行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告
□期中進度報告

利用化學演化技術產生對碳奈米球具特異性之 RNA/DNA aptamers 及其在生物感應器上之應用

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研究目的

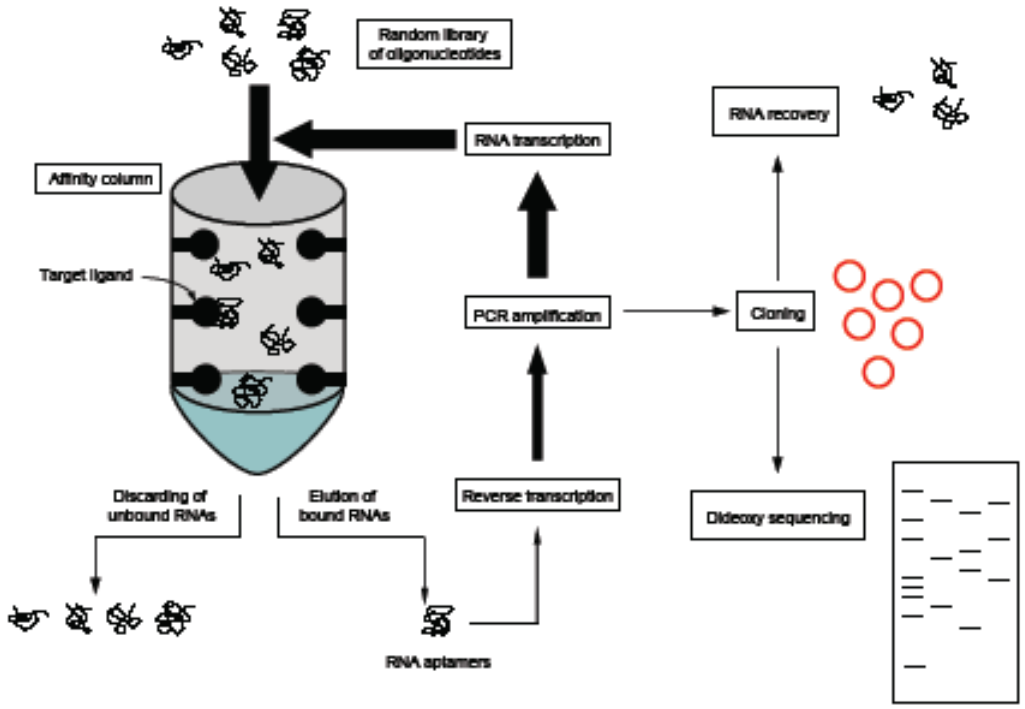
Nanotechnology has captured exceptionally attentions and appeared with extreme pace just in these few years. To date, we know very little about the health and environmental influences of nanomaterials, such as the properties of nanoparticles vary with their chemical composition, sizes, interactions, and virtually nothing about their synergistic impacts. Fullerene is one of the well known nanoparticle and a new type of carbon allotrope that was discovered in 1985. Due to the excessive hydrophobicity and poor solubility of the compounds in aqueous solution make the study of fullerene and its derivatives become difficult. Since the first water-soluble fullerene was reported in 1993, however, many papers have demonstrated on their interaction with biological target, (i) as antioxidants and neuroprotective agents (1-3), (ii) antiapoptotic activity (4-6), (iii) DNA photocleavage (7), (iv) enzyme inhibition (7-9), (v) anti-HIV activity (10,11), and (vi) antimicrobial activity (12,13).

Based on the most recent toxicology reports have shown that nanoparticles may pose a unique risk to everything from bacteria to mammals (14-16). These studies have demonstrated that fullerene when washed in a suspension into the lung tissue of rats, can form aggregates, causing tissue damage, respiratory problems, and even death. Therefore, practical applications of fullerenes as biological or pharmacological agents require that dosage and serum levels be capable of accurate measurement, preferably by sensitive and simple detection procedures. This requires some types of antibody for fullerene to be produced. As fullerene derivatives become useful clinically, anti-fullerene antibodies are ideally suitable for serum assays. Chen *et al.* have successfully prepared water-soluble fullerene derivatives of several proteins and peptides that were used for the immunization as well as for the isolation and characterization of the antibodies (17,18). Several mice derived monoclonal anti-C_{60} antibodies were prepared by standard procedure and the specificity for fullerene was determined by competitive inhibition.

There are several drawbacks of using anti-C_{60} antibodies assay, however, to detect the existence of C_{60} nanoparticle *in vivo* or in our environment. It's a time-consuming process to have production of anti-C_{60} antibodies through mice immunization, including the conjugation between C_{60} and bovine thyroglobulin, rabbit serum albumin, and pentalysine derivatives (17). In addition, only small quantities of anti-C_{60} antibodies can be produced, and also it's inconvenient to purify and characterize their specificity from each batch.

In order to (i) obtain different type of molecules other than classical antibody which are capable of specifically recognizing and binding to fullerene, (ii) overcome the problems by using anti-C_{60} antibodies assay, here we propose a novel method to accomplish this approach. *In vitro* selection, or SELEX (**S**ystematic **E**volution of **L**igands by **EX**ponential enrichment), is a technique that allows the simultaneous screening of highly diverse pools of different RNA/DNA molecules for a particular feature. In 1990, the laboratories of Joyce (Scripps Institute, CA), Szostak (Harvard, Boston), and Gold (U. of Colorado, Boulder) independently developed a technique which allows the simultaneous screening of more than 10^{15} individual nucleic acid molecules for different functionalities (22-24). This method is commonly known as "*in vitro* selection". This novel technique is gaining tremendous attentions as an extremely useful tool in chemical biology. With this technique, large random pools of nucleic acids can be screened for a particular functionality, such as the binding to small organic molecules, large proteins or the alteration or *de novo* generation of ribozyme- catalysis. Functional molecules ("aptamers" is composed of the latin *aptus* = to fit and the greek suffix *-mer*) are selected from the mainly

non-functional pool of RNA/DNA by affinity column chromatography or other selection techniques that are suitable for the enrichment of any desired property.



The method is conceptually straightforward: a starting pool is generated by a standard DNA synthesizer. The machine synthesizes an oligonucleotide with a completely random base-sequence which is flanked by defined primer binding sites. In this way, up to 10^{15} different DNA molecules can be synthesized at once, which is an incredibly complex pool, if one considers the number of antibodies a mouse can possibly generate between 10^{9} and 10^{11}. The immense complexity of the generated pool justifies the assumption that it contains a few molecules with the correct receptor structure or with tertiary structures which lead to catalytic activity; these can be se-lec-ted by affinity chromatography or filter binding assay. Because a pool of such high complexity can be expected to contain only a very small fraction of functional molecules, several purification steps are usually required. Therefore, the very rare active molecules are amplified by the polymerase chain reaction (PCR) or in a transcription-based step. In this way, iterative cycles of selection can be carried out. Successive selection and amplification cycles result in an exponential increase in the abundance of functional sequences, until they dominate the population.

The advantages of aptamers over other types of techniques include the comparatively simple techniques and apparatus required for their isolation, the reasonable number of molecules that can be screened, and their chemical simplicity. As bio-recognition elements in analytical devices, aptamers appear as alternative candidates to antibodies (Ab) often offering substantial advantages. Aptamers can be chemically produced and selected in extreme conditions, permitting study in a broad range of samples, while Abs in general are only stable in a physiological condition. Series of multi-selection processes can be performed by using a single oligonucleotide library simultaneously. Following selection, aptamers can be easily modified (to incorporate molecular markers, favor oriented immobilization on solid support or beads, or increase their stability to nucleases) without affecting their affinity, while chemical modifications of Abs often decrease their affinity. The animal-free production of aptamers not only avoid ethical problems but also allow generation of aptamers against toxic or poorly immunological molecule such as the limited genetic diversity characteristic of IgG and IgMs, and prevent the batch-to-batch variations of polyclonal Ab production.

In order to have fast methods to detect fullerene, we will develop fluorescein-tag labeled fullerene aptamer (see **研究方法)** and fullerene biosensor. In the design of fullerene biosensor, one of the most useful of the immunoassays, two-antibody "sandwich" ELISA, will be employed here. This assay is used to determine the antigen (fullerene) concentration in unknown samples, and it requires two antibodies (aptamers) that bind to epitopes that do not overlap on the antigen. To utilize this assay, one aptamer (the "capture" aptamer) is purified and bound to a solid support. Fullerene is then added and allowed to complex with the bound apatemr. Unbound products are then removed with a wash, and a labeled second aptamer (the "detection" aptamer) is allowed to bind to the fullerene, thus completing the "sandwich". The assay is then quantitated by measuring the amount of labeled second aptamer bound to the matrix, through the use of a colorimetric substrate. Unlike Western blots, which use preciptating substrates, ELISA procedures utilize substrates that produce soluble products. Ideally the enzyme substrates should be stable, safe and inexpensive. Popular enzymes are those which convert a colorless substrate to a colored product, e.g. p-nitrophenylphosphate (pNPP) which is converted to the yellow p-nitrophenol by alkaline phosphatase. Substrates used with peroxidase include 2,2'-azo-bis(3-ethylbenzthiazoline-6- sulfonic acid) (ABTS), o-phenylenediamine (OPD) and 3,3',5'-tetramethylbenzidine base (TMB), which yield green orange and blue colors, respectively.

The goal of this research project are to conduct series of structure-to-function experiments in the area of DNA/RNA-ligand interaction to illustrate the relationship between buckminsterfullerene C_{60} and specific DNA/RNA molecules (aptamers): (i) by using *in vitro* selection method, (ii) determine detailed structures of C_{60}-binding DNA/RNA aptamers, (iii) develop a C_{60} aptamer-based trace system to detect the existence of C_{60} or its derivatives *in vivo* and in our living environment.

研究方法

Design of DNA Template and Preparation of Initial Random RNA Pool. For the DNA template, a fragment containing the region from -17 to -1 of the strong T7 RNA polymerase promoter followed by specific primer-binding sequence and a random region was synthesized (25, 26). The 87-nucleotide DNA template consisted of a 40-nucleotide random sequence region flanked by defined 5' and 3' fixed regions, 5'-GCTAGATCCGGGCCTCATGTCGAA-$(N)_{40}$-TTGAGCGTTTATTCTGAGCTCCC-3'. Two DNA primers, 5'-TTAATACGACTCACTATAGGGAGCTCAGAATAAACGCTCAA-3' and 5'- GCTAGATCC GGGCCTCATGTCGAA-3'. A double-stranded DNA will be first generated from the single-stranded DNA template by using of Klenow fragment. To prepare material for the first round in the SELEX process, a large-scale transcription reaction will be carried out (in nmol scale). The initial RNA transcription is carried out on a 1 mL scale with 3 nmol of DNA template. Subsequent rounds of transcription are done in 500-µl volumes utilizing 250 pmol of DNA template. The reactions are incubated for 3-4 h at 37 ºC. On average, there will have 5-80 RNA copies of each template under the transcription conditions determined by RNA isolation and quantitation. The template DNA can co-migrate with the RNA band on the polyacrylamide gel, and may also act as a competitive binder during selection. Therefore, the template DNA can be removed by DNase I digestion, and the RNA is purified by phenol/chloroform extraction, ethanol precipitation, and gel electrophoresis on denaturing polyacrylamide.

In Vitro Selection Procedure for RNA Aptamers. Selections will be performed by using affinity columns containing 1 mM fullerene. To assure that the affinity column is in optimal condition, the column will be replaced after every 3-4 rounds of selection. In cycle 1, 5, and 10, the RNA pools will be pre-selected on

TentaGel S beads-only column to remove RNAs with affinity for the column matrix. The initial RNA pool contains 300 μg (10 nmol) with approximately $6x10^{15}$ molecules. Before applying to the column, the RNA pool will be first denatured, renatured, then pre-equilibrated on the column with binding buffer. The binding steps will be all carried out at 4°C and unbound RNAs are then removed with washing buffer. The bound RNAs will be eluted with affinity buffer, and quantitated by UV or binding of RiboGreen I fluorescent dye (purchased from Molecular Probe) then measured by fluorospectrometer. The eluted RNAs (bound species) are combined, desalted, dried, then resuspended in buffer. They will be reverse transcribed in 20 μl of a reaction. The reaction mixture is cycled at 95 ºC for 45 s, 52 ºC for 1 min, 72 ºC for 2 min for 10-15 cycles, and then the DNA is precipitated in ethanol and used for transcription. Transcription *in vitro* is performed at 37 ºC for 4 h with T7 RNA polymerase as described above. After the synthesis of RNA and treatment with DNase I, the reaction mixture will be fractionated on a 10% polyacrylamide gel in 8 M urea, RNA is eluted from gel and used for subsequent rounds of selection.

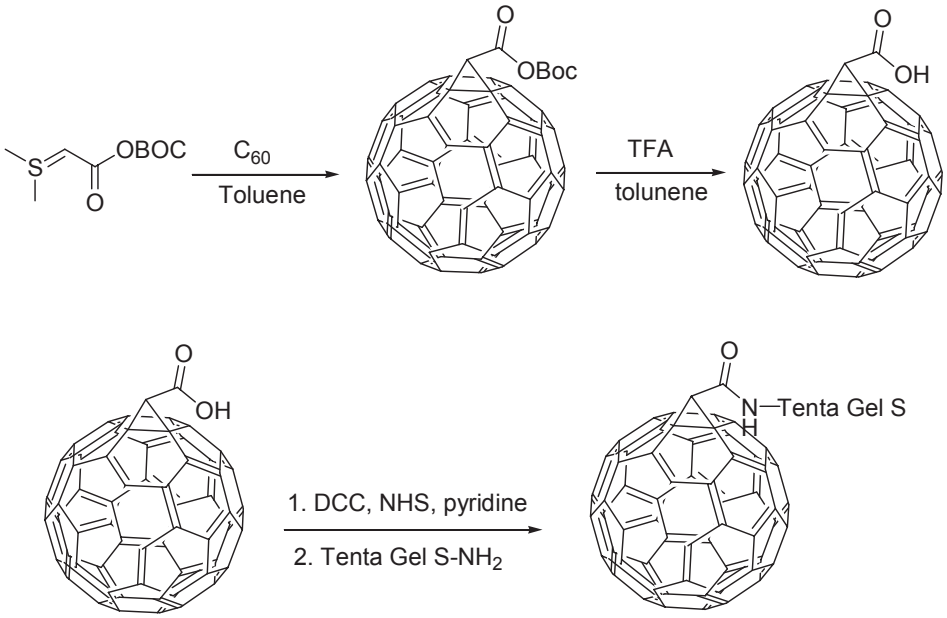
Cloning and Regeneration for Selected RNA Aptamers. PCR-amplified DNA pool from the final round of SELEX will be cloned into the pGEM-T vector (Promega), and subjected to transformation into JM109 competent cells. Sequencing of each clone will be done by automated sequencer using fluorescently-tagged primers. In order to regain the selected RNA aptamers from DNA clones, the DNA clones will be cleaved by *Eco*RI to obtain selected sequences. The cleaved plasmid will be gel purified, then used directly for RNA transcription reaction as described above.

Dideoxy DNA Sequencing for Selected RNA Aptamers. Sequencing of each clone was done by a modified protocol of SequiTherm Excel™ II CycleSequencing kit (Epicentre, WI) that was optimized for a LI-COR® 4200 *IR* series automated sequencer using fluorescently-tagged primers.. The PCR reaction will be set up by mixing 0.9 μl of master solution with 0.6 μl of plasmid, then aliquoting to four marked reaction tubes. The thermocycler will be programmed as follows: 94 ºC (2 min) for 1 cycle; 94 ºC (10 sec), 53 ºC (10 sec), and 72 ºC (60 sec) for 30 cycles; 94 ºC (30 sec) and 72 ºC (60 sec) for 20 cycles; hold at 4 ºC. The reaction is terminated by adding 1 μl of stop solution to each tube and mixing well. Denaturing 6% polyacrylamide sequencing gels (with sequencing grade of urea) will be prepared for a mid-sized set-up (for 250-400 bp range), and pre-run for at least 20 min. Each solution (1.5 μl) was loaded onto the gel by using microtips, and run on the gel for approximately 3 h. The DNA sequences will be determined by using Oligo™ software (Molecular Biology Insights, Inc).

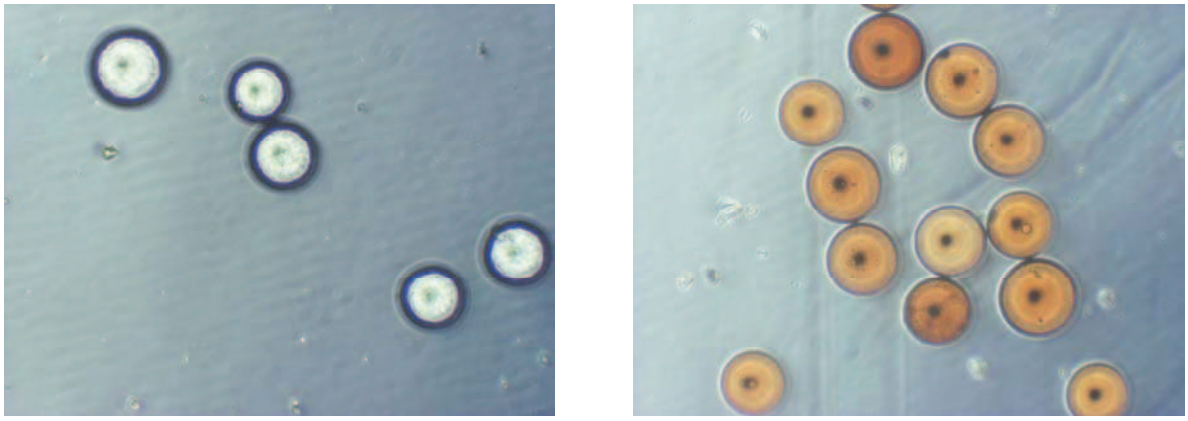
Direct Characterization of Positive Colonies by PCR. It is difficult to distinguish positive clones by using the standard blue-and white (IPTG/X-gal) method due to the fact that a short DNA fragment is inserted, therefore, an alternative screening method is necessary. The colonies will be resuspended in 0.5 ml H_2O and boiled in a water bath for at least 20 min. After centrifugation for 3 min at 9,000 rpm, 5 μl of the supernatant is used in a PCR reaction. For the amplification reactions, a 20 μl reaction mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg/ml gelatine, 0.25 mM dNTP, 10 pmol each primer oligonucleotide, 2 units *Taq* DNA polymerase. The reactions will be subjected to 25 rounds of temperature cycling (94 ºC 40 sec, 55 ºC 40 sec, 72 ºC 1 min), and a final 7 min 60 ºC step. The reaction mixture (10 μl) will be analyzed on a 1% agarose gel containing 0.3 μg/ml ethidium bromide. Reverse sequencing primer (5'-TCACACAGGAAACAGCTATGAC-3') and forward sequencing primer (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') will be used in the PCR reaction.

結果與討論

Preparation of Affinity Chromatography Columns. TentaGel S beads (90 μm) are obtained from Rapp Polymer Company. C_{60}-carboxylic acid (0.074g, 0.09 μmol) is dissolved in 3mL of dry pyridine. N-hydroxysuccinimide (0.01g, 0.9 μmol) is added and brought into solution with the fullerene compound (19-21). Dicycohexylcarbodiimide (0.29g, 0.9 μmol) dissolved in 1mL of dry pyridine was added, and the reaction allow to proceed at room temperature for 48 h. The reaction mixture the is added dropwise over a period of about 5 min to 0.3g of TentaGelS (0.3mmol/g of free amine) dissolved in 4mL of dry pyridine. The reaction is allowed to proceed overnight at room temperature. The mixture is centrifuged, and the pyridine is discarded. The beads are washed with toluene 20 mL twice, CH_2Cl_2 20 mL twice, EtOH 20 mL twice and water 20 mL twice. The wet beads are placed on 96 wells plates for digital photography (Figure 14, A: TentaGel S beads only; B: TentaGel S beads conjugated with fullerene).

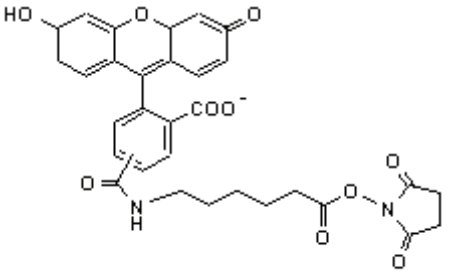


(A) (B)



Primary Sequence Alignments and Secondary Structure Prediction by RNA Folding. Oligo™ program can be used for sequence alignment. Alternatively, conserved sequences can be found by visually comparing and identifying repetitive 4- to 10-bp nucleotide-repeats in all of the sequences. The "find" option of the edit function in most word processing programs can also be used to facilitate and extract all possible RNAs that contain those sequences. Programs such as GeneWork and Mfold will be used to search sequences and predict secondary structures of fullerene-binding RNAs, and to calculate the thermodynamic parameters of optimal and suboptimal secondary structures. The Mfold program is based on the energy minimization algorithm and the energy values. Calculations are performed at 4 °C, which is the same condition used in the selection procedure. Mfold is available on the internet at the uniform resource locator http://bioinfo.math.rpi.edu/~zukerm/.

Fullerene-Fluorescein Conjugation and Determination of K_d by Using Fluorescence Spectroscopy. Adjust fullerene concentration to ~1 mg/ml. Prepare CFSE (Carboxyfluorescein succinimidyl ester, Molecular Probes C-1311), 1.5 mg/ml in anhydrous DMSO and dilute CFSE in anhydrous DMSO immediately before use. Add 100 µl CFSE per 1 ml of fullerene solution. Incubate for 90 minutes in the dark at room temperature with continuous gentle agitation. Alternatively, incubate overnight in the dark at 4 °C with continuous gentle agitation. Exact conjugate efficiency depends on temperature, length of incubation, concentration of fullerene, and concentration of CFSE. Separate labeled fullerene from free fluorescein compounds by filter filtration (Centriprep® and/or Centricon® concentrators) versus PBS solution.



NH_2-Reactive Fluorescein

The fluorescence assay described here will be used to determine the apparent binding constants for selected fullerene aptamers. In the titration assay, a constant concentration (0.5 µM) of Fullerene-Fluorescein conjugate (Fluo-F) will be used. All measurements will be performed in buffer solution containing 10 mM Tris-HCl (pH 7.6) at room temperature. The titration of the Fluo-F with RNA aptamers resulted in an overall quenching in the Fluorescein fluorescence emission. The titration will be continued until the binding sites were fully saturated (no further changes in fluorescence were observed). After data points were collected, the fluorescence values were corrected for dilution by using the following equation:

$$f_{corrected} = \left[f_{measured} \times \left(\frac{V_{initial}}{V_{final}} \right) - f_{blank} \right]$$

in which $f_{corrected}$ is the corrected fluorescence, $f_{measured}$ is the observed fluorescence intensity at the point in the titration, f_{blank} is the fluorescence intensity of the solvent, $V_{initial}$ is the initial volume of the Fluo-F solution, and V_{final} is the volume of the solution at the point in titration. The fraction of RNA bound (F_r) at a given point will be determined from the following equation:

$$F_r = \frac{f_{initial} - f_{corrected}}{f_{initial} - f_{final}}$$

in which $f_{initial}$ is the fluorescence intensity of Fluo-F without RNA, and f_{final} is the fluorescence intensity at saturation. The following equation is used to represent binding:

$$[\text{Fluo-F}]_{total} = [\text{Fluo-F}]_{unbound} + [\text{Fluo-F}]_{bound}$$

in which $[RNA]_{total}$ is the total concentration of RNA added at the point in the titration, $[RNA]_{unbound}$ is the concentration of RNA not bound to fullerene, and $[RNA]_{bound}$ = concentration of RNA bound to fullerene ($F_r \bullet [RNA]_{total}$).

Plotting F_r versus the concentration of total added RNA gives a curve that is fit according to equations above. The bound RNA concentration will be determined by assuming that $[RNA]_{bound} = [\text{Fluo-F}]_{bound}$. Plotting either $[RNA]_{total}$ or $[RNA]_{unbound}$ produced similar curves, therefore $[RNA]_{total}$ will

be used. The binding constant, K_b, is then determined by fitting the data to equations for first-order or second-order RNA binding, as represented below:

$$F_r = \frac{K_b \ [RNA]_{total}}{1 + K_b \ [RNA]_{total}} \qquad \text{(1st order)}$$

$$F_r = \frac{K_b^2 \ [RNA]^2_{total}}{1 + K_b^2 \ [RNA]^2_{total}} \qquad \text{(2nd order)}$$

The first-order binding equation assumes a 1:1 stoichiometry and single binding site, whereas the second-order equation assumes a more complex binding event.

Structural Probing of Aptamers by Enzymatic and Pb(II)-induced Cleavage. RNA was labeled with ^{32}P-5'-pCp and T4 RNA ligase as described by England & Uhlenbeck (*29*). The 3'-end labeled fullerene-binding RNAs will be used for enzyme and Pb(II) digestion experiments. Alkaline digestion will be performed in 50 mM sodium carbonate (pH 9.0), 1 mM EDTA, and 5 ng of *E. coli* tRNAs for 10 min at 90 ºC. To produce a nucleotide-specific ladder, 3'-end labeled RNA (25,000 cpm) will be pre-incubated at 50 ºC for 5 min in 5 µl containing 1 µg total tRNA, 20 mM sodium citrate, pH 5.0, 7 M urea, 0.02% xylene cyanol and bromophenol blue. The reactions will be initiated with 0.5 unit of RNase T1 (G-specific) for 5 min at 55 ºC, and then kept on ice for later use on the sequencing gel. Prior to the structure-probing experiments, all RNA samples will be renatured in selection buffer as described above. RNase V1 (double-strand specific) probing (0.5 unit) will be performed in 10 mM Tris-HCl, pH 8.0, 100 mM KCl, and 5 ng of *E. coli* tRNAs for 5 min at 20 ºC. RNase I (single-strand specific) probing (0.05 unit) will be performed in 20 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 10 mM $MgCl_2$, and 5 ng of *E. coli* tRNAs for 3 min at 37 ºC. In the Pb(II)-probing reactions, RNA aptamers are mixed with cleavage buffer (20 mM HEPES-KOH, pH 7.5, 50 mM KOAc) and incubated at 25 °C for 5 min, then hydrolysis reactions are initiated with 2 mM Pb^{2+} for 5 min. The RNA sample in each tube will be resuspended in 5 µl of loading buffer (95% (v/v) deionized formamide, 10 mM EDTA, 0.1% (w/v) bromophenol blue and xylene cyanol FF), denatured at 90 °C for 3 min, and then chilled on ice for 2 min prior to loading onto a 0.3-mm thick, 12% denaturing sequencing gel containing 7 M urea and 0.5x TBE buffer.

Development of Fullerene Detection Biosensor by Using of C_{60}/Aptamer Sandwich ELISA Format. Before the assay, both aptamer preparations should be purified (capture aptamer) and one must be labeled (detector aptamer). For most applications, a polyvinylchloride (PVC) microtiter plate is the most appropriate type of plate for binding. Bind the unlabeled fullerene to the bottom of each well by adding approximately 50 µL of antibody solution to each well (20 µg/mL in PBS). PVC will bind approximately 100 ng/well (300 ng/cm2). The amount of fullerene used will depend on the individual assay, but if maximal binding is required, use at least 1 µg/well, this is above the capacity of the well, but the binding will occur more rapidly, and the binding solution can be saved and used again. Incubate the plate overnight at 4 °C to allow complete binding. Wash the wells twice with PBS. The remaining sites for fullerene binding on the microtiter plate must be saturated by incubating with blocking buffer. Fill the wells to the top with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hrs to overnight at room temperature. (Note: Sodium azide is an inhibitor or horseradish peroxidase. Do not include sodium azide in buffers or wash solutions, if an HRP-labeled antibody will be used for detection.) Wash wells twice with PBS. Add 50 µL of the aptamer solution to the wells (the aptamer solution should be titrated). All dilutions should be done in the blocking buffer (3% BSA/PBS with 0.02% sodium azide). Incubate for at

least 2 hrs at room temperature. Wash the plate four times with PBS. Add the labeled second aptamer as detector. The amount to be added can be determined in preliminary experiments. For accurate quantitation, the second aptamer should be used in excess. All dilutions should be done in the blocking buffer. Incubate for 2 hrs or more at room temperature. Wash with several changes of PBS. Add substrate as indicated by manufacturer's manual. After suggested incubation time has elapsed, optical densities at target wavelengths can be measured on an ELISA reader.

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計畫成果自評

Having the proposed research outlined clearly as above, we expect to accomplish its objectives as follows:

(i) The goal of this research project are to conduct series of structure-to-function experiments in the area of DNA/RNA-ligand interaction to illustrate the relationship between buckminsterfullerene C_{60} and specific DNA/RNA molecules (aptamers) by using *in vitro* selection method.

(ii) Determine detailed structures of C_{60}-binding DNA/RNA aptamers

(iii) Develop and provide a C_{60} aptamer-based trace system for fast detection of C_{60} existence or its derivatives *in vivo* and in our living environment.

(iv) More sophisticated analyses can be done to understand the binding mechanism of the fullerene to DNA/RNA, or sequence-mutated DNA/RNAs in binding assays, it will help to understand the exact contributions of the hydrophobicity or π stacking in fullerene-RNA binding.

(v) Based on this high-throughput screening platform, we are able to generate novel aptamers for virtually all kind of nanoparticles which allow us quickly develop screening assay.