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 a 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

B

Time (minutes)
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

Effectors (fmole)

RNA37s (fmole)

Relative Effect

Effectors (fmole)
FIGURE 4

[Insert diagram showing RNA37s and RNA132 experiments with different conditions and protein concentrations.]
FIGURE 5

0 15 25 30 35 40

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

inactive $BCC-MS \rightarrow$ active $BCC-MS$

RNA132 $+ \rightarrow$ inactive $BCC-MS \rightarrow$ inactive $BCC-MS$

$RVV-X$ $+ \rightarrow$ VEGF $\downarrow$ $RVV-X$ $+ \rightarrow$ active $BCC-MS$

FIGURE 8
Inhibitory effect of RNA37s on the RVV-X-induced phase transition in the BCC-MS detection system

FIGURE 9
The effect of VEGF_{165} 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.

FIGURE 11
UNIVERSAL FIELDABLE ASSAY WITH UNASSISTED VISUAL DETECTION

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 (VEGF165) was created. RNA132 inhibits enzymatic activity of RVV-X. The effector molecule, VEGF165 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 VEGF165 could be detected by the naked eye within an hour. Similar results were obtained for another allosteric aptamer modified to detect molecules to which aptamers can be affinity for RVV-X and human vascular endothelial growth factor (VEGF115) was created. RNA132 inhibits enzymatic activity of RVV-X. The effector molecule, VEGF115 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 VEGF115 could be detected by the naked eye within an hour. Similar results were obtained for another allosteric aptamer modified to detect molecules to which aptamers can be.

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.

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 (VEGF165) was created. RNA132 inhibits enzymatic activity of RVV-X. The effector molecule, VEGF165 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 VEGF165 could be detected by the naked eye within an hour. Similar results were obtained for another allosteric aptamer modified to detect molecules to which aptamers can be.

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 (t50) for a given concentration of RVV-X. (B) Optical density at 405 nm measured on a microplate reader in the same wells at 5-minute intervals.

FIG. 2. Schematic representation of the secondary structure folding for the RNA aptamers. (A) RNA37s (SEQ ID NO:1), (B) VT144(−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 VEGF165 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 VEGF165 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. One hundred microliters of the BCC-MS reaction mix 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 µl 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 OD405 measurements at 5-10 minute intervals. T(50) is the time for a 50% decrease in OD405.

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 OD405 measurements at 5-10 minute intervals. T(50) is the time for a 50% decrease in OD405.

FIG. 10. The effect of VEGF165 on the inhibitory action of RNA132 on RVV-X in RNA132-controlled BCC-MS detection system. In the RNA132-controlled BCC-MS experiment, VEGF165 reversed inhibitory action of RNA132 on the RWV-X-triggered phase transition in a concentration-dependent mode. This effect implies competition between VEGF165 and RVV-X for binding to RNA132. Dynamic range of the detection system appeared to be narrow, i.e. about 10-fold range of VEGF165 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 VEGF165 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:
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.

**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 polystyrene 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 Hemaotechnology Technologies. SPECTROZYME®fXa (SPZXa) was from American Diagnostica. PCR and RT-PCR reagents and T7 RNA Polymerase kits were from Promega and Epicentre. Human VEGF, human VEGF121, murine VEGF165, zebrafish VEGF165, endocrine gland VEGF, and human VEGF/PIGF 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 nM Factor Va, 580 nM 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 to which 5-10 µL of a test solution, e.g. RVV-X, were added. After that the plate was immediately placed into a microplate reader (Genios, Tecan) and shaken for 10-30 seconds prior to OD405 measurements at 5-10 minute intervals. The relative effect (zero meaning no effect, and one meaning maximal effect) of VEGF165 on the inhibition of the phase transition by RNA132 was calculated according to the formula, which allowed graphic presentation for the dynamic range of the reaction:

$$\frac{t_{1/2_{-RVV-X}} - t_{1/2_{RVV-X}}}{t_{1/2_{VGGF165-RVV-X}} - t_{1/2_{VGGF165+VGGF165}}},$$

where $t_{1/2}$ is the time for a 50% reduction in absorbance. The same formula was applied for the calculation of the relative effect for PTPase.

The BCC-MS reaction can be “frozen” at any stage by adding EDTA to 10 mM final concentration.

SELEX Protocol and the Creation of Allosteric Aptamers. Sequences for the initial DNA template library (40N) and primers for the SELEX for aptamer to RVV-X were taken from (17). Large-scale PCR amplification and all other subsequent synthetic and selection steps were performed as described in (17-19) without modification. The initial selection involved 500 picomoles of random RNA library (3x10^14 molecules). After each round of selection RNA was tested in a nitrocellulose filter binding assay to determine the dissociation constant of aptamer-protein complexes (K_d) (19) and in a single point binding assay with equimolar concentrations of protein and RNA (50 nM) (20). In the latter experiments one set of filters was washed with IC buffer. Another set of filters was washed with IC buffer+300 mM NaCl (high salt buffer) to remove more of non-specifically bound RNA. After the fifth and ninth round of selection, RNA was reverse transcribed, DNA was amplified by PCR and cloned. Each time 45 clones were sequenced to determine the presence of consensus sequences. Cloning and sequencing were performed by Laragen.

Three VEGF165-binding RNA aptamers, 12t, 84t and 100t described in (21), and three other VEGF165-binding RNA aptamers, VP2, VP22 and VT144 described in (22), were synthesized using unmodified NTPs and tested in a gel-shift assay with VEGF165. Aptamer VT144 showed the highest affinity for VEGF165 in the IC buffer, since it was initially selected in the presence of calcium, which was absolutely required for high affinity binding of this aptamer to VEGF165 (22). Please note that Ca ions are essential for BCC-MS amplification cascade.

Allosteric aptamers were created either by directly fusing of the 5'-end of RNA37s with the 3'-end of an effector aptamer or by inserting an effector aptamer RNA a number of bases downstream into the RNA37s.

RVV-X Activity Determination with SPECTROZYME®fXa (RVV-X-SPZXa Assay). SPECTROZYME®fXa is a chromogenic substrate for the activated Factor X. RVV-X by itself (10 µL, 1.7 nM) or pretreated with aptamers (17 nM) was incubated with Factor X (170 nM) and mixed with 140 µL of SPECTROZYME®fXa (5 mM, IC buffer). Appearance of the chromophore, p-nitroanilide acetate, was monitored over time at 405 nm in a microplate reader. The final measurement 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:

\[
1 - \left( \frac{A_{RVV-X} - A_{RVV-X+aptamer}}{A_{RVV-X+aptamer}} \right) \times 100 
\]

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 Pseudoviewer2 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°C 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, I (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 substitution of the rabbit brain cephalin with the commercially available phospholipid vesicles. FIG. 1 shows the kinetics of the phase transition ( clot formation) caused by the RVV-X activity in the BCC-MS amplification cascade monitored both visually and spectrophotometrically at 405 nm. Snapshots of the wells taken at the time of 50% reduction in absorbance (t_f/2) for different RVV-X concentrations show distinct differences among the wells depending on the progress of the phase transition (FIG. 1A). At the beginning (time zero) all wells have a uniformly whitish, milky appearance, as the MS are in suspension. The well with no RVV-X added ( open circles) remains the same during the entire course of the experiment. After 12 minutes, corresponding to t_f/2 for 100 attomoles of RVV-X (solid diamonds), less opaque material is seen in the corresponding well. No changes are observed in the other wells at this time. At 26 minutes, corresponding to t_f/2 for 10 attomoles of RVV-X (open squares), less opaque material is seen in the well, as was at 12 minutes for the well with 100 attomoles of RVV-X. The MS in the well with the 100 attomoles of RVV-X is coagulated by this time, and the well has a clear black background. This coagulation pattern is repeated in subsequent wells. Thus, the homogeneous BCC-MS amplification cascade is adequate for the detection of small quantities of RVV-X by the naked eye.

Selection of an Aptamer to RVV-X. An RNA aptamer binding to RVV-X was obtained via standard SELEX protocol (17-19). The progress of the selection was monitored by determining the dissociation constant of the RNA-protein complex (K_d) and by determining the fraction of RNA that binds to the target protein at a same “high” concentration (50 nM) of both the aptamer and the target protein in a single point binding assay. The latter assay was performed using both the binding buffer and the high salt buffer (binding buffer+300 mM NaCl) to decrease the input of nonspecifically bound complexes (20). The initial pool of unselected RNA (RNA0) had a K_d value of 550±23 nM. In a single point binding assay, 6.3% of RNA0 preincubated with RVV-X was bound to the filter when the binding buffer was used for washing, and 2.2% of 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, K_d 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-PCRed 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_165 (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 adequate controls for the specificity of the detection system; and there are commercially available highly sensitive ELISA assays for this protein that can serve as reference standards.

An aptamer binding to VEGF_165, RNA ligand VT44 (22), with one base omitted from both 5’- and 3’-ends, VT44{−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 VT44{−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 VT44{−2} (ΔG_{col} = -24.0 kcal/mol versus ΔG_{col} = -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_{col} = -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±0.9 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 the effect 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, VEGF₁₆₅. Reverses Inhibition of the Phase Transition Caused by RNA132. As was shown in the RNA132-controlled BCC-MS detection system, VEGF₁₆₅ 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 VEGF₁₆₅ and RVV-X for binding to RNA132. Dynamic range for the effect of VEGF₁₆₅ 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 VEGF₁₆₅ concentrations (3-30 fmol). This was shown to be typical for the BCC-based detection systems (28). The sensitivity of the detection of VEGF₁₆₅ in the RNA132-controlled BCC-MS detection system is within the lower half of the linear range for the VEGF₁₆₅ 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 *Versinia enterococcolitica*, 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 VEGF₁₆₅.

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 VEGF₁₆₅. Human VEGF₁₆₅, murine VEGF₁₆₅ and zebrafish VEGF₁₆₅ have both the same biological function and contain 165 of amino acids. Murine VEGF₁₆₅ has an 89% identity 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 VEGF₁₆₅ 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 forms a photo-inducible cross-link to a uridine at position 14 of RNA V144 (22) (which corresponds to position 13 of RNA V144(2) and position 18 of RNA132) is missing in VEGF₁₂₁. Human VEGF/PIGF heterodimer is a protein artificially dimerized in vitro. It consists of human VEGF₁₆₅ 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 VEGF₁₆₅ and murine VEGF₁₆₅. In both cases, the coagulation process was complete within 40 minutes. This was not surprising, since human and murine VEGF₁₆₅ are highly homologous. At the same time point (40 minutes) when the wells containing human VEGF₁₆₅ demonstrated completed coagulation, the wells containing zebrafish VEGF₁₆₅ demonstrated the onset of coagulation. It can be explained by less homology (62%) between human VEGF₁₆₅ and zebrafish VEGF₁₆₅. Meanwhile in the wells containing human VEGF₁₂₁, endocrine gland VEGF, human VEGF/PIGF, 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 VEGF₁₆₅ 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 VEGF₁₆₅ 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 DNAII and DNA III 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 VI44 domain of RNA132 significantly decreases VEGF₁₆₅ binding to RNA132. When shorter DNA fragments corresponding to the VI44 domain of RNA132 (DNAV and DNA V1) were used for the competition, the impact of competing DNA on VEGF₁₆₅ 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. 2F) is involved in the binding of both proteins. This can result in direct steric 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 two 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 within 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 (VEGF_{165}) 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: 5.

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: 3.

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

10. A method for detecting a target molecule in a sample, comprising:

```
GGGAGCUU UGAGCGUCG AUUGUUAAG ACAGGAGCU CAC
```

<210> SEQ ID NO 2
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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 VEGF_{165} or a protein tyrosin phosphatase.

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