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Selection, identification, and characterization of aptamers for pro-gastrin-releasing peptide (31–98), a tumor marker for small cell lung cancer

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Pro-gastrin-releasing peptide (31-98) (ProGRP$_{31-98}$) is a highly reliable, sensitive, and specific tumor marker for small cell lung cancer (SCLC). DNA aptamers for ProGRP$_{31-98}$ were selected in this study from an 89-mer DNA library with a random region of 48 nucleotides (nt) flanked with two primer hybridization sites, by using the systematic evolution of ligands by exponential enrichment (SELEX) method. The DNA sequences binding to ProGRP$_{31-98}$ immobilized magnetic beads were selected by running 12 SELEX cycles and 3 negative selection cycles. The selected DNA sequences were separated by cell based DNA cloning, and then sequence analyzed. The DNA sequences obtained were screened and investigated for binding specificity to ProGRP$_{31-98}$ by using electrochemiluminescence (ECL) measurement with [Ru(bpy)$_2$dppz]$^{2+}$, a molecular light-switch, as the ECL probe. One DNA sequence (89 nt) and its random region (48 nt) were identified as specific aptamers for ProGRP$_{31-98}$. Based on the secondary structures of the obtained DNA aptamers, other two truncated DNA aptamers with 40 nt and 15 nt long respectively, were identified. The obtained aptamers have strong affinities to ProGRP$_{31-98}$, with Kd value of 16 nM, and can detect ProGRP$_{31-98}$ with a detection limit of 17 nM using the label-free ECL measurement.

Introduction

Aptamers are single-stranded DNA or RNA molecules that can bind to target molecules with high affinity and specificity, similar to antibodies. In addition, aptamers possess a number of advantages over antibodies, such as easily synthetic, wide range of molecular targets, low production cost and time, easily to be modified, strong chemical and thermal stability, and long storage life.[1–5] Since their discovery, aptamers have become increasingly important molecular tools for target validation, diagnostics, therapeutics, and drug discovery.[1,3,4]

Since the first aptamers against bacteriophage T4 DNA polymerase and several organic dyes were selected by using the method of systematic evolution of ligands by exponential enrichment (SELEX) in 1990,[6,7] a lot of specific aptamers affinitive to various targets, such as sialyl Lewis X,[2] HIV gp 120,[3] L-arginine,[8] adenosine triphosphate (ATP),[9] human thrombin,[10] prostate-specific antigen,[11] have been reported. It is highly desirable to select aptamers for more targets, to expand their applications.

Gastrin-releasing peptide (GRP) is a neuroendocrine peptide, secreted from the dense neurosecretory granules of small cell lung cancer (SCLC) cells.[12] GRP is rarely detected in the serum of patients with lung cancer, because of its short

half life. It is generated by the proteolytic cleavage of pro-gastrin-releasing peptide (ProGRP), the more stable precursor of GRP. The proteolytic cleavage of ProGRP occurs after the Lys-Lys sequence at positions 29–30 to release the C-terminal fragment.[13] The area between amino acids 43 and 97 is conserved and found only in mammals.[14] The 31–98 segment of ProGRP (ProGRP$_{31–98}$) has been clearly identified as a highly reliable, sensitive, and specific tumor marker for SCLC,[15–17] which grows quickly and spread early in the course of the disease, and accounts for about twenty-five percent of lung cancer. Measurement of serum ProGRP$_{31–98}$ levels enables diagnosis, prediction of prognosis, and treatment monitoring in SCLC.[18,19] Recently, a 25-mer DNA aptamer for ProGRP$_{1–98}$ with dissociation constant (K_d) of ~1.2 μM was selected.[20] ProGRP$_{1–98}$ is the 1–98 segment of ProGRP. To our knowledge, no aptamer selection against ProGRP$_{31–98}$, the 31–98 segment of ProGRP, has been reported. Compared to ProGRP$_{1–98}$, ProGRP$_{31–98}$ is a more reliable, sensitive, and specific tumor marker for SCLC.[14-17]

Aptamers usually fold into unique three dimensional structures to ensure their specific binding to targets. [Ru(bpy)$_2$dppz]$^{2+}$ (bpy = 2,2'-bipyridine, dppz = dipyrido[3,2-a:2',3'-c] phenazine), a molecular light switch, shows negligible electrochemiluminescence (ECL) in aqueous solution, but exhibits significantly enhanced ECL intensity when it intercalates into the base pairs of the aptamer three-dimensional structures.[21,22] The aptamer ligand, e.g. ATP, can cause the three-dimensional structure changes to the aptamer/[Ru(bpy)$_2$dppz]$^{2+}$ complex, leading to ECL intensity

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changes.[21] This technique has been applied for DNA interaction study and a label-free ATP aptasensor.[21]

This paper reports the selection of DNA aptamers for ProGRP$_{31-98}$ from an 89-mer DNA library with a random region of 48 nucleotides (nt) flanked with two primer hybridization sites using the SELEX technique. The DNA sequences binding to ProGRP$_{31-98}$ immobilized magnetic beads were selected by running 12 SELEX cycles. The selected DNA sequences were amplified by polymerase chain reaction (PCR), and then cloned into *pEASY*-T3 cloning vector, followed by transformation into *Escherichia Coli* (*E. coli*) Trans5α chemically competent cells. The DNA sequences of some positive clones were determined. The binding activity and selectivity of the selected DNA sequences towards ProGRP$_{31-98}$ were evaluated by using the ECL technique with $[Ru(bpy)_2dppz]^{2+}$ as the probe. The most active DNA sequences were truncated according to their secondary structure; and the affinity and the selectivity of the truncated DNA sequences were also evaluated. The obtained affinitive and selective DNA aptamers were applied in label-free aptasensors using the ECL technique.

Experimental

Materials

Silica coated magnetic beads modified with carboxylic groups (SM3-P100, diameter: 1150 nm) were purchased from Shanghai Allrun Nano Science & Technology (Shanghai, China). Streptavidin was obtained from, and all DNA sequences were custom synthesized by Sangon Shanghai Biotech (Shanghai, China). Concanavalin A (ConA), bovine serum albumin (BSA), bovine insulin, and 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC) were obtained from Sigma-Aldrich (St. Louis, MO). ProGRP$_{31-98}$ (Mw: ~7.5 KD) was kindly provided by Meike BioMeditech Wuxi (Wuxi, China). *Trans*Taq-T DNA polymerase, deoxynucleotide triphosphates (dNTPs), Trans 2K Plus DNA marker, ampicillin (Amp), isopropyl β-D-thiogalactoside (IPTG), 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), EcoR 1, *pEASY*-T3 cloning vector, and *E. coli* Trans5α chemically competent cells were purchased from Beijing TransGen Biotech (Beijing, China).

$[Ru(bpy)_2dppz]^{2+}$ was synthesized according to the method reported.[23] Briefly, $[Ru(bpy)_2]C1_2\bullet2H_2O$ (1.54 g, 2.97 mmol) and dipyridophenazine (0.93 g, 3.19 mmol) were dissolved in methanol-water mixture (1:2, v/v, 380 mL) and refluxed for 4.5 h. The reaction mixture was then filtrated and the filtrate was precipitated with the addition of 10% $NaBF_4$. A deep red product was finally recrystallized from ethanol. Dipyridophenazine was synthesized from o-phenylenediamine and l,10-phenanthroline-5,6-dione;[23,24] and l,10-phenanthroline-5,6-dione was synthesized from 1,10-phenanthroline monohydrate.[23]

$[Ru(bpy)_2]C1_2\bullet2H_2O$, o-phenylenediamine, and 1,10-phenanthroline were obtained from Aladdin Industrial (Shanghai, China). Other chemicals, such as 4-morpholine ethanesulfonic acid (MES), were obtained from Sinopharm

Chemical Reagent (Shanghai, China). All chemicals were of analytical grade and used as received. Deionized water obtained from a Millipore water system was used throughout the experiment.

Instrumentation

HB-1000 hybridizer (UVP, Upland, CA, USA) was used for conjugating proteins/peptides onto the magnetic beads. UV 2450 UV-Vis spectrometer (Shimadzu, Japan) and F-4500 fluorescence spectrophotometer (Hitachi, Japan) were employed for protein/peptide and DNA concentration measurement, respectively. PCR was performed using Eppendorf PCR instrument (Mastercycler Gradient, Germany). Electrophoresis experiments were realized using DYY-6C electrophoresis power supply (Beijing Liuyi Instrument, China) and JY04S-3C gel imaging system (Beijing Junyi-Dongfang Electrophoresis Equipment, China). The magnetic beads were processed using MS-12 Otzi magnetic separator (Shanghai Allrun Nano Science & Technology, China). A conventional three-electrode system consisting of an Ag/AgCl/(3M KCl) reference electrode, a platinum wire counter electrode and a glassy carbon working electrode (GCE) was used in the ECL experiment. Prior to the experiment, the GCE was sequentially polished with aqueous slurries of 1 μm, 0.3 μm and 0.05 μm alumina powders respectively, and then sonicated in water and ethyl alcohol bath each for 5 min. ECL intensities were measured using BPCL-1-TIC Ultra-Weak luminescence analyzer (Beijing Jianxin Lituo Science & Technology, China) equipped with Ingsens1010s electrochemical workstation (Guangzhou Ingsens Sensor Technology, China). The PMT in the ECL measurement was biased at 1200 V. All the measurements were performed at room temperature (~25 °C).

Conjugation of proteins/peptides onto the magnetic beads

The proteins/peptides, including ProGRP$_{31-98}$, streptavidin, ConA, BSA, or insulin were conjugated onto the magnetic beads through covalent reaction between –COOH on the magnetic beads and –NH_2 of the proteins/peptides, using EDC and N-hydroxysuccinimide (NHS) as the coupling agents. In brief, the magnetic beads (2 mg) were washed with 0.01 M MEST buffer (2.13 g L^{-1} MES·H$_2$O, 0.05% Tween 20, pH = 6.0) twice, and then incubated in a 400 μL solution containing 2.5 mg mL^{-1} EDC and 5 mg mL^{-1} NHS at 37 °C for 1 h to activate the –COOH group. The magnetic beads were then washed with 0.01 M MES buffer (2.13 g L^{-1} MES·H$_2$O, 0.31 g L^{-1} boric acid, pH = 9.0) twice, and then mixed with 100 μL 0.05 M phosphate buffer solution (PBS) (pH = 7.4) containing 1 mg mL^{-1} protein/peptide. The reaction mixture was adjusted to a total volume of 500 μL with the MES buffer and agitated in the HB-1000 hybridizer at 37 °C overnight. The amount of conjugated proteins/peptides was determined as the protein/peptide diminishment in the supernatant after the reaction by using Bradford method.

In vitro selection of aptamers using the SELEX method

For vitro selection of aptamers for ProGRP$_{31-98}$, the 89-mer DNA library (sequence: 5'-CTTCTGCCCGCCTCCTTCC-48 nt-

GGAGACGAGATAGGCGGACACT-3') (2.4 nmol) was dissolved in 200 μL binding buffer (100 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, 20 mM Tris-HCl, 0.02% Tween 20, pH = 7.6) and then denatured at 95 °C for 10 min. After being cooled in ice bath for 10 min, the DNA library was incubated with 300 μL 0.1 mg mL^{-1} $ProGRP_{31-98}$ coated magnetic beads in the binding buffer at 37 °C for 2 h in the HB-1000 hybridizer. The beads were then washed with sterilized water and taken as the template for PCR (94 °C for 5 min; 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, 15-30 cycles; 72 °C for 10 min) using F-FAM (5'-FAM-CTTCTGCCCGCCTCCTTCC-3') and R-Biotin (5'-Biotin-AGTGTCCGCCTATCTCGTCTCC-3') as the forward and the reverse primer, respectively, in the presence of *Trans*Taq-T DNA polymerase and dNTPs. FAM is a fluorescence molecule. The double-stranded DNA (dsDNA) (250 μL) obtained from the PCR was verified using agarose gel electrophoresis (AGE) analysis and then incubated with 200 μL 10 mg mL^{-1} streptavidin coated magnetic beads in TE-NaCl solution (10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl, pH = 8.0) at room temperature for 30 min, when the dsDNA bound to the streptavidin coated magnetic beads' surface. The bound dsDNA was then denatured by incubating the beads in 100 μL 0.15 M NaOH for 10 min. The supernatant was neutralized with 0.3 M HCl, and then diluted to 200 μL with the binding buffer, producing a FAM-labeled DNA library.

The above SELEX procedure was repeated for 12 cycles. From the second SELEX cycle, the fluorescence intensity of the $ProGRP_{31-98}$ coated magnetic beads after incubation with the FAM-labeled DNA library was measured to monitor the DNA enrichment. To enhance the specificity of the selected aptamers for $ProGRP_{31-98}$, 3 negative selection cycles of incubating the DNA library with insulin or BSA coated magnetic beads were inserted after the 6th SELEX cycle, and repeated every 1 to 3 SELEX cycles.

Separation and sequence analysis of the DNA aptamers

The obtained final DNA library was incubated with $ProGRP_{31-98}$ coated magnetic beads, and then the beads were served as PCR template (94 °C for 5 min; 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 min, 15 cycles; 72 °C for 20 min) using F (5'-CTTCTGCCCGCCTCCTTCC-3') and R (5'-AGTGTCCGCCTATCTCGTCTCC-3') as the forward and the reverse primer, respectively. Finally, the PCR product was purified using AGE (1.5%) and spin column separation methods, and verified using AGE analysis. The purified dsDNA product was cloned into *pEASY*-T3 cloning vector and then transformed into Trans5α *E. coli* chemically competent cells. The cells were then cultured on solid LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, 15 g L^{-1} agar, pH = 7.4) containing 100 μg mL^{-1} Amp and coated with IPTG and X-gal, at 37 °C overnight to screen positive clones. The white colonies were chosen and subjected to colony PCR (94 °C for 10 min; 94 °C for 30 s, 58 °C for 30s, 72 °C for 2 min, 30 cycles; 72 °C for 10 min) using M13F (5'-TGTAAAACGACGGCCAGT-3') and M13R (5'-CAGGAAACAGCTATGACC-3') as the forward and the reverse universal primer, respectively. After the PCR product was verified by AGE analysis, the selected positive

recombinants were inoculated into liquid LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, pH = 7.4) containing 100 μg mL^{-1} Amp. The culture was shaken at 37 °C overnight. The plasmids were then extracted from the positive recombinants and digested by restriction endonuclease EcoR 1. The digested plasmids were subjected to AGE analysis to confirm the positivity of the recombinants. The positive clones were sent to Beijing Liuhe Greatness Technology Co. for DNA sequence analysis using M13F (5'-TGTAAAACGACGGCCAGT-3') and M13R (5'-CAGGAAACAGCTATGACC-3') as the universal forward and reverse primer, respectively. The secondary structures of the selected DNA sequences were analyzed using OligoAnalyzer.

Binding specificity assay of the selected aptamers

The DNA aptamers selected using the SELEX method were custom-synthesized and verified for their binding specificity to $ProGRP_{31-98}$ by ECL measurement with $[Ru(bpy)_2dppz]^{2+}$ as the probe. Briefly, 10 μL 100 μM DNA aptamer in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0) was denatured at 95 °C for 10 min, and then added to 1 mL 20 μM $[Ru(bpy)_2dppz]^{2+}$ in 5 mM oxalate/oxalic acid solution (pH = 5.5). The ECL intensity was monitored until the ECL peak value (denoted as ECL_0) was stabilized in ~50 min. With the addition of 50 μL 1.2 mg mL^{-1} protein/peptide, the ECL intensity was continuously monitored in ~40 min and the stabilized ECL peak value was denoted as ECL_1. The $(ECL_0-ECL_1)/ECL_0$ value was calculated.

Affinity assay of the selected aptamers

The affinity of the selected DNA aptamers to $ProGRP_{31-98}$ was investigated by measuring the amount of the bound aptamers onto the $ProGRP_{31-98}$ coated magnetic beads in various concentrations (18–244 nM, denoted as C_0) of the aptamers in the binding buffer. The aptamers were labeled with fluorescence molecule FAM at their 5' ends. The aptamer solution (400 μL) was denatured at 95 °C for 10 min, and then incubated with 10 μL 1 mg mL^{-1} $ProGRP_{31-98}$ coated magnetic beads for 2 h at 37 °C in the HB-1000 hybridizer. The aptamer concentration was estimated by measuring the fluorescence intensity at the emission wavelength of 520 nm. The concentration of the bound aptamer (C_b) was calculated based on the aptamer concentrations before and after the binding. Following the Lineweaver-Burk equation (Eq. 1):

$$1/C_b = K_d/B_{max}C_0 + 1/B_{max} \quad \text{Eq. 1}$$

, where Bmax denotes the maximum value of C_b, the K_d value of the aptamer–$ProGRP_{31-98}$ complex was estimated from the $1/C_b$ versus $1/C_0$ linear plot.

Aptamer-based $ProGRP_{31-98}$ assay by ECL titration

The selected aptamers were applied in label-free aptasensors using the ECL measurement with $[Ru(bpy)_2dppz]^{2+}$ as the probe in 5 mM oxalate/oxalic acid solution (pH = 5.5). Similar with the binding specificity assay experiment, a $[Ru(bpy)_2dppz]^{2+}$/aptamer complex was formed and its ECL peak value (denoted as ECL_0) was recorded. The $ProGRP_{31-98}$ solution was then added gradually in a batch mode. The ECL

peak value (ECL_1) was recorded for different ProGRP$_{31-98}$ concentrations after the chemiluminescence was stabilized in ~40 min. The changes of the ECL peak values ($\Delta ECL = ECL_0 - ECL_1$) with the addition of ProGRP$_{31-98}$ was calculated as the signal.

Results and discussion

In vitro selection and sequence analysis of DNA aptamers

The conjugation of proteins/peptides onto the magnetic beads and the DNA enrichment in the SELEX procedure were confirmed using the UV-Vis and the fluorescence measurement, respectively (data not shown). The agarose electrophoresis gel image (Figure 1) of the PCR product amplified from the final SELEX cycle showed an ~100 base pair (bp) DNA band, a length slightly longer than the synthesized DNA length (i.e., 89 bp), because the DNA polymerase *Trans*Taq-T added poly-nucleotides A to the DNA ends. The final DNA library and the PCR product had an expected and specific DNA length.
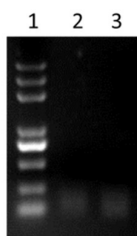

Figure 1. The 1.5% agarose electrophoresis gel image of the PCR product from the final SELEX cycle (lane 2 and 3). Lane 1 is the DNA marker containing 5000, 3000, 2000, 1000, 750, 500, 250, and 100 bp of DNA sequences.

After the PCR product from the final SELEX cycle was purified, cloned and then transformed into the *E. coli* chemically competent cells, the white colonies were subjected to colony PCR. Most of the white colonies had positive PCR products of 345 bp, the right size from the positive vectors (Figure 2). The map and the construct of the *pEASY*-T3 cloning vector are shown in Figure S-1.
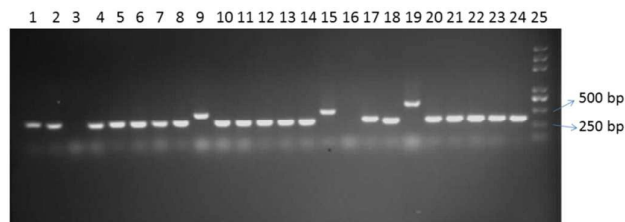

Figure 2. The 1.5% agarose electrophoresis gel image of the PCR products from the white colonies numbered C1–C11, C13–C17, C12, C18–C24 (corresponding to lane 2–25, respectively). Lane 1 is the DNA marker containing 5000, 3000, 2000, 1000, 750, 500, 250, and 100 bp of DNA sequences.

The plasmids of the positive colonies were extracted and then digested by EcoR I. The agarose electrophoresis gel analysis results of some digestion products are illustrated in Figure 3. Most of the digested plasmids clearly exhibited two electrophoresis bands with the size of ~126 bp and 3004 bp respectively, consistent with theoretic values. Twenty-nine of the positive colonies with confirmed plasmid digestion products were subjected to DNA sequence analysis. Eight colonies were successfully sequenced with the right length of random region (i.e. 48 nt). The obtained DNA sequences, denoted as DNA aptamer candidates for ProGRP$_{31-98}$, are listed in Table 1. The DNA aptamer candidates were named as A2, A12, etc, followed after their corresponding colony number C2, C12, respectively. Among the 8 DNA aptamer candidates obtained, A19 and A21 have exactly the same oligonucleotide sequence (i.e., the sequence occurrence is 2).
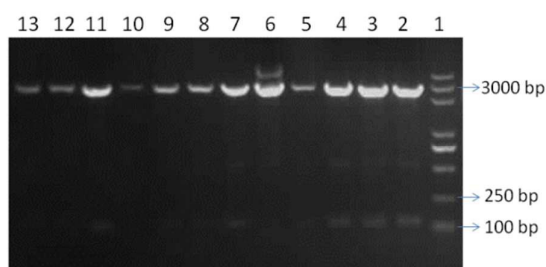

Figure 3. The 1.5% agarose electrophoresis gel image of the plasmid EcoR I digestion products from some white colonies (lane 2–13). Lane 1 is the DNA marker containing 5000, 3000, 2000, 1000, 750, 500, 250, and 100 bp of DNA sequences.

Table 1. Sequences of DNA Aptamer Candidates for ProGRP$_{31-98}$ Obtained in This Study

Name	Sequence
A2	5'AATGCCGTGGAGACGAGATAGGCGGACACTAACCCCTCTAGGACGAGA-3'
A12	5'-TGGGTCATCTAGCCTTCTCTGCGTCGCAGCACTATTGCTTCGGAGTTT-3'
A16	5'-AAACTCTTGTAATCATAACGGTTTTATCAACGGAGCTCCTACGCACAT-3'
A18	5'-CATGCGGAGTAGATTCGAGCCCAGATAGTCCCTGGTTATTTCCTTAGG-3'
A19	5'-ATCCTCTGTCGCCTTTCTACACGCGCGCTGGAGGCACCATTATTTGCC-3'
A21	5'-ATCCTCTGTCGCCTTTCTACACGCGCGCTGGAGGCACCATTATTTGCC-3'
A31	5'-TACCATACCTTGGTATTCGCAGTTATAAAGCGTCTGGGTCTCCCGGTG-3'
A41	5'-CCCGGTTCTATACGACTGGCTAGATTTTACTTTGACCATACAGTGACG-3'

Binding specificity screening of the DNA aptamer candidates

The obtained DNA aptamer candidates were chemically synthesized and screened for their binding specificity to ProGRP$_{31-98}$ by ECL measurement with $[Ru(bpy)_2dppz]^{2+}$ as the probe. $[Ru(bpy)_2dppz]^{2+}$ is a remarkable luminescent reporter of DNA structure, described as a molecular light switch for DNA, exhibiting a negligible ECL intensity in aqueous solution. The ECL intensity of the molecular light switch enhances for at least 10^4 times when it intercalates into the base pairs of double-strand DNA, such as the hairpin DNA stem.[21,22] Aptamers usually fold into unique three-dimensional

structures to bind to targets specifically. The folded structures of the aptamers allow $[Ru(bpy)_2dppz]^{2+}$ to intercalate to produce strong ECL.[21] Introduction of target molecules may compete with $[Ru(bpy)_2dppz]^{2+}$ to bind to aptamer,[21] or causing three-dimensional structure changes to the aptamer, leading to the ECL intensity changes.

Among the 8 DNA aptamer candidates, A18 can specifically interact with $ProGRP_{31-98}$. The ECL intensity peak value decreased for 46.9% (from 1023 counts to 543 counts) with the presence of $ProGRP_{31-98}$ in the oxalate/oxalic acid solution containing 20 µM $[Ru(bpy)_2dppz]^{2+}$ and 1 µM A18 (Figure 4A). In contrast, the ECL intensity had no obvious changes upon the addition of the interference protein bovine serum albumin (BSA) (Inset in Figure 4A). The interference proteins/peptides tested, including insulin, BSA, and concanavalin A (ConA), caused only ~6.9%, 7.2%, and 16.6% ECL intensity changes respectively, significantly lower than $ProGRP_{31-98}$ did (Figure 4B), indicating that the A18 sequence is a specific DNA aptamer for $ProGRP_{31-98}$.
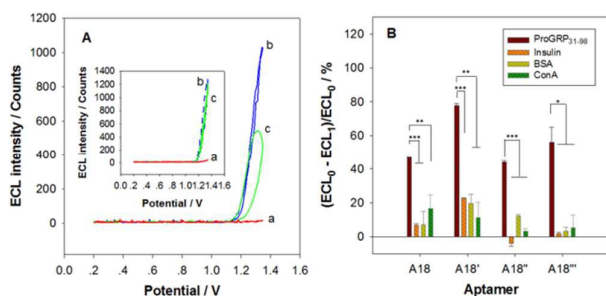

Figure 4. (A, Inset in A) The ECL intensity versus the applied potential curves at a glassy carbon electrode in 5 mM oxalate/oxalic acid solution (pH = 5.5) containing (a) 20 µM $[Ru(bpy)_2dppz]^{2+}$, (b, c) 20 µM $[Ru(bpy)_2dppz]^{2+}$ and 1 µM A18, (b) without, and (c) with 57 µg mL^{-1} (A) $ProGRP_{31-98}$, or (Inset in A) BSA. The PMT was biased at 1200 V. (B) The relative changes of the ECL peak values of the $[Ru(bpy)_2dppz]^{2+}$/aptamer complex (ECL_0) upon the addition of 57 µg mL^{-1} proteins/peptides (ECL_1). *, **, and *** represents that the p value is less than 0.05, 0.01, and 0.001 respectively, in the t test (n = 3–5).

Sequence modification of DNA aptamers

The A18 sequence flanked with the two primer hybridization sites, a DNA sequence of 89 nt long (denoted as A18', Table 2) was then verified for binding specificity to $ProGRP_{31-98}$. Similarly, the ECL peak value of the $[Ru(bpy)_2dppz]^{2+}$/aptamer complex decreased for ~77.6% with the addition of $ProGRP_{31-98}$, significantly higher than those with the introduction of insulin, BSA, and ConA (~22.7%, 19.7%, and 11.3%, respectively) (Figure 4B), indicating that the A18' sequence is also a specific DNA aptamer for $ProGRP_{31-98}$.

Table 2. Sequences of DNA Aptamers for $ProGRP_{31-98}$ Obtained in This Study

Aptamer	Sequence
A18	5'-CATGCGGAGTAGATTCGAGCCCAGATAGTCCCTGGTTATTTCCTTAGG-3'
A18'	5'-CTTCTGCCCGCCTCCTTCC-CATGCGGAGTAGATTCGAGCCCAGATAGTCCCTGGTTATTTCCTTAGG-GGAGACGAGATAGGCGGACACT-3'
A18''	5'-CATGCGGAGTAGATTCGAGCCCAGATAGTCCCTGGTTATT-3'
A18'''	5'-CCAGATAGTCCCTGG-3'

The secondary structure of the A18 aptamer contains two stem-loop hairpins (Figure 5A), with 4 bp for one hairpin stem, and 2 bp for the other. In contrast, the A18' aptamer has four stem-loop hairpins (Figure 5B), with one hairpin the same with the 4 bp stem one of A18. Assuming that the hairpin areas are the main binding domains, the A18 aptamer sequence was truncated at their two ends to a DNA sequence of 40 nt long (A18'', Table 2), without changing its secondary structure (Figure 5C). The ECL peak value of the $[Ru(bpy)_2dppz]^{2+}$/aptamer complex of A18'' decreased for ~44.1% with the addition of $ProGRP_{31-98}$, but only for about -3.8%, 12.3%, and 3.4% with the addition of insulin, BSA, and ConA respectively (Figure 4B), indicating that the A18'' sequence is a specific DNA aptamer for $ProGRP_{31-98}$.
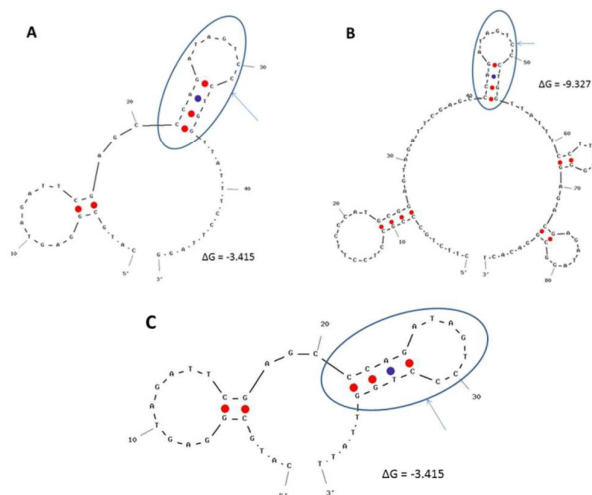

Figure 5. The secondary structures of the DNA aptamer (A) A18, A18', and (B) A18'' under the conditions of C_{Na+}= 100 mM, C_{Mg2+} = 2 mM, T = 25 °C. The circled area is the DNA sequence of the DNA aptamer A18'''.

The A18, A18', and A18'' aptamers have a common 4 bp stem hairpin (the circled area in Figure 5, 15 nt long), denoted as A18''' (Table 2). Assuming that the 4 bp stem hairpin is a main domain in binding with the target, the binding specificity of the hairpin DNA sequence to $ProGRP_{31-98}$ was verified. The ECL peak value of the $[Ru(bpy)_2dppz]^{2+}$/aptamer complex of A18''' decreased for ~56.1% with the introduction of $ProGRP_{31-}$

$_{98}$, significantly larger than those with the introduction of insulin, BSA, and ConA (~1.8%, 3.2%, and 5.5%, respectively) (Figure 4B), indicating that the A18''' sequence is also a specific DNA aptamer for ProGRP$_{31-98}$, and is indeed a main binding domain.

Affinity assay and aptamer-based ProGRP$_{31-98}$ assay

The affinities of the aptamer A18, A18', A18'', and A18''' to ProGRP$_{31-98}$ were investigated. The calibration plot of the fluorescence intensity of the FAM-labeled A18 versus the aptamer concentration, and the plot of $1/C_b$ versus $1/C_0$ of A18 are illustrated in the Supplementary Information (Figure S-2, S-3, respectively). The K_d value of the aptamer A18 binding to ProGRP$_{31-98}$ was estimated to be 49 nM, and those of the aptamer A18', A18'', and A18''' were estimated to be 306, 87, and 16 nM, respectively. The K_d values of the aptamers to ProGRP$_{31-98}$ are in the middle-nanomolar range, 2 order of magnitude smaller than the 25-mer one reported for ProGRP$_{1-98}$ (K_d: ~1.2 μM),[20] and similar to those of antibody-antigen interactions (K_d = 0.01–1000 nM), indicating that these aptamers have high affinity to ProGRP$_{31-98}$. It should be noted that the selected aptamers in this work are totally different in sequence with the 25-mer one reported for ProGRP$_{1-98}$, a different target from ProGRP$_{31-98}$.[20]

The obtained aptamers were applied in label-free aptasensors using ECL measurement with $[Ru(bpy)_2dppz]^{2+}$ as the probe. The ECL intensity–potential curves of the $[Ru(bpy)_2dppz]^{2+}$/aptamer complex under the titration with ProGRP$_{31-98}$ were recorded, and those for A18 and A18' are illustrated in Figure 6. Those for A18'', and A18''' are shown in the Supplementary Information (Figure S-4). The ECL intensity decreased monotonically with the increase of ProGRP$_{31-98}$ concentration. The ECL peak value changes (i.e. ΔECL) versus the ProGRP$_{31-98}$ concentration for A18 is linear with 0.48–3.36 μM target (regression coefficient = 0.9969), with a limit of detection (LOD) of 17 nM (signal to noise ratio = 3). The LOD values for the aptamer A18', A18'', and A18''' were obtained to be 440, 770, and 210 nM, respectively. The LOD value of the A18 aptamer-based ProGRP$_{31-98}$ assay was in the middle-nanomolar range, ~2 order of magnitude smaller than that of a surface plasmon resonance aptasensor reported for ProGRP$_{1-98}$ (LOD: 1 μM), a different target from ProGRP$_{31-98}$.[20] To our knowledge, no aptasensors for ProGRP$_{31-98}$ have been reported.
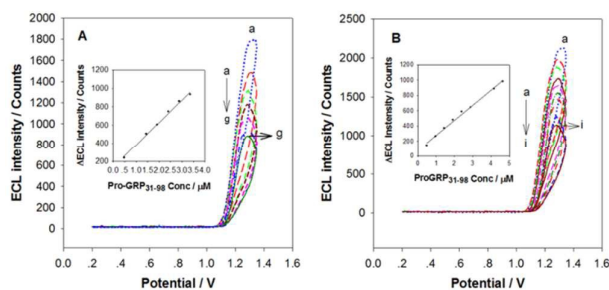

Figure 6. The ECL intensity versus the applied potential curves at a glassy carbon electrode in 5 mM oxalate/oxalic acid solution (pH = 5.5) containing (A) 1 μM A18 and 20 μM $[Ru(bpy)_2dppz]^{2+}$, or (B) 1 μM A18' and 40 μM $[Ru(bpy)_2dppz]^{2+}$, under the titration with ProGRP$_{31-98}$. The ProGRP$_{31-98}$ concentration gradually increased from curve 'a' to 'g' or 'l' .The PMT was biased at 1200 V. Insets: The plots of the ECL peak value changes of the $[Ru(bpy)_2dppz]^{2+}$/aptamer complex versus the ProGRP$_{31-98}$ concentration.

Conclusions

One 48 nt DNA aptamer and one 89 nt DNA aptamer, specifically binding to ProGRP$_{31-98}$, a highly reliable, sensitive, and specific tumor marker for SCLC, have been selected and characterized. By analyzing the secondary structures of the two DNA aptamers, other two highly specific DNA aptamers with 40 nt and 15 nt long respectively, have been obtained. The aptamers exhibit strong affinity to ProGRP$_{31-98}$, with K_d values in the middle-nanomolar range. By using $[Ru(bpy)_2dppz]^{2+}$ as a probe, the ECL intensity of the $[Ru(bpy)_2dppz]^{2+}$/aptamer complex decreased significantly and specifically with the addition of ProGRP$_{31-98}$. The aptamers can sensitively and selectively response to ProGRP$_{31-98}$ in a label-free mode in the presence of the $[Ru(bpy)_2dppz]^{2+}$ probe, by using the ECL measurement. The obtained aptamers may be promisingly applicable in clinical diagnosis.

Acknowledgements
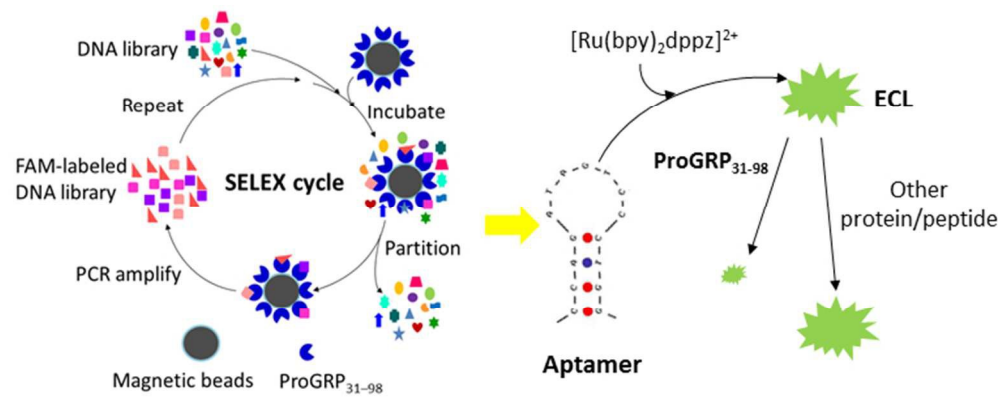
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