Aptamer Directly Evolved from Live Cells Recognizes Membrane Bound Immunoglobulin Heavy Mu Chain in Burkitt’s Lymphoma Cells*

Prabodhika Mallikaratchy, Zhiwen Tang, Sefah Kwame, Ling Meng, Dihua Shangguan, and Weihong Tan‡

The identification of tumor related cell membrane protein targets is important in understanding tumor progression, the development of new diagnostic tools, and potentially for identifying new therapeutic targets. Here we present a novel strategy for identifying proteins that are altered in their expression levels in a diseased cell using cell specific aptamers. Using an intact viable B-cell Burkitt’s lymphoma cell line (Ramos cells) as the target, we have selected aptamers that recognize cell membrane proteins with high affinity. Among the selected aptamers that showed different recognition patterns with different cell lines of leukemia, the aptamer TD05 showed binding with Ramos cells. By chemically modifying TD05 to covalently cross-link with its target on Ramos cells to capture and to enrich the target receptors using streptavidin coated magnetic beads followed by mass spectrometry, we were able to identify membrane bound immunoglobulin heavy mu chain as the target for TD05 aptamer. Immunoglobulin heavy mu chain is a major component of the B-cell antigen receptor, which is expressed in Burkitt’s lymphoma cells. This study demonstrates that this two step strategy, the development of high quality aptamer probes and then the identification of their target proteins, can be used to discover new disease related potential markers and thus enhance tumor diagnosis and therapy. The aptamer based strategy will enable effective molecular elucidation of disease related biomarkers and other interesting molecules. Molecular & Cellular Proteomics 6:2230–2238, 2007.

Membrane proteins play crucial roles in all living organisms. These include cell signaling, cell-cell interactions, ion/solute transport that facilitates the exchange of membrane impermeable molecules between the outer environment to the cells, and communication between cells through signal transduction. Recently, much interest has been focused on identifica-

From the Center for Research at Bio/nano Interface, Department of Chemistry, Shands Cancer Center, University of Florida Genetics Institute and McKnight Brain Institute, University of Florida, Gainesville, Florida 32611.

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1 The abbreviations used are: SELEX, Systematic Evolution of Ligands by EXponential Enrichment (SELEX), we have generated DNA
Aptamers that can identify the differences in relative levels of membrane protein expression patterns of a given set of cells in a complex biological mixture (14). These probes can then be used to identify the up-regulated membrane proteins in a given set of cells. We think that these profiling studies (13, 14) would possibly assist in finding disease markers specific for one group of cell type. For example, in our previous studies, we have demonstrated that aptamer probes selected against a T-cell leukemia line, CCRF-CEM, can be used to identify T-cell leukemia in patient samples as well as CEM cells spiked into human bone marrow aspirates, suggesting the applicability of the cell specific aptamers in real samples. In addition, we have demonstrated that each aptamer has its own binding patterns when recognizing molecular signatures on cells, further suggesting that there is a correlation between aptamer mediated cell recognition and different protein expression patterns in diseased cells.

In our recent studies with B-cell leukemia, we have identified an aptamer TD05 that recognizes Ramos cells, a Burkitt’s lymphoma cell line. We reason that observed binding ability of this aptamer with Ramos cells can be used to identify a membrane protein related to Ramos cells. Through the work in this paper, we have found that TD05 binds selectively to membrane bound immunoglobin heavy mu chain (IGHM), which is the heavy chain portion of the IgM protein. IgM is a major component of B-cell receptor complex expressed in Burkitt’s lymphoma that has been widely studied as a potential marker for Burkitt’s lymphomas (15, 16). Also, some studies emphasize its active role in Burkitt’s lymphoma cell proliferation and survival (17–20). Following the initial binding studies with different cell lines, the identification of IGHM using the aptamer TD05 was done in a four step process. First, the aptamer probe was chemically modified with a photoactive uracil derivative for covalent binding of the aptamer with cells. Second, the target protein was enriched by magnetic extraction with a biotin-streptavidin interaction. Third, the collected protein was identified by MS followed by a database search. Last, the identity of the target protein was confirmed using an existing antibody and the selected aptamer. Scheme 1 exemplifies the protein-aptamer separation procedure used in this work. To our knowledge, this is the first report on the use of aptamers in identifying Burkitt’s lymphoma membrane proteins by the covalently cross-linking of photochemically modified aptamer to its target entity using a whole cell.

### EXPERIMENTAL PROCEDURES

A panel of aptamers targeting Burkitt’s lymphoma cells has been selected by using cell-based SELEX, among which the aptamer TD05 showed significant and specific binding with Ramos cells. The aptamer sequence TD05 was modified with photo-active 5-iododeoxyuridine (5-dUI) nucleotides.

**TD05:** 5’-FITC-5’-ACCCGAGGAATGGCTCGTGGCCGGTGTTCA-GTCCGCTCCGGGTG-3’

**TD05-SSBiotin:** 5’-ACCCGAGGAATGUCGGTGGCGTTCA-GGQUCTCUCGCCGGTG-S-S-T-PEG (3’)-Biotin

**TD02-SSBiotin:** 5’-fluorescence-ATCUAAGCGCCGGCG- GAAATACGTACGGTTAGAGS-S-PEG (3’)-biotin

All DNA synthesis reagents were from Glen Research. Biotin controlled pure glass was used for all the biotin-labeled aptamer synthesis. All oligonucleotide sequences were synthesized using standard phosphoamidite chemistry using an ABI3400 DNA synthesizer. Three PEG units were introduced to avoid the interference of biotin with the spatial formation of the aptamer. Mild deprotection conditions were used to avoid reduction of disulfide bond. Following the ethanol precipitation, precipitated DNA was purified using HPLC. The HPLC purification was performed on a ProStar HPLC station (Varian, CA) equipped with a photodiode array detector. A C18 reverse phase column (Alltech, C18, 5 μm, 250 × 4.6 mm) was used.

The conditions for aptamer probes containing disulfide bond were further optimized by streptavidin-biotin interaction followed by gel electrophoresis. Briefly, a 500-nm aptamer probe was passed through a mini column packed with streptavidin-Sepharose (Amersham Biosciences) three times. After washing with 1x phosphate-buffered saline (PBS), the beads were placed into a microcentrifuge tube. The probe’s attached beads were treated with 50-100 mM Tris(2-carboxyethyl)phosphoamidite chemistry using an ABI3400 DNA synthesizer.

### Competition Assays with the Modified Aptamer Probes—CCRF-CEM (CCL-119, T-cell line, human acute lymphoblastic leukemia), Ramos (CRL-1596, B-cell line, human Burkitt’s lymphoma), CA46 (CRL 1648, B-cell line, human Burkitt’s lymphoma), Jurkat (TIB-152, human acute T-cell leukemia), Toledo (CRL-2631, B-cell line, human diffuse large cell lymphoma), K562 (CCL-243, chronic myelogenous leukemia, and NB-4), and HL-60 (CCL-240, acute promyelocytic leukemia) were obtained from the American Type Culture Collection.

All of the cells were cultured in RPMI 1640 medium (American Type Culture Collection) supplemented with 10% fetal bovine serum (heat-inactivated; Invitrogen) and 100 units/ml penicillin–streptomycin (Cellgro). Cells were washed before and after incubation with wash buffer (4.5 g/liter glucose and 5 mM MgCl2 in Dulbecco’s PBS with calcium chloride and magnesium chloride; Sigma). Binding buffer used for selection was prepared by adding yeast RNA (0.1 mg/ml; Sigma) and 1 mg/ml sheared salmon sperm DNA into wash buffer to reduce nonspecific binding.

The effect of replacement of thymine base with 5-dUI was investigated by conducting competition assays. Briefly, 1 μM TD05 mixed with 10 μM modified aptamer probe were incubated with 1 × 10⁵ cells for 30 min at 4 °C in the binding buffer. After washing with wash buffer, the cells were then washed and analyzed by determining the decrease in the fluorescence of TD05 using a FACScan cytometer (BD Immunocytometry Systems) by counting 30,000 events. A FITC-labeled unselected single-stranded DNA library was used as a negative control.

### Aaptamer Labeling with ATP²³P—Samples containing oligonucleotide (3.74 nmol) and [γ-³²P]ATP (8.3 pmol) were incubated overnight at room temperature with 50 units of polynucleotide kinase (Promega). Unincorporated ATP was removed using a G-25 column (Amersham Biosciences).

### Protein-Nucleic Acid Photocross-linking—Counts per minute of 5’ × 10⁴ of 5’-²³P-labeled aptamers was added to 400 × 10⁶ cells in binding buffer. The cells and the aptamers were incubated at 4 °C for 30 min to allow the complete binding. A control with the TE02 aptamer sequence was performed in parallel. The unbound aptamers were washed with wash buffer until no radioactivity was detected in the washings. Cells along with bound aptamer were resuspended in the wash buffer in a cuvette possessing a 10-cm path length. During
the irradiation, the cell suspension was constantly stirred to allow the maximum cross-linking. The sample was irradiated with 55 pulses of 308-nm light from a Lambda Physik model LPX240 XeCl excimer laser operating at 16.065 mJ per pulse for 20 s.

**Cell Lysis and Protein-Aptamer Extraction.**—The cells were suspended in lysis buffer containing a protease inhibitor mixture (Sigma), 1 mg/ml salmon sperm DNA (Eppendorf), 1 mg/ml tRNA (Fisher), 500 mM HEPES, 1 mM EGTA, and homogenized using a Dounce homogenizer with 75 strokes per min. Water soluble protein extracts were separated from the crude membrane by centrifugation at 4000 rpm for 30 min at 4 °C. Pelleted crude membrane was solubilized in solubilizing buffer containing 1× PBS (Fisher), 0.5% Nonidet P-40 (Sigma), 1.25% cholesteryl-hemisuccinate (Tris-HCl salt, Sigma), and 2.5% n-dodecyl-β-D-maltoside (Sigma) with gentle agitation at 4 °C.
The solubilized membrane proteins were separated from cell debris by centrifuging at 3000 rpm for 30 min at 4 °C. Crude cell lysate was stirred with 2 mg/ml of magnetic beads (Invitrogen) at 4 °C for 2 h 30 min. Captured probes were magnetically separated and washed with washing solutions by stirring for 15 min at 4 °C. Harsh washing steps were used to ensure cross-linked proteins remained and to minimize the nonspecific binding. Order of the washing was as follows: 1 × PBS containing 1% Nonidet P-40, 10 mM EDTA containing 1% Nonidet P-40 and 0.05% SDS.

**Release of Captured Complex and Protein Gel Electrophoresis**—Captured complexes along with magnetic beads were heated at 75 °C for 30 min with 10 mM Tris(2-carboxyethyl)phosphine and 2× sample loading buffer. The solution was loaded on to 10%-bis-Tris PAGE and a voltage of 200 V was applied and run for 55 min. Protein bands were visualized using Bio-Rad gel-code blue staining reagents according to the manufacturer’s instructions. Then the gel was exposed to Bio-Rad personal phosphorimager screens overnight and visualized by Bio-Rad personal phosphorimager. A single band appeared in the gel. It was excised, digested in situ, and analyzed by QSTAR LC-MS/MS and a Mascot database search at the Protein Chemistry Core Facility, University of Florida.

**Characterization of Aptamer Binding Protein on Ramos Cells**—Alexa Fluor 488-labeled anti-IgM heavy chain (Molecular Probes, Invitrogen) was used to further confirm the protein target. Competition experiments were carried out using a previously described procedure. Briefly, 0.5 μM of FITC-TD05 incubated with Ramos, Toledo, CEM cells at 4 °C for 15 min and washed off the unbound probe. Cells were then incubated with 2 μg/μl of Alexa Fluor 647 labeled anti-IgM heavy mu chain for 15 min at 4 °C, washed of the excess antibody and analyzed by Fluorescence Activated Cell Sorting (FACScan, Becton Dickinson).

**RESULTS**

**Cell Specific Aptamer Probes for Protein Identification**—The cell-SELEX process was used to select aptamers, which targeted Ramos cells using a procedure similar to one described previously (14). One difference in this selection procedure, however, was the elimination of the counter selection step. Interestingly, among a significant number of selected aptamers, TD05 recognizes target Ramos cells (Fig. 1A and supplemental Table I) (14). We later carried out experiments by partially digesting cell surface proteins with proteinase K to confirm that TD05 binds with a cell surface protein. After the treatment with proteinase K, the TD05 did not recognize Ramos cells anymore, suggesting that the target was a transmembrane or extracellular membrane protein (14).

**Probe Modification and the Effect of Modification on Aptamer Affinity and Specificity**—We have designed a simple and sensitive method for the identification of a target protein by a four step process: covalent binding of the aptamer with the target via photocross-linking, magnetic bead conjugation and magnetic extraction and enrichment, MS analysis to identify target proteins, and confirmation analysis of the target protein.

Modification of TD05 with photoactive 5-dUI facilitates its covalent cross-linking with the target protein on the cell membrane. Therefore, only the cross-linked target protein with the aptamer would be extracted from the cell lysate. First, to achieve high cross-linking efficiency, the position of the photoactive nucleotide 5-dUI in the TD05 was optimized. Since 5-dUI has a similar covalent radii compared with deoxycytidine, we initially hypothesized that the modification would not affect the aptamer binding with Ramos cells. However, when the TD05 aptamer was fully modified with 5-dUI, it no longer recognized Ramos cells (data not shown). It is common that aptamer-protein interactions can be affected by modifications of the probe (21, 22) because the aptamer-protein interaction mainly depends on unique spatial folding of the aptamer that allows a specific binding in protein binding pockets. Therefore, as shown in Fig. 2, the aptamer was modified with 5-dUI in alternating positions of the first four and final four deoxycytidine bases. This modified aptamer did show binding, suggesting the modification did not affect its affinity (Fig. 3).

Next, biotin was linked through a disulfide bridge at the 3’ end of the aptamer. Introduction of the disulfide bridge assisted the efficient cleavage of extracted complex from the streptavidin coated magnetic beads. After this, the modified TD05 was analyzed for binding with target cells through a

**Fig. 1.** A, TD05 recognizes Ramos cells. Flow cytometry analysis of binding of 0.5 μM FITC-labeled TD05 binding with Toledo, CEM, and Ramos cells. B, FITC-TD05 and Alexa Fluor 488 anti-IGHM antibody show similar binding patterns with control cells. Alexa Fluor 488-labeled anti-IGHM antibody with Ramos cells, CEM, and Toledo cells. Cells were incubated with 2 μg/μl anti-IGHM antibody at 4 °C and washed three times prior to flow cytometer analysis. FL-1, fluorescence channel 1.
competition assay with unmodified FITC-labeled aptamer. The results indicate that the aptamers compete with each other because replacing the unmodified FITC-TD05 results in a decrease in fluorescence intensity. This suggests that the binding ability of the modified TD05 was retained (Fig. 3). We have also conducted studies to confirm the effect of 5-dUI on TD05 specificity using a control cell line: T-cell CCRF-CEM cells (data not shown). Finally, the conditions pertaining to concentration, temperature, and time were optimized to achieve effective release of captured aptamer probe from the magnetic beads using Tris(2-carboxyethyl)phosphine.

Separation of Captured Complex from the Crude Cell Lysate and MS Analysis of Captured Protein—To trace the aptamer-protein complex during its isolation, we have labeled the TD05 using $^{32}$P at the 5’ end. The resulting $^{32}$P-labeled photoactive TD05 bound to Ramos cells were irradiated with nanosecond pulses of a XeCl excimer laser to initiate cross-linking of the aptamer with the cell surface protein. Chemical cross-linking of TD05 with its target protein was introduced to maximize the specificity of the extraction and to minimize contamination caused by nuclear proteins. In addition to covalent cross-linking, the nonspecific interactions were further minimized by using a mixture of sheared salmon sperm DNA and RNA in the cell lysis buffer. To avoid contamination caused by plasma proteins, water soluble proteins were removed prior to magnetic extraction using an aqueous lysis buffer. The resulting crude membrane was dissolved in membrane solubilization buffer containing a mixture of cationic and anionic detergents. To separate the complex, which was rich in hydrophobic proteins along with the TD05-protein complex, the mixture was incubated with streptavidin coated magnetic beads to extract aptamer sequences that bear biotin at the 3’ end. Use of streptavidin coated magnetic beads allowed for the specific extraction of the biotin bearing TD05 along with the covalently bound target protein. The extraction was repeated 3 times until ~90% of the complex was efficiently extracted onto the beads. Since the TD05 was labeled with $^{32}$P, we detected the efficiency of the extraction by measuring the radioactivity on the beads compared with cell extract using a Geiger counter.

One of the challenges in isolating protein from cell lysates is the interference of nuclear and other nonspecific proteins with the aptamer. Therefore, the insufficient washings of the complex had led to contamination with nuclear proteins, which resulted in a smeared band after gel electrophoresis. Therefore, harsh washing conditions ranging from PBS to 0.05% SDS were introduced to ensure the removal of nonspecific nuclear proteins absorbed onto the magnetic beads. Due to the 5-dUI covalent cross-linking of the protein target and the aptamer, such harsh washing conditions did not allow the
Confirmation of the Protein IGHM as the Binding Target for TD05—With the MS results to suggest that IGHM is the target for TD05, we next investigated the binding of Alexa Fluor 488-labeled anti-IGHM antibody with Ramos and the number of T-cell acute lymphoblastic leukemia cell lines, myeloid leukemia cells, and a Burkitt’s lymphoma cell line that express surface IgM (CA-46 cell line) and a cell line that does not express surface IgM (Toledo cells) (Fig. 1B, Supplemental Table I and Supplemental Fig. 1). Even though Toledo cells closely resemble Burkitt’s lymphoma cells, these cells do not contain the typical chromosomal translocations of Burkitt’s lymphoma. For this reason, these cells do not express cytoplasmic or membrane immunoglobulins (23). Next, we investigated the interaction of FITC-labeled TD05 aptamer and Alexa Fluor 488-labeled anti-IGHM antibody with surface IgM positive CA-46 (Supplemental Fig. 1). As expected, TD05 and anti-IGHM antibody showed binding with the CA-46 cell line, suggesting that IGHM could be the target protein. After this, TD05 aptamer and anti-IGHM antibody interaction was investigated with Toledo cells, and TD05 aptamer and anti-IGHM antibody did not bind to Toledo cells. Taken together, these results suggest that the IGHM is the target for TD05 aptamer. Next, we hypothesized that, if both TD05 and anti-IGHM bind to IGHM, we should observe a competition between the two in binding. We investigated the effect of TD05 binding with Ramos cells upon the addition of anti-IGHM antibody. Interestingly, TD05 did not affect anti-IGHM binding. However, anti-IGHM antibody reduced the interaction between TD05 with Ramos cells as shown in Fig. 5, suggesting both probes bind to same target entity with different affinities. In addition, we found that TD05 most likely binds to a membrane protein based on our study (14) of the aptamer binding upon treatment of trypsin and proteinase K. Interestingly, during this study, we observed that TD05 binding with Ramos cells was not lost by partial digestion of trypsin. It has been reported that the mild conditions employed in partial digestion of membrane proteins using trypsin can be interfered with the neighboring masking proteins. Thus, a limited cleavage was observed (5). We next investigated whether similar patterns could also be observed with the anti-IGHM antibody interaction with Ramos cells upon partial digestion with trypsin. The anti-IGHM antibody did not lose its ability to recognize Ramos cells with trypsin digestion (Fig. 6), further confirming that both TD05 and anti-IGHM bind to the same protein.

**DISCUSSION**

We have presented a simple strategy for identifying differentially expressed proteins by employing aptamer probes selected against target tumor cells. This method integrates the selection of molecular probes targeting specific cells and

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**Fig. 4. Phospho images for 10% Tris-bis PAGE analysis of the captured complex.** A, Lane 1, captured aptamer-protein target shifted band corresponds to cross-linked AP-TD05-protein complex; Lane 2, control: aqueous cell lysate lane. No DNA is seen because none of the aptamer is extracted. B, gel analysis of control TE02 sequence: Lane 1, cell lysate without any extraction; Lane 2, with modified TE02 extraction. TE02 is a control sequence. No shifted band is seen in either lane.
uses the cell specific aptamers for effective identification of target proteins. Our previous work established that aptamer binding signatures pertaining to each cell type could be logically interpreted as alerted expression patterns of protein candidates. Also, it has been demonstrated that each aptamer candidate has its own identity when recognizing target proteins in complex biological specimens (13, 14, 24). Furthermore, we have shown that it is feasible to recognize up-regulated proteins that may have a role in transforming unhealthy cells into diseased cells (14). Notably, the elimination of the counter-selection in this study’s modified selection protocol shows that, even with no negative selection, it is feasible to identify up-regulated protein candidates in response to immunological, chemical, or genetic mutations in/or around the cells that may lead to cancer.

We also have exploited the remarkable versatility for chemical modification of DNA aptamers by incorporating different functionalities to improve their performance as molecular probes. These modifications improved two important features: probe stability and collection efficiency. This is one of the advantages for DNA aptamers where easy modification can be done to increase desired features without altering probe binding ability. Enhanced stability of the aptamer-protein complex through incorporation of 5-dUI allowed the complex to sustain harsh washing conditions in extraction, which is important in purification and enrichment of targeted proteins from a cell lysate sample. Enhanced efficiency of the biotin aptamer removal from the streptavidin support occurred through introduction of a readily cleavable disulfide bond.

The typical approach for solubilizing membrane proteins from the cell membrane is to use carefully optimized ratios of detergents that mimic the membrane in an artificial environment. However, poor optimization of detergent compositions could result in misfolding of the receptor and/or irreversible denaturation of the receptor molecule because the native conformation of these receptor molecules depends on the hydrophobic lipid bilayer of the cell membrane. This may lead to possible disruption of the aptamer-protein complex when the target protein misfolds or denatures. These protein alterations caused by changes in the lipid composition of buffers is one of the major concerns in identification of membrane proteins using protein-specific aptamers and can possibly be one of the limitations when using these probes in identifying molecular markers. We have addressed this issue by modifying the aptamer with photoactive 5-dUI to facilitate covalent cross-linking of the probe with the target protein, which increases the stability of the complex.

Conjugations using biotin-streptavidin have been exploited for many applications in immunology, affinity chromatography, and other separation applications (25, 26). However, one of the major disadvantages of such interactions is that such affinity requires harsh conditions to elute the biotin bearing ligand off of the streptavidin coated solid support. Also, these harsh conditions result in either damaging the protein-ligand complex or in requiring larger sample volumes for additional steps in sample pre-concentration prior to SDS-PAGE analysis (7). The aptamer was modified with the disulfide functional group prior to the biotin bearing ligand off of the streptavidin coated solid support. Also, these harsh conditions result in either damaging the protein-ligand complex or in requiring larger sample volumes for additional steps in sample pre-concentration prior to SDS-PAGE analysis (7). The aptamer was modified with the disulfide functional group prior to the biotin bearing ligand off of the streptavidin coated solid support. Also, these harsh conditions result in either damaging the protein-ligand complex or in requiring larger sample volumes for additional steps in sample pre-concentration prior to SDS-PAGE analysis (7).
IGHM is one of the major components of B-cell receptor complex expressed in mature Burkitt’s lymphoma cells. It has been known that IGHM expression on premature B-lymphocytes is closely related to Burkitt’s lymphoma development (20). The discovery of IGHM on the Ramos cells is correlated with our initial expectations of this study for the following reason: binding of TD05 with Ramos cells correlated with reported IGHM expression patterns of these cells compared with other cell lines and in real bone marrow samples. Furthermore, IGHM is a receptor that has a role in development of Burkitt’s lymphoma, and this protein is a marker for Burkitt’s lymphomas. Accordingly, these findings demonstrate the adaptability of this approach in identifying cell membrane receptors that have altered expression levels in tumor cells.

In conclusion, we have shown that cancer cell-specific aptamers provide an effective tool in identifying target proteins that show increased expression levels on a chosen pool of diseased cells. The ease in chemical modification of the DNA aptamer probe has lent needed binding stability and strength for the effective capture, enrichment, and identification of corresponding target receptors on cell membrane surface. In addition, findings of the approach show that the generation of aptamers using Cell-SELEX followed by identification of binding entity of each aptamer can be useful in discovering disease-specific marker proteins in a given cell type. In contrast to conventional methods, such as phage display antibody production targeting a previously known specific protein, the novelty of cell-SELEX based protein discovery is rooted in its focus on finding cell surface membrane markers with no prior knowledge of the molecular contents of the cell surface. Also, owing to their easy chemical manipulation and reproducible generation of DNA aptamers by automated synthesis, this method is more universal and technically feasible. Finally, apart from the ability of identification of disease markers that may play key roles in cancer progression, this method can also be useful in early diagnosis, targeted therapy, and as molecular tools in recognition as well as mechanistic studies of diseased cells.

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†To whom correspondence should be addressed: Center for Research at Bio/nano Interface, Dept. of Chemistry, Shands Cancer Center, University of Florida Genetics Institute and McKnight Brain Institute, University of Florida, Gainesville, FL 32611. Tel.: 352-846-2410; Fax: 352-846-2410; E-mail: tan@chem.ufl.edu.

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