controlled BCC-MS detection system. In the RNA132-controlled BCC-MS experiment, VEGF_{165} reversed inhibitory action of RNA132 on the RVV-X-triggered phase transition in a concentration-dependent mode. This effect implies competition between VEGF_{165} and RVV-X for binding to RNA132. Dynamic range of the detection system appeared to be narrow, i.e. about 10-fold range of VEGF_{165} concentrations (6-60 fmol/reaction mix), which is typical for BCC-based assays. The above sensitivity of the assay is within the lower half of the linear range of the VEGF_{165} concentrations detected by ELISA assays commercially available from, e.g., R&D Systems or Cell Sciences. But components of the BCC-MS detection system presented here allow adjusting their relative concentrations to detect the molecule of interest within a wider range of concentrations. The formula to calculate relative effect of different concentrations of VEGF165 is as follows:

\[
\frac{C_{RNA132_{165} + VEGF165} - C_{RNA132_{165} + VEGF165_{null}}}{C_{RNA132_{165} + VEGF165_{null}} - C_{RNA132_{165} + VEGF165}}
\]

T(50)|RNA132_{165} + VEGF165_{null} - T(50)|RNA132_{165} + VEGF165

T(50) is the time for a 50% decrease in OD_{405}.

5
10
15
20
25
30
35
40
45
50
55
60
65
FIG. 11. Visual presentation of the kinetics of phase transition in the RNA132-controlled BCC-MS detection system in the presence of different proteins. Five femtomoles of different proteins (except for 100 fmole of RNase) were incubated with a mix of RVV-X and RNA132 for 15 minutes at RT in 5-10 uL of IC buffer, followed by the addition of the components of the BCC-MS detection system. At the indicated time points, wells of the 96-well plate were scanned on a flatbed scanner. The results are presented in duplicates.

Human VEGF165, Murine VEGF165, and Zebrafish VEGF165 have both the same biological function and contain 165 of amino acids. Murine VEGF165 has an 89% homology with Human VEGF165 where 147 of 165 amino acids are identical. Zebrafish VEGF165 has about a 62% amino acid sequence identity with Human VEGF165. Human VEGF121 is a truncated version of Human VEGF165, with 44 amino acids truncated downstream from position 110 of the polypeptide chain. Thus cysteine 137 of VEGF165 that forms a photo-inducible cross-link to a uridine at position 14 of RNA VTt44 (which corresponds to position 13 of RNA VT144(--2)) and position 18 of RNA132 is missing in VEGF121. Human VEGF/P/GF heterodimer is a protein artificially dimerized in vitro. Both full length and truncated variants of Human VEGF165 and Placental growth factor Endocrine gland VEGF is a protein of irrelevant amino acid sequence consisting of 86 amino acids.

Detailed description of the invention

Aptamers play an increasingly important role in biotechnology and nanotechnology. They can acquire allosteric properties similar to enzymes. There is no established terminology in the field, and these aptamers are variously called allosteric, ligandregulated, ligand-dependent, structure-switching signaling, or modular. Armed with fluorophores, these aptamers can be used for detecting "effector" molecules, where the readout is performed using the spectrofluorometer. In order to free the readout procedure from any kind of instrumentation, an approach utilizing biochemical amplification is explored.

The biochemical amplification, resulting in an eye-detectable phase transition, is accomplished using the components (Factors X, Va, Prothrombin, and Fibrinogen) of the blood coagulation cascade (BCC). When a specific metalloproteinase, the Russell's viper venom factor X activator (RVV-X), is added to the components of BCC, the cascade is triggered. Triggering of BCC in the context of polystyrene microspheres (MS) results in the eye-detectable clot formation (precipitate of MS bound to fibrin). An RNA aptamer (RNA37s) was selected to RVV-X. When RVV-X is mixed with RNA37s, and the resulting product is added to the components of BCC, clot formation is delayed. The fusing of RNA37s to another aptamer (RNAVT144), which binds to human Vascular Endothelial Growth Factor (VEGF165), produced a fusion-aptamer RNA132. When RVV-X is mixed with RNA132 and the resulting product is added to the components of BCC, clot formation is also delayed. However, when RVV-X is mixed with RNA132 and then VEGF165 is added, the resulting product does not delay clot formation when added to the components of BCC. Hence RNA132 is an allosteric aptamer. The sensitivity of the above assay for VEGF165 is essentially the same as the sensitivity of commercially available ELISA. Similar results were obtained for another allosteric aptamer (RNA37sN71 (SEQ ID NO:5)) that was created by fusion of RNA37s with an aptamer for a protein tyrosine phosphatase from *Yersinia enterocolitica*. Presumably this method can be used to create detection systems for other molecules to which aptamers can be selected.

Advantages of the above detection system include: only two pipetting cycles (compared with approximately 10 in ELISA), no washes, no reagents immobilized on solid support, and visual readout without any instrumentation.

SELEX

An RNA aptamer binding to RVV-X was obtained via standard SELEX protocol. The progress of the selection was monitored by determining the dissociation constant of the RNA-protein complex (K_D) and by determining how much RNA binds to a single "high" concentration (50 nM) of the protein target in a single point binding assay. Initial pool of unselected RNA (RNA0) had a K_D value of 550±30 nM with the binding of 6.3% in a single point binding assay when the IC binding buffer was used for washing and 2.2% when IC binding buffer+300 mM NaCl (high salt) was used for washing, with 1.2% background noise. After five rounds of selection, K_D for RNA5 dropped to 22±3.0 nM; binding in a single point binding assay increased to 23% and 7% respectively. An attempt to clone and sequence at this point failed to produce a consensual sequence of reasonable length. After the ninth round of selection, K_D for RNA9 went down to 2.2±0.9 nM, and the results for the single point binding assay were 55% and 43% respectively, with 2% background noise. A consensual sequence for RNA9c was derived from the analysis of 45 clones.

The full length RNA9c (87 bases) containing a consensual 40-mer sequence flanked by the primers showed inhibitory activity of 43% in the RVV-X-SPZxa test. A systematic deletion mutagenesis applied to RNA9c allowed to isolate 43-mer RNA37s. RNA37s showed 84% inhibitory activity in the RVV-XSPZxa test. It also showed concentration-dependent inhibitory effect on the RVV-X-induced clot formation in the BCC-MS detection system. An aptamer binding to VEGF165, RNA VT144, with one base snipped from both 5'- and 3'-ends (VT144(--2), was inserted into RNA37s five bases downstream from its 5'-end to create an allosteric aptamer RNA132. This structure appeared to be more stable with AG=−24.0 kcal/mol, compared with the "head to tail" version, when RNA37s was directly linked with the 3'-end of RNA VT144(--2), AG=−18.0 kcal/mol (28-30). It is important to note that the putative RNA binding domains for both proteins stayed unharmed in the chimeric RNA132. A possible conformation including pseudoknots was also predicted with AG=−27.0 kcal/mol.

The following examples are intended to illustrate, but not to limit, the scope of the invention. While such examples are typical of those that might be used, other procedures known to those skilled in the art may alternatively be utilized. Indeed, those of ordinary skill in the art can readily envision and produce further embodiments, based on the teachings herein, without undue experimentation.

Examples

This invention presents an approach for a broadly applicable detection system without using any instrumentation for both processing and readout.

In the last several years aptamers have become widely used as sensors and diagnostic agents with a high level of specificity and sensitivity (1-10). Among other advantages, aptamers can be used in homogeneous assays, thus eliminating reagent immobilization and reducing processing time and manipulation steps (4, 10). Aptamers can acquire allosteric properties similar to enzymes (8, 11-16), and therefore they can be used for detecting ‘effector’ molecules (8, 15). To achieve the level of sensitivity that rivals more conventional immunoassays (1, 2, 15), aptamer-based detection systems...
also have to use sensitive and sophisticated instrumentation for processing and readout. In order to free the readout and the processing procedure from any kind of instrumentation, an approach is explored based on allosteric aptamers controlling the biochemical amplification cascade. The biochemical amplification cascade is based on the components of the blood coagulation cascade (BCC.) in the presence of poly-styrene microspheres (MS). BCC-MS amplification cascade, results in an eye-visible phase transition, i.e. the precipitation of MS bound to the clotted fibrin. Controlled by an allosteric aptamer, BCC-MS amplification cascade becomes BCC-MS detection system.

Experimental Procedures

Materials. All plasticware including 96-well plates (flat bottom) used in this study were non-stick or low binding from Ambion and Corning. RNase-free water was from Ambion. Reagents for the human blood coagulation cascade: Fibrinogen, Prothrombin, Factor Va, Factor X and also snake venom protease RVV-X and phospholipid vesicles (PCPS) were purchased from American Diagnostics Technologies. SPECTROZYME® Fxa (SXPZxa) was from American Diagnostics, PCR and RT-PCR reagents and T7 RNA Polymerase kits were from Promega and Epicentre. Human VEGF165, human VEGF121, murine VEGF165, zebrafish VEGF65, endocline gland VEGF, and human VEGF/PiGIF heterodimer were from R&D Systems and USB. Polystyrene microspheres were from Bangs Laboratories. Ready-made polycrylamide gels were from Invitrogen. Oligonucleotides were synthesized and purified by IDT or Qiagen.

All reactions took place in 50 mM imidazole-HCl, 3 mM CaCl2 buffer, pH 7.8, (IC buffer). Snapshots of the 96-well plates were taken by a Memorex flatbed scanner, model 6142c, BCC-MS Amplification Cascade. A typical reaction mix contained 600 nM PCPS, 230 nM Fibrinogen, 170 nM Prothrombin, 870 FM Factor Va, 580 pM Factor X and 1/450-1/350 dilution of polystyrene microspheres (10% solids) 0.77-1.0 µm in diameter in IC buffer. One hundred microliters of the reaction mix were dispensed into wells of a 96-well plate. Volumes for the 5'-end of RNA37s with the 3'-end of an effector aptamer was taken 10 minutes after the onset of the reaction. The inhibitory effect of an aptamer on the RVV-X activity was calculated according to the formula:

RNA Folding and Minimum Free Energy Calculations. Predictions of the RNA folding patterns with or without pseudoknots and calculations of minimum free energy (AG) were done according to algorithms and software presented in (23, 24). Visual representation of the RNA secondary structure based on the above predictions was done using Pseudoview2 software (25).

DNA Competition Assay for Mapping of RNA132 Binding Sites for RVV-X and VEGF165. DNA molecules complementary to various segments of RNA132 were synthesized (see the list in Table 1) and used as competitors to RVV-X and VEGF165 in binding to 32P-labeled RNA132. The complexes were analyzed in a gel-shift assay utilizing 6% PAGE (0.5x TBE). RNA132 (100 fmol) was annealed with DNA (200 fmol) by incubating the mix at 70° for 1 minute with slow cooling to room temperature. Proteins were added to the complex at room temperature and after 15 minutes of incubation the samples were subjected to PAGE. Volumes for bands, determined by Phosphorimager “Storm” (Molecular Dynamics), corresponding to RNA132 complexes with RVV-X or VEGF165, with no competing DNA added, were taken as 100% binding. When a full-length DNA complement (DNA I) was competing with the proteins for binding to RNA132, no bands corresponding to the RNA-protein complexes were detected for either of the proteins, thus binding for them was 0%. In a control experiment proteins were annealed with the DNA used at tenfold higher concentrations. No binding was detected between competing DNA and the proteins.
Results

Homogeneous BCC-MS Amplification Cascade. A biochemical signal amplification cascade, utilizing components of the BCC cascade in the presence of MS was described as a part of a heterogeneous ELISA assay (26). The BCC portion of the amplification cascade consisted of Factors X, Va, (Prothrombin), and I (Fibrinogen). BCC was triggered by a specific metalloproteinase, the Russell’s viper venom factor X activator (RVV-X) (27). RVV-X-initiated BCC resulted in an eye-visible phase transition, i.e., precipitation of MS bound to the clotted fibrin. The assay showed sensitivity of 10-100 fg/ml for RVV-X, which corresponds to 10-100 zeptomoles of the protein detected in 100 µL of a test solution in 60 minutes. The disadvantages of the above assay include its basis on a standard solid support ELISA format that requires multiple washes and reagent transfers. Furthermore, the above assay relies on several specific, not readily available reagents, one of which is a monoclonal antibody conjugated to RVV-X (26).

This invention provides an effective approach overcoming these issues. An allosteric aptamer would substitute for antibodies and conjugates, and would render the assay homogeneous, eliminating washing and minimizing reagent transfer steps. An allosteric aptamer will bind to RVV-X and inhibit its enzymatic activity, while subsequent binding of an effector molecule to the aptamer will modulate the inhibitory effect by reversing it. Thus, an on/off switch for the RVV-X activity will be created depending on the presence of an effector molecule. The allosteric aptamer-controlled detection system based on BCC in the presence of MS (BCC-MS) will require only one variable component—a part of the allosteric aptamer specific to the effector molecule.

Components for the homogeneous BCC-MS amplification cascade were derived from (26, 28). Modifications included substitution of the Factor V with the Factor Va, and there are commercially available highly sensitive ELISA adequate controls for the specificity of the detection system; several natural analogs of the human VEGF 165 that can be used for washing, and 2.2% of the RNA0 stayed bound to the filter when the high salt buffer was used for washing, with 1.2% of background noise. After the ninth round of selection, Kd for RNA9 decreased to 2.2±1.9 nM, and the results for the single point binding assay were 55% and 43% respectively, with 2% background noise. A consensus sequence (RNA9c) was derived from the analysis of 45 clones of RT-PCRd RNA9.

The full length RNA9c (87 bases) containing the consensus 40-mer sequence flanked by the primers showed a 43% inhibitory effect on the RVV-X enzymatic activity in the RVV-X-SPZXa assay. A systematic deletion mutagenesis applied to RNA9c enabled the isolation of a minimal 43-mer RNA37s (FIG. 2A) with 84% inhibitory effect on RVV-X in the RVV-X-SPZXa assay. RNA37s also showed a concentration-dependent inhibitory effect on the RVV-X-induced clot formation in the BCC-MS detection system (FIG. 3A). Binding of RNA37s to RVV-X was confirmed in a gel-shift experiment (FIG. 4).

Allosterizing of the Aptamer to RVV-X Human VEGF 165 was chosen as an effector molecule for the construction of an allosteric aptamer. The choice of VEGF 165 was based on several criteria, including the availability of high affinity aptamers to VEGF (21, 22), one of which was shown to be an efficient anti-VEGF 165 agent in vivo (29). Also there are several natural analogs of the human VEGF 165 that can be used as reference standards.

An aptamer binding to VEGF 165, RNA ligand VTt44 (22), with one base omitted from both 5’- and 3’-ends, VTt44(−2), (FIG. 2B), was inserted into RNA37s five bases downstream from its 5’-end to create the allosteric aptamer RNA132 (FIG. 2C). This structure appeared to be more stable when compared with the “head to tail” version, where the 5’-end of RNA37s was directly fused with the 3’-end of VTt44(−2) (ΔG=−24.0 kcal/mol versus ΔG=−18.0 kcal/mol) (23, 24). Presumably, putative RNA binding domains stayed accessible for both proteins in the chimeric RNA132. A possible conformation including pseudoknots was also predicted for RNA132 with ΔG=−27.0 kcal/mol (FIG. 2D) (23).

In a gel-shift assay, RVV-X and VEGF 165 were shown to bind to RNA132 at about equal concentrations (FIG. 4). The dissociation constants for both proteins, 5.7±2.9 nM, appeared to be identical within experimental error. RNA132 was also tested in the RVV-X-SPZXa assay and showed 86% inhibitory effect similar to that of RNA37s within
experimental error. As tested in the BCC-MS detection system, concentration-dependent inhibitory effect of RNA132 on the phase transition was also similar to RNA37s within experimental error.

The Effector Molecule, VEGF<sub>165</sub>. Reverses Inhibition of the Phase Transition Caused by RNA132. As was shown in the RNA132-controlled BCC-MS detection system, VEGF<sub>165</sub> reversed the inhibitory effect of RNA132 on the RVV-X-triggered phase transition in a concentration-dependent mode (FIG. 3B). This effect suggests a competition between VEGF<sub>165</sub> and RVV-X for binding to RNA132. Dynamic range for the effect of VEGF<sub>165</sub> on the reversal of the phase transition in the RNA132-controlled BCC-MS detection system appeared to be narrow, i.e. about 10-fold range of VEGF<sub>165</sub> concentrations (3-30 fmol). This was shown to be typical for the BCC-based detection systems (28). The sensitivity of the detection of VEGF<sub>165</sub> in the RNA132-controlled BCC-MS detection system is within the lower half of the linear range for the VEGF<sub>165</sub> concentrations detected by commercial ELISA assays.

Another allosteric aptamer (RNA37sN71) modulated by a different effector molecule was created by fusing RNA37s with an aptamer to a protein tyrosine phosphatase (PTPase) from *Yersinia enterocolitica*, N71 (30). In the RNA37sN71-controlled BCC-MS detection system, the PTPase also reversed the inhibitory effect of RNA37sN71 on the RVV-X-triggered phase transition in a concentration-dependent mode (FIG. 3B), though the curve describing the relative effect of the PTPase was shifted to higher concentrations of PTPase as compared with VEGF<sub>165</sub>.

Specificity of the BCC-MS Detection System. FIG. 5 presents the data on the specificity of the RNA132-controlled BCC-MS detection system for human VEGF<sub>165</sub>. Human VEGF<sub>165</sub>, murine VEGF<sub>165</sub> and zebrafish VEGF<sub>165</sub> have both the same biological function and contain 165 of amino acids. Murine VEGF<sub>165</sub> has an 89% identity with human VEGF<sub>165</sub>, where 147 of 165 amino acids are identical. Zebrafish VEGF<sub>165</sub> has about a 62% amino acid sequence identity with human VEGF<sub>165</sub>. Human VEGF<sub>165</sub> is a truncated version of human VEGF<sub>165</sub> with 44 amino acids truncated downstream from position 110 of the polypeptide chain. Thus cysteine 137 of VEGF<sub>165</sub> forms a photo-inducible cross-link to a uridine at position 14 of RNA VTt44 (22) (which corresponds to position 13 of RNA VT44 (22) and position 18 of RNA132) is missing in VEGF<sub>165</sub>. Human VEGF/P1GF heterodimer is a protein artificially dimerized in vitro. It consists of human VEGF<sub>165</sub> and Placental growth factor. The amino acid sequence of endocrine gland VEGF is unrelated to that of human VEGF. The kinetics of the clot formation were similar in the wells containing human VEGF<sub>165</sub> and murine VEGF<sub>165</sub>. In both cases, the coagulation process was complete within 40 minutes. This was not surprising, since human and murine VEGF<sub>165</sub> are highly homologous. At the same time point (40 minutes) when the wells containing human VEGF<sub>165</sub> demonstrated completed coagulation, the wells containing zebrafish VEGF<sub>165</sub> demonstrated the onset of coagulation. It can be explained by less homology (62%) between human VEGF<sub>165</sub> and zebrafish VEGF<sub>165</sub>. Meanwhile in the wells containing human VEGF<sub>121</sub>, endocrine gland VEGF, human VEGF/P1GF, and BSA, phase transition has not been observed by that time. The results in FIG. 5 clearly demonstrate the high specificity of the detection system.

Exploration of the Possible Mechanism of the Allosteric Effect. An attempt was made to find a mechanism underlying the competition between VEGF<sub>165</sub> and RVV-X for the interaction with RNA132. DNA molecules complementary to different segments of RNA132 were used in the competition binding experiments of RVV-X and VEGF<sub>165</sub> to RNA132 (see Table 1). When a full-length DNA complement to RNA132 (DNA1) was used, no binding was observed for either of the proteins. When DNAI and DNAII compete for the RVV-X binding to the 37s domain of RNA132, predictable low levels of RVV-X binding were detected. Annealing of DNAIV to the VT44 domain of RNA132 significantly decreases VEGF<sub>165</sub> binding to RNA132. When shorter DNA fragments corresponding to the VT44 domain of RNA132 (DNAV and DNAVI) were used for the competition, the impact of competing DNA on VEGF<sub>165</sub> affinity for RNA132 is decreased. An unexpected inhibitory effect of DNAIV and DNAV on RVV-X binding to RNA132 was also observed. A possible explanation is that the putative stem located between positions 2-8 and 28-34 in RNA132 (FIG. 2C) is involved in the binding of both proteins. This can result in direct stochastic competition between the proteins. One can also speculate that the decrease of RVV-X binding in the presence of DNAIV and DNAV can be explained by assuming an alternative folding of RNA132 into a structure containing pseudoknots (FIG. 2D).

Further theoretical and experimental analysis is required to more clearly describe the molecular interactions resulting in allosteric properties of RNA132.

Discussion

In this study, an allosteric RNA aptamer-based competitive homogeneous detection system with readout by the naked eye was achieved. This became possible by coupling an allosteric RNA aptamer with the biochemical signal amplification cascade, BCC-MS. The signal amplification cascade results in an eye-visible phase transition, i.e. formation of the precipitate of polystyrene microspheres bound to clotted fibrin. The allosteric aptamer contains a domain that binds to RVV-X thus inhibiting BCC. There is another domain on the allosteric aptamer, which binds to an effector molecule reversing the effect of the first domain. The latter domain of the aptamer is the only variable part of the detection system. Therefore, adjusting the detection system to a new effector molecule will involve only one molecular component—an aptamer to the effector molecule. Further, the detection system requires only 2 pipetting cycles compared with 10 in the case of ELISA, and it requires less than an hour for its completion, compared with 4.5 hours for ELISA. Most importantly, the naked eye is used as a readout instrument instead of expensive and rarely available equipment. These features make the detection system a good candidate as a platform for fieldable detection systems and make it competitive with ELISA.

While the present invention has been described herein with specific details by way of illustrations and examples, those of ordinary skill in the art will readily recognize that numerous modifications, substitutions, and alterations are possible without departing from the spirit and scope of the invention. It is intended that all of these modifications and variations be within the scope of the present invention as described and claimed herein, and that the invention be limited only by the scope of the claims which follow, and that such claims be interpreted as broadly as reasonable.

REFERENCES

The following references are cited herein. The entire disclosure of each reference is relied upon and incorporated by reference herein.


What is claimed is:

1. A detection system comprising:
   a) a fusion aptamer formed by fusing a first aptamer to a second aptamer;
   b) Russell’s viper venom factor X activator (RVV-X); and
   c) a biochemical signal amplification subsystem comprising blood coagulation components and a signaling agent,

wherein said first aptamer is one specific for RVV-X, said second aptamer is one specific for a target molecule to be detected, and said signaling agent is one capable of interacting with the product of blood coagulation cascade reaction to give a visual signal readable by the naked eye.

2. The detection system according to claim 1, wherein the first aptamer is one having a sequence according to SEQ ID No: 1.

3. The detection system according to claim 1, wherein said signaling agent is a polystyrene microsphere.

4. The detection system according to claim 1, wherein the second aptamer is one specific for human vascular endothelial growth factor (VEGF₁₆₅) or a protein tyrosine phosphatase from Yersinia enterocolitica.

5. The detection system according to claim 1, wherein said fusion aptamer is one having a sequence according to SEQ ID No: 4.

6. A fusion aptamer formed by fusing a first aptamer specific for RVV-X and a second aptamer specific for a target molecule.

7. The fusion aptamer of claim 6, wherein said first aptamer is one having a sequence according to SEQ ID No: 5.

8. The fusion aptamer of claim 6, wherein said fusion aptamer having a sequence according to SEQ ID No: 6.

9. The fusion aptamer of claim 6, wherein said fusion aptamer having a sequence according to SEQ ID No: 7.

10. A method for detecting a target molecule in a sample, comprising:
contacting the sample with RVV-X, a fusion aptamer, blood coagulation components and a signaling agent, wherein said fusion aptamer is formed by fusing a first aptamer specific for RVV-X and a second aptamer specific for said target molecule, said signaling agent is one capable of interacting with the product of blood coagulation cascade reaction to give a visual signal readable by the naked eye, and whereby presence of the target molecule in the sample initiates a blood coagulation cascade reaction to give a visual signal.

11. The method of claim 10, wherein said RVV-X, fusion aptamer, blood coagulation components, and signaling agent are pre-mixed together prior to contacting the sample.

12. The method of claim 10, wherein said sample, blood coagulation components and signaling agent are brought in contact first before a mixture of RVV-X and the fusion aptamer are added.

13. The method of claim 10, wherein said first aptamer specific for RVV-X is one having a sequence according to SEQ ID No: 1.

14. The method of claim 10, wherein said fusion aptamer is one having a sequence according to SEQ ID No: 3 or SEQ ID No: 5.

15. The method of claim 10, wherein said target molecule is one selected from VEGF₁₆₅ or a protein tyrosin phosphatase.

16. The method of claim 10, wherein said signaling agent is a polystyrene microsphere.