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G-quadruplex DNA aptamers generated for systemin

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ABSTRACT

Ligands specific to bioactive molecules play important roles in biomedical researches and applications, such as biological assay, diagnosis and therapy. Systemin is a peptide hormone firstly identified in plant. In this paper we report the selection of a group of DNA aptamers that can specifically bind to systemin. Through comparing the predicted secondary structures of all the aptamers, a hairpin structure with G-rich loop was determined to be the binding motif of these aptamers. The G-rich loop region of this binding motif was further characterized to fold into an antiparallel G-quadruplex by truncation–mutation assay and CD spectrum. The apparent equilibrium dissociation constant (K_d) of one strong binding sequence (S-5-1) was measured to be 0.5 μM. The specificity assay shows that S-5-1 strongly bind to whole systemin, weakly bind to truncated or mutated systemin and does not bind to the scrambled peptide with the same amino acid composition as systemin. The high affinity and specificity make S-5-1 hold potentials to serve as a molecular ligand applied in detection, separation and functional investigation of systemin in plants.

1. Introduction

Systemin is the first peptide hormone identified in plant in 1991, which has been found in the plant species of the solanaceae family including tomato, potato, bell pepper, and black nightshade.[1,2] As a primary signal that is released into the vascular system of tomato plants at sites of herbivore and pathogen attacks, systemin initiates a signaling cascade that activates over 20 defensive genes and induces the synthesis of defense-related proteins in leaves throughout the plants.[1,3,4] Systemin is active in the nM range, similar to those of animal peptide hormones. The activity of systemin is commonly reflected by the activity of downstream enzymes, expression of related genes or/and the concentration of small molecules regulated by systemin. Although considerable progress has been made towards a better understanding of the systemic defense responses recently, the systemin-associated molecular mechanisms remain poorly understood.[5,6] Systemin is an 18-amino acid peptide (Ala-Val-Gln-Ser-Lys-Pro-Pro-Ser-Lys-Arg-Asp-Pro-Pro-Lys-Met-Gln-Thr-Asp) that is processed from a 200-amino acid precursor protein, called prosystemin. The enzymes involved in the processing of prosystemin have not been identified yet.[7]

High quality affinity reagents to bioactive molecules play important roles in biomedical researches and applications, such as biological assay, diagnosis and therapy. Antibodies are well known with high affinity and specificity and have been widely used in biomedicine. However, only a small portion of bioactive molecules have antibodies that are available for use in routine application. Even less common are antibodies with high affinity and specificity to specific bioactive peptides. Until now, no antibody to systemin has been reported. Therefore, the development of molecular ligand to systemin would be helpful for the detection of systemin and study of systemin-associated molecular mechanism.

Aptamers are artificial DNA or RNA ligands, which are evolved from combinatorial nucleic acid libraries by an in vitro selection technique, named SELEX (Systematic Evolution of Ligands by EXponential enrichment).[8,9] The selected aptamers can recognize a wide variety of targets, including small molecules, proteins, cells and tissues[10–12] by folding into well-defined three-dimensional shapes via the intramolecular interaction.[13] Compared with antibodies, aptamers have low molecular weight, fast tissue penetration, high stability and low immunogenesis.[13] They can be synthesized chemically with low cost and labeled easily with various reporters[14]; furthermore they can be ligated and/or amplified by enzymes in vitro.[15] Owing to these advantages, aptamers have attracted intense research efforts on their potential biomedical applications, such as recognition, inhibition, diagnosis and therapy.[16,17]

Here we describe the selection of DNA aptamers that specifically bind systemin with high affinity. The binding motif of these

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aptamers was determined by comparison of the secondary structure of aptamers. A strong systemin binding sequence, S-5-1 was optimized by truncation–mutation assay. The specificity of S-5-1 to systemin in relation to its peptide analogues was characterized.

2. Results and discussions

2.1. *In vitro* selection of systemin binding aptamers

The key step of aptamer selection is separation of the target-bound sequences from the nucleic acid pool. Since systemin is a relatively small molecule (an 18-amino acid peptide), it is hard to collect the systemin–aptamer complex from the 85-mer DNA pool except to fix systemin on a support. Therefore an affinity chromatographic SELEX protocol[18,19] was optimized for the selection of systemin binding aptamer. Three kinds of support were tried to immobilize systemin. The first attempt was to immobilize systemin on home-made monodisperse, non-porous, cross-linked polyglycidylmethacrylate (PGMA) beads (4 μm) bearing free epoxide groups[20], but we failed to get any enrichment after 39 rounds of selection with a variety of optimizations (data not shown). Then we tried to use the streptavidin-coated Sepharose beads fixed with N-terminal biontinylated systemin as the affinity support, however, we only got the aptamers that bind to streptavidin as we described previously.[10] Finally we coupled systemin on Epoxy-activated Sepharose 6B beads through the reaction between its amino groups and the epoxy group, and used them to evolve aptamers (see Section 4.4 for details). In order to reduce the non-specific DNAs that bind to sepharose beads, a counter selection step was performed from the second round of selection. The enrichment of aptamers in each round of selection was monitored by measuring the fluorescence intensity of bound DNAs on beads. Significant enrichments of aptamers binding to systemin were observed until the 17th round of selection (Fig. S1). The enriched pool of round 17 only bind to systemin immobilized beads and did not bind to beads without systemin, this binding could be competed completely by adding systemin in binding buffer, suggesting that aptamers specific to systemin were enriched. After 17 rounds of selection, the enriched DNA pool was cloned and 103 clones were sequenced (Table 1).

Table 1
Core region sequences of the selected aptamers (5′–3′ direction)

Clone	Sequence (from 5′ to 3′)	Copies
Sys#5	GCACTAACGGGTTTCGGGGGGGTAGGGAGGTTTTGCTATCGCGCC	×16
Sys#98	GCACTAACGGGTTTCGGGGGGG-AGGGAGGTTTTGCTATCGCGCC	×1
Sys#57	GCACTAACGGGTTTCGGGGGGGTAGGGAGGTTTTGCTATCGTGCC	×1
Sys#85	GCACTAACGGGTTTCGGGGGGGTAGGGAGGTTTTGCTATCGCGCC	×1
Sys#8	GCACTAACGGGTTTCGGGGGGT-AGGGAGGTTTTGCTATCGCGCC	×10
Sys#4	GCACTAACGGGTTTCGGGGGGT-AGGGAGGTTTTGCTATCGCACC	×1
Sys#96	GCACTAACGGGTTTCGGGGGGGTAGGGAGGTTTTCCTATCGCGCC	×1
Sys#61	TTCGGGACTCGGGGGGGTAGGGGAATGACTCAAACGTTGATCTC-	×1
Sys#37	-GTTCCGGCTAGGGGGGGAAGTGGGGGAACTTTATCGCTCGGCCTA	×3
Sys#99	GTTCCGGCTAGGGGGGGAGGTGGGGGAGCTTTATCGCTCGGCCTA	×1
Sys#56	-CGGCTTTGGGGGGGTGTGGGGTAAC-CGGTACTGCCTCCTTTCCGT	×1
Sys#91	CCGCGGCTTGGGGGGGTGTGGGGCGGAGACTTTTGCTACTGATCC–	×1
Sys#102	-GCGGTGTAGGGGGGCAGTGGGGCATTACCCTACGGCTGCTCATG-	×1
Sys#101	-TGCGGTGTAGGGGGGCAGTGGGGCATTAACCTACGGCGCTCATG-	×1
Sys#2	-TGCGGTGTAGGGGGGCAGTGGGGCATTACCCTACGGCTGCTCATG-	×2
Sys#1	TCAATGGTAGGGGGGTCTTTGGGAGATTGTTA–TCCTTGCGGGGAC	×1
Sys#90	TCAATGGTAGGGGGGTCTTTGGGAGATTGTTA–TCCTTGCTGGGAC	×1
Sys#26	TCAATGGTAGGGGGGTTTTTGGGAGATTGTTA–TCCTTGCGGGGAC	×1
Sys#89	TCAATGGTAGGGGGGTTTTTGGGAGATTGTTA–TCCTCGCGGGGAC	×1
Sys#28	CCAATGGTAGGGGGGTTTTTGGGAGATTGTTA–TCCTTACGAG	×1
Sys#83	ATAATGGTTAGGGGGGGATTTGGGAGATTATTGGCCTCCTGACGCG	×2
Sys#31	TTAGTGAGCTGGTTAGGGGGGGCTTCGGGAGAGCTCACTATCTGC-	×7
Sys#46	TTAGTGAGCTGGTTAGGGGGGGCTTCGGGAGAGCTCACTGTCTGC	×1
Sys#14	TTAGTGAGCTGGTTAGGGGGGGTTTTGGGAGAGCTCACTATCTGC-	×14
Sys#6	–TGGTTAGGGGGGTCTTTGGGATATATCACGGATTCAGCATGCGCA	×1
Sys#95	–CCTGGTTAGGTGGGGATTCGGGAAAGGTATAGGTTTCGCTGCTGG-	×1
Sys#100	-ACCTGGTTAGGTGGGGATTCGGGAAAGGTATAGGTTTCGCTGCTGG-	×1
Sys#103	-CGCGGGTTAGGGGGGTCTTTGGGAACGCGTAATCCCCTCTGTTTG	×1
Sys#3	-GCACGGTAGGGGGGTATTTGGAAGGTGCAGTTCTGCC-CTGATCTC	×1
Sys#35	-CTGGCGCACGGTTAGGGGGGTTTTTGGG-GTGCTGCGTATCTCTTG-	×1
Sys#30	-TGCTGCACTAGGTGGGTGGGTGGGAGGATAGCTGCAGCATACTG-	×1
Sys#33	-CGTTGACTAGACGCCGGTAGGTGGGGTTATTGGGAGGGCGTCTAA–	×2
Sys#88	TGCGAACTCATCAATCACGCGGTAGGTGGGTATTTGGGAGGCGTG—	×1
Sys#9	–ATTCGCGGTAAGTGATGGTAGGTGGGTATTTGGGAGATCACGGCA-	×4
Sys#19	–ATTCGCGGTAAGTGATGGTAGGTGGGTATTTGGGAGATCACGGCG-	×3
Sys#97	GAATTCGCGGTAAGTGACGGTAGGCGGGTATTTGGGAGAGCACGGCA-	×1
Sys#7	–CAGGCTATCGTGTTGATTGGGTTTGTAGGGAGGGCCTTCACGCTG-	×20
Sys#10	–CAGGCTATCGTGTTGATTGGGTTTGTAGGGAGGGCCTTCACGCTA-	×1

5′-AAG GAG CAG CGT GGA GGA TA-$(N)_{45}$-TTA GGG TGT GTC GTC GTG GT-3′, the full length sequences include two primer hybridization sites and the center random sequence. The underlined bases are in the loop region and the italic bases are in the stem region.

2.2. Characterization of selected aptamer sequences

The sequences of 103 clones are distributed in five groups based on their sequence similarity. All of them are rich in guanosine. Moreover a hairpin structure with a highly conserved guanosine-rich loop (Fig. S2) was found in all the sequences except Sys#7 and Sys#10 by comparing the predicted secondary structures.[10,14] According to our previous research,[10,14] this hairpin structure might be the binding motif of these sequences. Therefore a truncated hairpin structure of sequence Sys#14 (S-14-2) was synthesized and labeled with carboxyfluorescein (FAM) at its 5′ ends. As we expected, binding assay showed that S-14-2 bound to systemin-immobilized beads selectivity (Fig. 1) and did not bind to sepharose beads without systemin. The control sequences St-2-3 (Table S1) was found not to bind to systemin-immobilized beads. These results suggest that S-14-2 is the binding motif of sequence Sys#14.

Then several hairpin structures (S-14-1, S-5-1, S-26-1, S-30-1, S-33-1, S-37-1 and S-95-1) in different groups were synthesized (unlabeled). Twenty micromolars of each sequence was mixed with 0.4 μM FAM-labeled S-14-2 and then incubated with systemin-immobilized beads (see Section 4.6 and Supplementary data for details). The bound ratio of total DNA to FAM-labeled S-14-2 was used to estimate their binding ability. As shown in Figures 2A and S3, sequences S-5-1, S-14-1, and S-26-1 have better binding ability than unlabeled S-14-2. S-14-1 is the same hairpin structure as S-14-2 but with a longer stem; its higher binding ability suggests that the longer stem is important for binding. S-5-1 derived from Sys#5 shows the highest binding ability. In addition, the sequences in the group containing Sys#5 nearly occupy 1/3 of the 103 clones, among which Sys#5 has 16 repeats. Therefore S-5-1 was chosen for further characterization. A FAM-labeled S-5-1 was synthesized and its apparent equilibrium dissociation constants (K_d) was measured to be 0.5 ± 0.3 μM (Fig. 2B) by static adsorption method.

In order to further understand the binding of S-5-1 to systemin, a truncation and mutation assay was performed. As shown in Figures 3 and S4, the original hairpin sequence (S-5-0) existing in sequence Sys#5 has two mismatched base-pairs (T–T, C–T) in the middle of the stem region, S-5-1 is a mutated sequence of S-5-0 by replacing the unpaired bases with perfect matched base-pairs (T–A, C–G), and S-5-2 is another mutated sequence of S-5-0 by removing the mismatched base pairs. It is well-known that the mismatched base-pairs in stem region would cause much lower stability of a hairpin structure. The competitive binding assay shows that S-5-0 possesses much lower binding ability compared

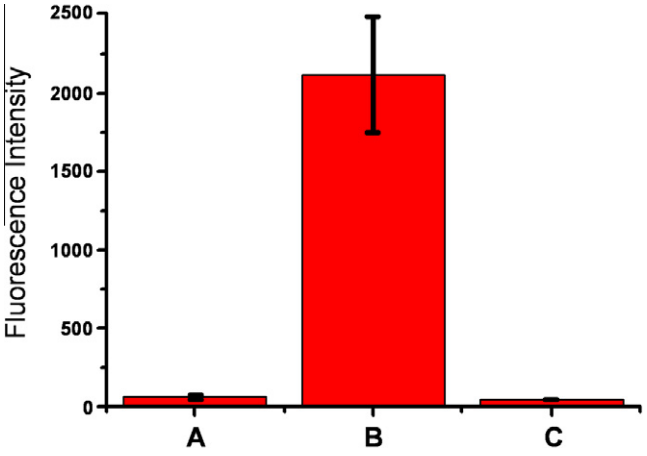

Figure 1. Binding of FAM labeled S-14-2 on systemin-coated beads or control beads. A: S-14-2 bound to control beads; B: S-14-2 bound to systemin-coated beads; C:St-2-3 (control sequence) bound to systemin-coated beads. Each data point represents an average ± the standard deviation of three replicates.

with S-5-1 and S-5-2. Gradually truncating the stem region of S-5-1 (S-5-T1, S-5-T2, S-5-T3, S-5-T4 and S-5-T5) gradually decreased the binding ability of the resulted sequences. The above results indicate that a stable hairpin structure is critical for the binding of S-5-1 to systemin. Replacing 1 or 3 base pairs in the stem region of S-5-T2 (S-5-3 and S-5-4) greatly decreases their binding ability. However replacing the whole stem of S-5-T2 with the stem of S-37-1 (same length with S-5-T2) enhanced its binding ability. Replacing the whole stem of S-5-1 with the stem of S-14-1 (one base-pair longer than with S-5-1) reduced its binding ability. These results suggest that the stem region not only stabilizes the binding structure of the aptamer but also interacts with systemin in a certain way.

A notable feature of the hairpin structures in all the cloned sequences is that their loop region contains three or four blocks of G residues, the G tracts have 2–7 G residues in a row. This arrangement is characteristic of DNAs that form G-quadruplex structures.[21] For investigating the structure of the loop region in S-5-1, a CD spectrum analysis was carried out. It is well known that antiparallel G-quadruplexes have a CD spectrum characterized by a positive band around 295 nm and a negative band around 260 nm, while the parallel quadruplexes have a positive band around 264 nm and a small negative band around 240 nm. CD spectra with peaks at both 264 and 295 nm could indicate either the

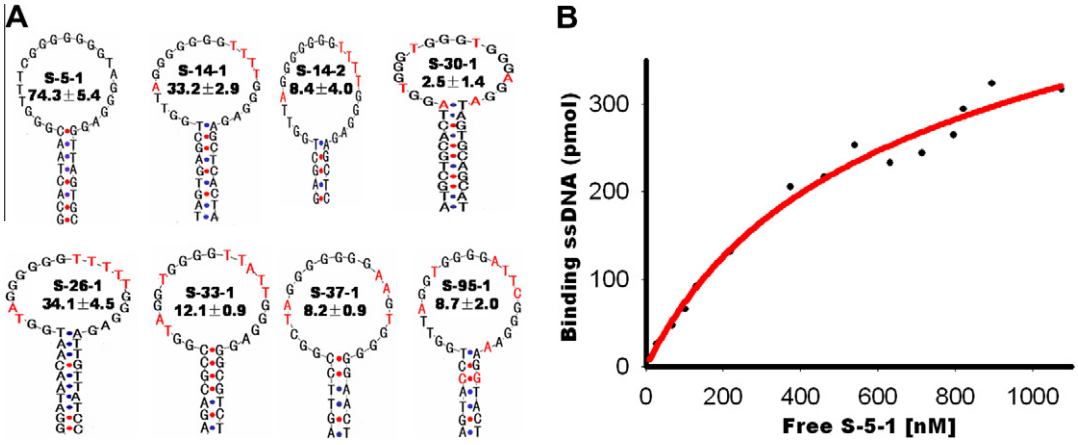

Figure 2. (A) Hairpin structure of truncated aptamers. The numbers shown in each sequence denote the binding ratio of total DNA to FAM labeled S-14-2. Each data point represents an average ± the standard deviation of three replicates. (B) Binding curves of S-5-1 to systemin-coated beads.

Figure 3. Truncted and mutated sequences of aptamer S-5-1. Nucleotides different from S-5-1 are shown in red. The numbers shown in each sequence denote the binding ratio of total DNA to FAM labeled S-14-2. Each data point represents an average ± the standard deviation of three replicates.

coexistence of distinct parallel and antiparallel folded species in solution or a single mixed-type hybrid quadruplex structure.[21,22] As shown in Figure 4A, the CD spectrum (in 150 mM Na^+) of sequence S-5-T6 (loop region of S-5-1) is consistent with an antiparallel G-quadruplex characterized by a positive band at 295 nm and a weak negative band near 260 nm. However the addition of K^+ (10 mM) caused a decrease in the positive band at 295 nm, an appearance of a large positive band at 265 nm and a negative band near 240 nm, which is consistent with the coexistence of parallel and antiparallel folded species, suggesting that K^+ causes the structural transformation from antiparallel structure to parallel structure. Similar structural transformation of G-quadruplex formed sequences has been well documented.[23–28] The CD spectrum of aptamer S-5-1 (Fig. 4B) exhibit a positive band at 290 nm, a negative band at 255 nm, and a shoulder band between 270–280 nm that could attribute to the duplex stem,[29] suggesting that the loop region of the hairpin structure still fold into antiparallel G-quadruplex. However the addition of K^+ only slightly increased the positive CD bands of S-5-1, suggesting that the stem of S-5-1 restrict the structure transformation of the loop region from antiparallel structure to parallel structure. The addition of systemin did not cause significant change of the CD spectra of S-

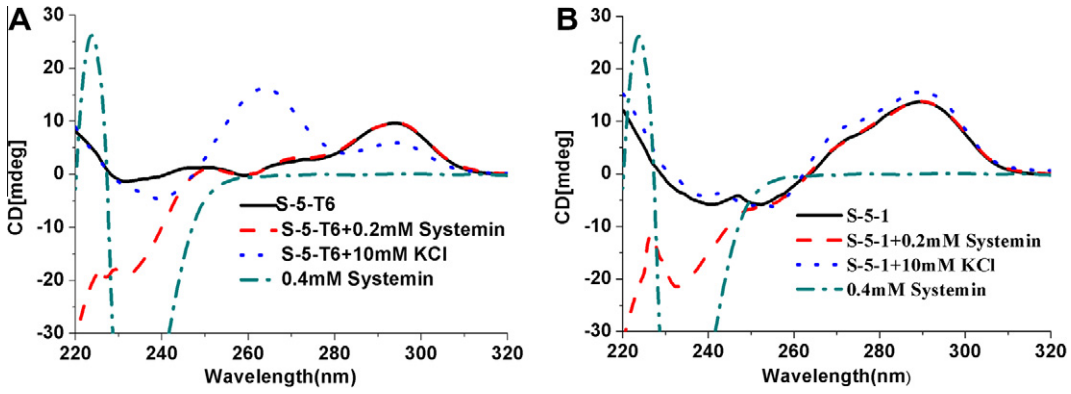

Figure 4. CD spectral changes of sequence S-5-T6 (A) and sequence S-5-1(B).

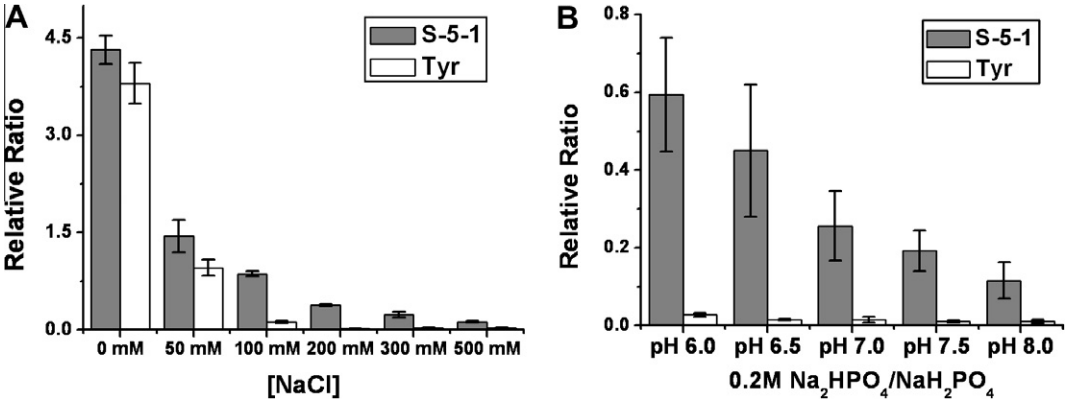

Figure 5. (A) Effect of ionic strength on the binding ability of S-5-1. The experiments were carried out in 20 mM Na_2HPO_4/NaH_2PO_4, pH 6.5 plus different concentration of NaCl. (B) Effect of pH on the binding ability of S-5-1. The experiments were carried out 0.2 M Na_2HPO_4/NaH_2PO_4 with different pH.

5-T6 and S-5-1 between 255 and 300 nm, suggesting that the binding of systemin to S-5-1 could not cause the change of antiparallel G-quadruplex structure of the loop region.

The mutation assay of the loop region of S-5-1 is shown in Figure 3. Respective mutation of G_{11}, G_{17} or G_{18} in the loop region to T (S-5-M3, S-5-M4 or S-5-M5) almost destroyed their binding ability, indicating that these G residues are critical for the binding. However mutation of G_{10} to T_{10} (S-5-M2) only partly decreased its binding ability, in which this T_{10} separate a seven-G tract in S-5-1 into a two-G tract and a four-G tract. Similar cases of that a two-G tract and a four-G tract are separated by a T were also found in aptamer S-33-1 and S-95-1. Since two-G tracts also appear in other obtained systemin binding aptamers (S-14-1, S-26-1, S-33-1, S-95-1), it is likely that S-5-1 may fold into an intramolecular G-quadruplex with two layer of G-quartets while not three. Mutation of the TTTC between the first two G-tracts to TA (S-5-M6) greatly decreased the binding ability, but mutation of this TTTC to TATC (S-5-M1) only partly decreased the binding ability, implying that the length and the base composition of the sequence between the first two G-tracts are important for systemin binding. A new sequence constructed by the stem of S-5-1 and the loop of S-14-1 (S-5-M7) shows similar binding ability (Fig. 3) with S-14-1, suggesting that the loop regions of these aptamers may play a major role in the binding.

2.3. The effect of ionic strength and pH on aptamer binding

pH and ionic strength are well known to play important roles in maintaining the binding of aptamers.[30,31] In order to have a better insight into the interaction between S-5-1 and systemin, the influences of pH and ionic strength on aptamer binding were evaluated. Usually, hydrogen bonds, electrostatic interactions, stacking interactions, molecular shape complementarity and hydrophobic effect are considered to be involved in the binding between an aptamer and its target molecule.[30–32] Systemin contains four positively charged amino acid residues (3 lysines + 1 arginine) and one negatively charged amino acid residue (aspartic acid), and carries two net positive charges at the physiological pH (7.4). Therefore it can be expected that systemin can bind to any nucleic acid at low ion strength via the electrostatic interaction. As expected, the binding of both aptamer S-5-1 and the control sequence (Tyr) (Table S1) to systemin coated beads are strong at low ionic strength, and the selectivity between them is indistinct (Fig. 5A). With the increase of ionic strength, the binding abilities of both sequences were gradually weakened, which indicates that increase of ionic strength decreases the interaction between the nucleic acids and their ligands by screening the charges on the phosphate backbone of nucleic acids and ligands. However, aptamer S-5-1 shows obvious selectivity to systemin over the control sequence at high ionic strength (Fig. 5A and B), suggesting that besides electrostatic interaction, other interactions such as hydrogen bonds and molecular shape complementarity may involve in the binding between S-5-1 and systemin. Since no aromatic amino acid in systemin,

Name	Sequences (From N to C)
Sys	AVQSKPPSKRDPPKMQTD
Sys-bio	Biotin-AVQSKPPSKRDPPKMQTD
VD	VQSKPPSKRDPPKMQTD
AT	AVQSKPPSKRDPPKMQT
AAD	AVQSKPPSKRD
AA	AVQSKAASKRDPPKMQTD
MS	MKDQTRVPSKPDQPKPAS

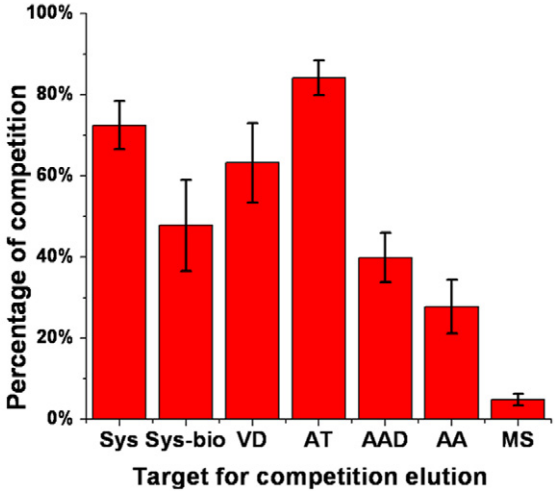

Figure 6. Competitive binding assays of S-5-1 to systemin-coated beads with analog peptides. The percentage of competition was calculated on the basis of the binding of the S-5-1 to systemin-coated beads without competitor. Each data point represents an average ± the standard deviation of three replicates. The sequences of analog peptides are shown above the diagram.

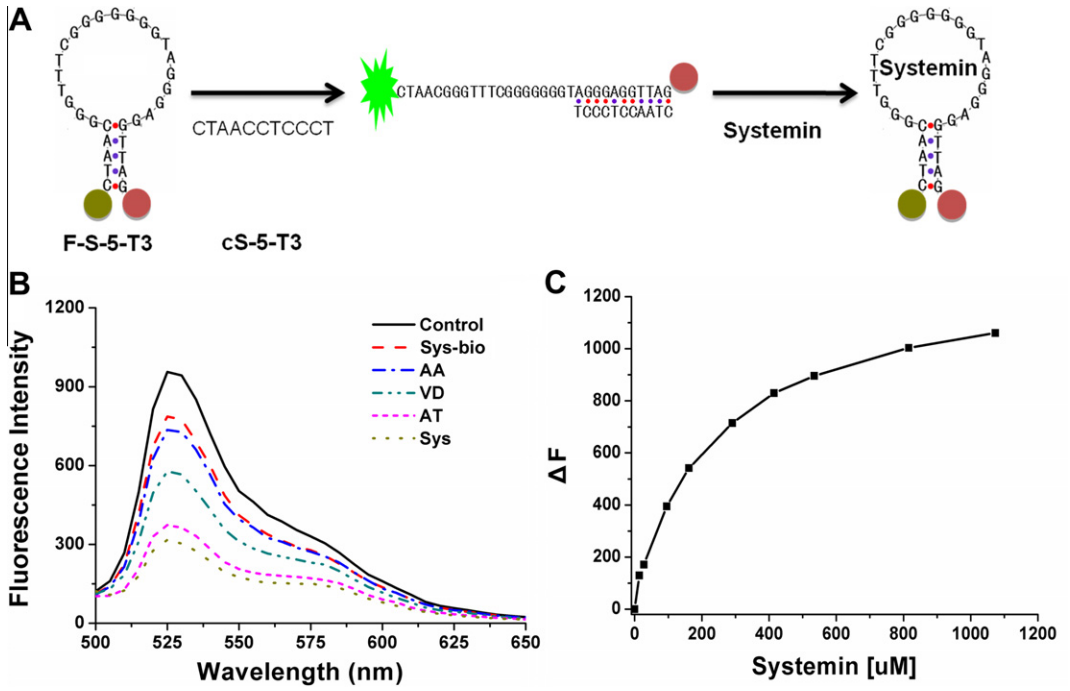

Figure 7. (A) Illustration of aptamer beacon. (B) Fluorescence emission spectra of aptamer beacon in the absence or presence of systemin (Sys) and peptide analogs (Sys-bio, AA, VD, AT). (C) The response curve of aptamer beacon against the concentration of systemin.

the molecule stacking interactions is not the driving force. The affinity of aptamer S-5-1 was decreased with the rise of pH value (Fig. 5B), which due to the deprotonation of amino groups in systemin resulted in the decrease of the electrostatic interaction between aptamer and systemin.[31–33]

2.4. Selectivity of aptamer S-5-1

In order to further demonstrate the specific interaction between systemin and S-5-1, seven peptides derived from systemin were synthesized and used to compete with systemin (Fig. 6). A scrambled peptide with the same amino acid composition as systemin (peptide MS) does not show any competition ability to systemin at all, indicating the specific interaction between S-5-1 and systemin. There are two proline doublets in the central region of systemin (Pro6–Pro7, Pro12–Pro13) that make it adopt a Z-like-β-sheet structure.[34–36] Displacement of the prolines at Pro6–Pro7 by two alanines (peptide AA) greatly decreased its competition ability to systemin, suggesting that the proline doublet play a role in maintaining the molecular shape of systemin that is complementary to aptamer S-5-1. Truncation of six amino acid residues from the C-terminal of systemin (AAD) partly decreased the binding ability, and truncation of the N-terminal alanine residue (peptide VD) slightly decreased its competition ability, suggesting that the binding is contributed by the whole peptide (including the N-terminal alanine residue). Labeling a biotin on the N-terminal amino group (peptide Sys-bio) partly decreased the competitive ability, which may be due to the loss of a positive charged amino group. On the contrary, removing the C-terminal aspartic acid residue of systemin (peptide AT) slightly enhanced its competition ability, which may be due to the loss of a negative charged carboxyl on the peptide. These results indicate that aptamer S-5-1 has good specificity to systemin. This specificity is contributed by the integrative interaction of the amino acid residues in systemin with S-5-1, and the spatial distribution of amino acid residues on the Z-like-β-sheet structure.

2.5. Aptamer beacon for recognition of systemin

In order to further demonstrate that the obtained aptamers can recognize free systemin in solution, an aptamer beacon[37,38] was designed as shown in Figure 7A. A truncated sequence (S-5-T3) of S-5-1 was labeled with FAM at its 5′end and Carboxytetramethylrhodamine (TAMRA) at its 3′end (named F-S-5-T3), then it was hybridized with an 11-nt complementary sequence of S-5-T3 (cS-5-T3). In the absence of target, the hybridized form of this beacon showed strong fluorescence at 530 nm (Fig. 7B) because FAM was separated from TAMRA. Upon the addition of systemin, aptamer F-S-5-T3 bound to systemin and formed a stem-loop structure, which resulted in the quenching of fluorescence of FAM by the approached TAMRA. The responses of this aptamer beacon to analogs of systemin are shown in Figure 7B, in which systemin and peptide AT caused the largest decrease of fluorescence intensity at 530 nm, which approximately agree with the specificity of S-5-1 to these peptides. The titration experiment showed that the response of this aptamer beacon depended on the concentration of systemin (Fig. 7C). These results indicate that systemin can compete with cS-5-T3 for binding to aptamer S-5-T3.

3. Conclusion

In summary, specific DNA aptamers to an important plant hormone, systemin, have been generated. A hairpin structure with G-rich loop has been found to be the binding motif of these aptamers. A 37-mer sequence, S-5-1 possesses the strong binding ability to systemin. S-5-1 adopt a hairpin secondary structure, in which the loop region further fold into an antiparallel G-quadruplex structure that is essential for aptamer binding. The competitive binding assay of peptide analogs of systemin shows that S-5-1 has high specificity to systemin, which is contributed by the integrative interaction of the amino acid residues spatially distributed in systemin and the specifically folded aptamer sequence. The highly specific aptamers hold the potential to serve as

molecular ligands applied in detection, separation and functional investigation of systemin in plants.

4. Materials and methods

4.1. Materials

All DNA sequences (Table S1) were synthesized by Sunbiotech Co. Ltd (Beijing, China), and unless otherwise indicated, were purified with PAGE by Sunbiotech Co. Ltd. All FAM or biotin-labeled sequences were purified by HPLC in our laboratory. All the peptide sequences were synthesized by GL Biochem (Shanghai) Ltd (Shanghai, China) and were purified by HPLC in our laboratory (FL2200, Wenling, China) with a C_{18} column (Agela, 5 μm, 100 Å, 4.6 × 250 mm, China). Streptavidin Sepharose High Performance and Epoxy-activated Sepharose 6B were purchased from GE Healthcare Bio-Sciences AB (GE Healthcare, Sweden), Taq polymerase and dNTPs were obtained from TAKARA Biotechnology (Dalian) CO., Ltd (Dalian, China), and all other chemicals were purchased from Beijing chemical plant (Beijing, China). The buffer used for all experiments, unless otherwise indicated, was binding buffer (140 mM NaCl, 2.5 mM KCl, 1.6 mM KH_2PO_4, 15 mM Na_2HPO4, 2.5 mM $MgCl_2$, 0.02% Tween20, pH 7.4). All solutions were prepared with deionized water purified by a Milli-Q system (Millipore, USA). All the fluorescence measurements were recorded by SpectraMax M5 (Molecular. Devices Corporation, USA).

4.2. Immobilization of systemin

Systemin was immobilized to Epoxy-activated Sepharose 6B™ beads as described by the manufacturer via the reaction between epoxy and amino groups. Briefly, 0.5 g dried powder of epoxy-activated Sepharose 6B™ was swelled and incubated with 50 mM systemin in 3 ml of 0.2 M $NaHCO_3/Na_2CO_3$ buffer (pH 11) at 37 °C for 48 h by mild shaking. After washing, the systemin-coated beads were deposited in 20% (v/v) ethanol solution. The density of the immobilized systemin was calculated to be 80 μmol/g dried beads based on the nitrogen content determined by elemental analysis.

4.3. DNA library and primers

The HPLC-purified library contained a central randomized sequence of 45 nucleotides flanked by two 20-nt primer hybridization sites (5′-AAG GAG CAG CGT GGA GGA TA-$(N)_{45}$-TTA GGG TGT GTC GTC GTG GT-3′). A FAM labeled 5′ primer (5′-FAM-AAG GAG CAG CGT GGA GGA TA-3′) and a biotin labeled 3′ primer (5′-Biotin-ACC ACG ACG ACA CAC CCT AA-3′) were used in the PCRs for the preparation of double-labeled and double-stranded DNA molecules. After denaturing in alkaline condition (0.1 M NaOH), the FAM-conjugated sense strands were separated from the biotinylated antisense strands with streptavidin-coated Sepharose beads and used for next-round selection.

4.4. Selection of systemin binding aptamers

Each ssDNA pool dissolved in binding buffer was heated to 95 °C for 5 min, and cooled on ice for 15 min, then kept at 25 °C for 5 min prior to use. For the initial selection round, 100 μl of washed systemin-coated beads were incubated with 10 nmol DNA library in 1 ml binding buffer at 25 °C for 30 min by mild shaking, then the mixture was transferred into a small empty column and washed with 2 × 150 μl of binding buffer. The beads bound with DNAs were directly applied to 10 parallel PCR reactions (50 μl). Each reaction contained 1×PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 300 μM dNTPs, 1.2 μM each primer, and 2.5 units Taq DNA in a 50 μl of reaction. The cycling protocol was 10–20 cycles of: 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min. Then the FAM-labeled sense ssDNA was separated by streptavidin-coated Sepharose beads, desalted by NAP-5 column (GE Healthcare, UK) and used as a new DNA pool for the next round of SELEX process. In order to acquire aptamers with high specificity, a counter selection step was performed from the second round of selection, namely, 100 μl of sepharose high performance beads (GE Healthcare, Sweden) without systemin were incubated with 20 pmol of ssDNA pool dissolved in 200 μl of binding buffer. After centrifuge, the supernatant was incubated with systemin-coated beads at 25 °C for 30 min by mild shaking, then the mixture was transferred into a small empty column and washed with n×150 μl of binding buffer containing different concentration of NaCl. The bound ssDNA on beads were eluted by heating in 100 μl of water containing 10 mM systemin and used for PCR directly. In order to obtain aptamers with high affinity, the volume of the systemin coated beads were scaled down gradually from 30 to 2 μl; the wash strength was enhanced gradually by extending wash time (from 0.5 to 3 min), increasing the number of washes (from 2–5) or increasing the concentration of the salt (from 150 to 500 mM). The selection process was monitored by recording the fluorescence intensity eluted from the systemin-coated beads. After 17 rounds of selection, the selected ssDNA pool was PCR-amplified by using unlabeled primers and cloned into *Escherichia coli* by using the TA cloning kit. 103 clones were sequenced by Beijing Genomics Institute (BGI) (Beijing, China).

4.5. Characterization of systemin binding aptamer

Predicted secondary structures of all the obtained sequences[39] (Fig. S2), were compared carefully to identify structure similarities. Then a common secondary structure motif found in this way, named S-14-2 was synthesized and labeled with FAM at its 5′end. 0.4 μM FAM-labeled S-14-2 or a control sequence (St-2-3) was incubated with 20 μl of systemin-coated beads or control beads in 100 μl of binding buffer. After washing with 100 μl of binding buffer, the bound DNAs were eluted by 0.5 M NaOH, and the fluorescence intensities of the eluates were measured by excitation at 488 nm and emission at 530 nm with cutoff at 515 nm. The binding of other sequences were characterized by competitive binding assay as shown in Section 4.6.

4.6. Competitive binding assay of truncated or mutated sequences

All truncated and mutated sequences were diluted in binding buffer and heated to 95 °C for 5 min, then cooled on ice for 15 min and kept at 25 °C prior to use. For competition assay: 0.4 μM FAM-labeled S-14-2 was incubated with 20 μM competitive sequences in 100 μl at 25 °C for 30 min. Then 10 μl of systemin-coated beads were added. After 60 min incubation, the sample was transferred into an empty column and washed with 100 μl of binding buffer, then eluted with 100 μl of 0.5 M NaOH. The fluorescence intensities of the eluates were measured on a 96-well plate (Costar) by excitation at 488 nm and emission at 530 nm with cutoff at 515 nm; and the absorptions were measured at 260 nm in a quartz cell with an optical path length of 10 mm. In order to eliminate the error in beads pipetting, binding ratio of total DNA to FAM-labeled DNA was used to estimate the binding ability of DNA competitors. The binding ratio was calculated as:

$$\text{Binding ratio} = \text{DNA}_{\text{total bound}}/\text{FAM} - \text{S} - 14 - 2_{\text{bound}},$$

where, $\text{DNA}_{\text{total bound}}$ is the total DNAs eluted from the systemin-coated beads calculated based on the UV absorption at 260 nm, and the S-14-2_{bound} was the S-14-2 eluted from the systemin-coated beads calculated based on the fluorescence intensity.

The affinity of S-5-1 to systemin was measured by incubating increasing amounts of FAM labeled S-5-1 with 20 μl of systemin-coated beads in 6 ml of binding buffer at 25 °C for 15 min, after centrifuge, the concentrations of free S-5-1 were determined by measuring the fluorescence intensity of the supernatant. The amount of bound aptamers ($Aptamer_{bound}$) was calculated as:

$$Aptamer_{bound} = aptamer_{total} - aptamer_{free}$$

The apparent equilibrium dissociation constant (K_d) of the aptamer to systemin was obtained by fitting the amount of bound aptamers on the concentration of the free aptamers to the equation $Y = B_{max}X/(K_d + X)$, using SigmaPlot (Jandel, San Rafael, CA).

4.7. CD spectra assays

Measurements were taken in 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 2 mM $MgCl_2$, DNA at certain concentrations 5 μM. Before measurement, all the samples were heated at 95 °C for 10 min, cooled quickly on ice, and then added systemin or KCl at terminate concentration of 0.2 and 10 mM respectively, stored at 4 °C for 4 h. To facilitate comparisons, the CD spectra were background subtracted, smoothed, and calibrated for concentration so that molar ellipticities could be obtained.

4.8. The influence of ionic strength and pH on aptamer binding

0.4 μM FAM-labeled S-5-1 or a control sequence (Tyr) was incubated with 10 μl of systemin-coated beads in 100 μl of binding buffer for 30 min. The mixture was transferred into an empty column, and the flow out solution was collected. The drained column was washed with 100 μl of binding buffer, and then eluted with 100 μl of 0.5 M NaOH. The fluorescence intensities of the eluates and flow out solutions were measured on a 96-well plate (Costar) by excitation at 488 nm and emission. The relative ratio was calculated as:

$$\text{Relative ratio} = FI_{eluted}/FI_{flow},$$

where, FI_{eluted} is the fluorescence intensity of eluates and FI_{flow} is the fluorescence intensity of the flow out solution.

For evaluating the influence of NaCl on the binding of S-5-1 to Systemin, all binding experiments were performed in phosphate buffer (20 mM KH_2PO_4/Na_2HPO_4, pH 6.5) plus different concentration of NaCl.

For evaluating the influence of pH value on aptamer binding, all binding experiments were performed in phosphate buffer (0.2 M). The flow out solution was adjusted to pH 13 using 5 M NaOH.

4.9. Competition assays of peptide analogs

The binding ability of the peptide analogs to aptamer was tested by competition assays. 0.4 μM FAM-labeled S-5-1 was mixed with 3 mM systemin or peptide analogs in 100 μl of binding buffer at 25 °C for 30 min, then incubated with 10 μl of systemin-coated beads for 60 min by mild shaking, the mixture was transferred into an empty column and washed with 100 μl of binding buffer, then eluted with 100 μl of 0.5 M NaOH. The fluorescence intensities of the eluates were measured on a 96-well plate (Costar) by excitation at 488 nm and emission at 530 nm. The percentage of competition was calculated on the basis of the binding of the S-5-1 without competitor:

$$\text{Percentage of competition} = (FI_0 - FI_c)/FI_0 \times 100\%,$$

where, FI_0 is the fluorescence intensity of the eluate without competitor, FI_c is the fluorescence intensity of the eluate with competitive sequence.

4.10. Recognition of systemin by aptamer beacon

Hundred microliters of 300 nM F-S-5-3T was incubated with 300 nM cS-5-T3 at 25 °C for 30 min in PBS buffer (140 mM NaCl, 2.5 mM KCl, 1.6 mM KH_2PO_4, 15 mM Na_2HPO4, pH 7.4) in a 96-well plate (Costar). Then 1 mM systemin or peptide analogs were added. After 30 min incubation, the fluorescence emission spectra of the samples were collected by excitation at 488 nm on SpectraMax M5.

Two hundred microliters of 300 nM F-S-5-3T was incubated with 300 nM cS-5-T3 at 25 °C for 30 min. Then different concentrations of systemin were added. After 30 min incubation, the fluorescence intensities of samples were measured in a 0.2 ml quartz cuvette by excitation at 488 nm and emission at 530 nm with cut-off at 515 nm on SpectraMax M5.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.05.061.

References and notes

1. Pearce, G.; Strydom, D.; Johnson, S.; Ryan, C. A. *Science* **1991**, *253*, 895.
2. Constabel, C. P.; Yip, L.; Ryan, C. A. *Plant Mol. Biol.* **1998**, *36*, 55.
3. Ryan, C.; Pearce, G. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 14577.
4. Bahyrycz, A. *J. Pept. Sci.* **2007**, *13*, 787.
5. Schmidt, S.; Baldwin, I. T. *Oecologia* **2009**, *159*, 473.
6. Schilmiller, A. L.; Howe, G. A. *Curr. Opin. Plant Biol.* **2005**, *8*, 369.
7. Ryan, C. A. *Biochim. Biophys. Acta* **2000**, *1477*, 112.
8. Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818.
9. Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505.
10. Bing, T.; Yang, X.; Mei, H.; Cao, Z.; Shangguan, D. *Bioorg. Med. Chem.* **2010**, *18*, 1798.
11. Shangguan, D.; Li, Y.; Tang, Z.; Cao, Z. C.; Chen, H. W.; Mallikaratchy, P.; Sefah, K.; Yang, C. J.; Tan, W. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 11838.
12. Li, S.; Xu, H.; Ding, H.; Huang, Y.; Cao, X.; Yang, G.; Li, J.; Xie, Z.; Meng, Y.; Li, X.; Zhao, Q.; Shen, B.; Shao, N. *J. Pathol.* **2009**, *218*, 327.
13. Jayasena, S. D. *Clin. Chem.* **1999**, *45*, 1628.
14. Shangguan, D.; Tang, Z.; Mallikaratchy, P.; Xiao, Z.; Tan, W. *Chembiochem* **2007**, *8*, 603.
15. Fredriksson, S.; Gullberg, M.; Jarvius, J.; Olsson, C.; Pietras, K.; Gústafsdóttir, S.; Östman, A.; Landegren, U. *Nat. Biotechnol.* **2002**, *20*, 473.
16. Wilson, D. S.; Szostak, J. W. *Annu. Rev. Biochem.* **1999**, *68*, 611.
17. Bunka, D. H.; Stockley, P. G. *Nat. Rev. Microbiol.* **2006**, *4*, 588.
18. Vianini, E.; Palumbo, M.; Gatto, B. *Bioorg. Med. Chem.* **2001**, *9*, 2543.
19. Boese, B. J.; Breaker, R. R. *Nucleic Acids Res.* **2007**, *35*, 6378.
20. Cheng, X.; Liu, X.; Bing, T.; Zhao, R.; Xiong, S.; Shangguan, D. *Biopolymers* **2009**, *91*, 874.
21. Burge, S.; Parkinson, G. N.; Hazel, P.; Todd, A. K.; Neidle, S. *Nucleic Acids Res.* **2006**, *34*, 5402.
22. Bugaut, A.; Balasubramanian, S. *Biochemistry* **2008**, *47*, 689.
23. Phan, A.; Patel, D. *J. Am. Chem. Soc.* **2003**, *125*, 15021.
24. Casals, J.; Viladoms, J.; Pedroso, E.; Gonzćlez, C. *J. Nucleic Acids* **2010**, doi:10.4061/2010/468017.
25. Parkinson, G.; Lee, M.; Neidle, S. *Nature* **2002**, *417*, 876.
26. Marathias, V.; Bolton, P. *Biochemistry* **1999**, *38*, 4355.
27. Marathias, V.; Bolton, P. *Nucleic Acids Res.* **2000**, *28*, 1969.
28. Smargiasso, N.; Rosu, F.; Hsia, W.; Colson, P.; Baker, E.; Bowers, M.; De Pauw, E.; Gabelica, V. *J. Am. Chem. Soc.* **2008**, *130*, 10208.
29. Berova, N.; Nakanishi, K.; Woody, R.; Woody, R. *Circular dichroism: principles and applications*, second ed.; Vch Verlagsgesellschaft Mbh, 2000.
30. Yang, X.; Bing, T.; Mei, H.; Fang, C.; Cao, Z.; Shangguan, D. *Analyst* **2011**, *136*, 577.
31. Lin, P.; Yen, S.; Lin, M.; Chang, Y.; Louis, S.; Higuchi, A.; Chen, W. *J. Phys. Chem. B.* **2008**, *112*, 6665.
32. Hermann, T.; Patel, D. *Science* **2000**, *287*, 820.
33. Hianik, T.; Ostatà, V.; Sonlajtnerova, M.; Grman, I. *Bioelectrochemistry* **2007**, *70*, 127.
34. Slosarek, G.; Kalbitzer, H. R.; Mucha, P.; Rekowski, P.; Kupryszewski, G.; Giel-Pietraszuk, M.; Szymanski, M.; Barciszewski, J. *Struct. Biol.* **1995**, *115*, 30.

35. Mucha, P.; Szyk, A.; Rekowski, P.; Kupryszewski, G.; Slósarek, G.; Barciszewski, J. *Collect. Czech. Chem. Commun.* **1999**, *64*, 553.
36. Toumadje, A., Jr.; Johnson, W. C. *J. Am. Chem. Soc.* **1995**, *117*, 7023.
37. Zhang, Z.; Guo, L.; Tang, J.; Guo, X.; Xie, J. *Talanta* **2009**, *80*, 985.
38. Nutiu, R.; Li, Y. *Chem. Eur. J.* **2004**, *10*, 1868.
39. Zuker, M. *Nucleic Acids Res.* **2003**, *31*, 3406.