Molecular Aptamer Beacons for Real-Time Protein Recognition

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One of the most pressing problems facing those attempting to understand the regulation of gene expression and translation is the necessity to monitor protein production in a variety of metabolic states. Thus far, there is no easy solution that will either identify or quantitate proteins in real time. Here we introduce a novel protein probe, molecular aptamer beacon (MAB), for real time protein recognition and quantitative analysis. The MAB combines the signal transduction mechanism of molecular beacons and the molecular recognition specificity of aptamers. An MAB based on a thrombin-binding aptamer was prepared as a model to demonstrate the feasibility. Significant fluorescent signal change was observed when MAB was bound to thrombin, which is attributed to a significant conformational change in MAB from a loose random coil to a compact unimolecular quadruplex. The MAB recognizes its target protein with high specificity and high sensitivity (112 picomolar thrombin concentration) in homogeneous solutions. Ratiometric imaging has been conducted with MAB labeled with two fluorophores, which makes it feasible for protein quantitation in living specimen. The unique properties of the MAB will enable the development of a class of protein probes for real time protein tracing in living specimen and for efficient biomedical diagnosis in homogeneous solutions.

In the postgenomic era, scientists are increasingly turning to the task of converting DNA sequences into information that will potentially revolutionize medicine and molecular biology. One of the major challenges ahead is to understand protein functions. Effective protein recognition mechanisms are thus necessary, especially those to monitor proteins in real time and in homogeneous solutions. Real time protein detection is important for two major reasons: clinical diagnosis in homogeneous solution and protein studies in living specimen like living cells. For clinical diagnosis, most of the current standard methods such as protein blotting and affinity chromatography require electrophoresis separation, solid-substrate capture or immobilization, multiple washing, blocking and usually use radiolabeling [1]. These methods are discontinuous, time-consuming and laborious. Moreover, sensitivity is limited by nonspecific absorption of target proteins onto the solid substrates. These problems would be circumvented by using methods for protein detection in real time and in homogeneous solution (detection without separation). Aside from clinical diagnosis, monitoring proteins inside living cells is critically important for a better understanding of cellular behavior. Tracing proteins inside living cells would provide revolutionary means for studying many biological pathway studies [2]. Although fluorophore-labeled antibodies have been used for living cell protein detection, usually only large aggregation of proteins can provide enough signal contrast, as the unbound antibodies are also fluorescent [3]. Tracking target proteins using fluorophore-labeled antibodies is difficult, because the fluorescence signal of the antibody remains the same irrespective of whether the antibody is bound with the target protein or not. The green fluorescence protein (GFP) family can help to solve this problem by fusing GFP with host proteins [4]. However, this requires using gene clone techniques and not all fusions are successful. It is thus clear that there is an increasing demand for the development of new molecular probes for real-time protein recognition in solution and in living specimen.

Recent effort has seen molecular beacon (MB), a hairpin-shaped oligonucleotide with a fluorophore and a quencher linked to each end of its stem, emerging as a novel probe for real time detection of nucleic acids and proteins [5–9]. The signal transduction mechanism for molecular recognition is based on resonance fluorescence energy transfer (FRET) and the conformational change of a MB. The MB acts like a switch that is normally closed to bring the fluorophore/quencher pair together to turn fluorescence “off” [10]. When binding
to a target biomolecule, it undergoes a conformational change that opens the hairpin structure and separates the fluorophore and the quencher, thus turning "off" the fluorescence. This feature makes MB an extremely useful probe in a variety of applications such as the real time monitoring of PCR processes and the detection of mRNA in living cells [7]. Although the original goal for developing the MB is for nucleic acids detection, recently MB has found promising applications in the homogeneous detection of DNA-binding protein [8]. The MBs can be used for non-specific protein-DNA binding studies, which opens a new approach to detect protein in homogeneous solution with high sensitivity, excellent reproducibility, fast speed and convenience. However, there is one apparent and critical disadvantage of MBs in protein recognition: the lack of sequence specificity in protein binding.

In another front, aptamers have shown to be an effective approach for protein recognition. Aptamers are DNA or RNA oligonucleotides isolated to bind to various biomolecules with high specificity [10]. Aptamers are screened from a randomly generated population of DNA/RNA sequences for their ability to bind with desired molecular targets. The screening process mimics natural selection and therefore, theoretically, it is possible to obtain aptamers that can recognize virtually any target molecules with high affinity and specificity. Using aptamers for specific protein binding studies has drawn much interest recently [10, 11]. Unlike MB, aptamers have the advantage in binding specificity and also generality, but lack the built-in mechanism for signal transduction, especially the capability of "detection without separation" [6]. It is thus desirable to combine the binding specificity and generality of aptamers with the excellent signal transduction capability of MB to develop novel protein-reporting probes for quantitation in homogeneous solutions and for real time protein monitoring. Such a probe is called molecular aptamer beacon (MAB).

There have been three reports on the development of using aptamers for protein recognition with a similar signal transduction as that used in MBs [12-14]. In the first report, we briefly presented the preliminary result on the construction of a DNA MAB for protein thrombin detection [12]. In the second report, Yamamoto et al. developed an aptamer to detect Tat protein of HIV-1 [13]. A duplex RNA aptamer was split into two portions, with one portion transformed into a DNA probe, and with the other portion partly complementary to the DNA probe. The two portions could not hybridize until Tat protein was present, and the hybridization caused restoration in the otherwise quenched fluorescence. In the most recent report, a thrombin-binding aptamer was constructed by Hamaguchi et al. [14]. A half stem sequence was added to the thrombin aptamer so as to change the aptamer into a MB form. Protein binding changes the MB form back to an aptamer form, restoring the quenched fluorescence.

In this report, we will detail our study on thrombin-binding MAB. Two types of MABs have been constructed for thrombin binding studies. One is a quenching type MAB, which is labeled with a fluorophore-quencher pair. The other one is a FRET-type MAB, which is labeled with two fluorophores (a donor-acceptor pair), showing an increased fluorescent signal in the presence of target protein. Real-time observations of protein-aptamer recognition, protein quantitation in homogeneous solution, and ratiometric imaging of proteins are tested using these MABs.

MATERIALS AND METHODS

Synthesis of dual labeled and single labeled DNA oligonucleotides. Four labeled DNA oligonucleotides were synthesized: fluorophore-quencher labeled MAB (quenching type MAB), fluorophore-quencher labeled scrambled sequence, donor-acceptor labeled MAB (FRET type MAB), and single labeled aptamer. All DNAs were synthesized on ABI 391 Model DNA synthesizers. For the fluorophore-quencher labeled MAB, 6-carboxyfluorescein (6-FAM: Molecular Probes, Eugene, OR) and 4-(4'-dimethylaminophenylazo) benzonic acid (DABCYL: Molecular Probes) are conjugated to the ends of the thrombin-binding aptamer as fluorophore and quencher, respectively. The synthesis is similar to published reports [6, 8, 9]. The aptamer sequence is 5'TGGTGTGGTTGTTGTTGT3'. For the donor-acceptor labeled MAB, the synthesis is the same as that of fluorophore-quencher labeled MAB, except that DABCYL is replaced by coumarin (Molecular Probes). We also synthesized a fluorophore-quencher labeled scrambled sequence, GTGTTGTGTTGTTGG, which was used to replace the DNA sequence in the MABs. For the single labeled aptamer, 3' end labeling was omitted, and 5' end was labeled with 6-FAM through the same spacer.

Time course measurements of aptamer beacon-thrombin binding. Binding between MABs and thrombin was monitored by measuring the changes in fluorescence intensity of the MAB solution. The fluorescence intensity of the MAB-thrombin mixture solution was recorded over time. The equipment used was a LS-50B Luminescence Spectrophotometer (Perkin-Elmer Instruments, Norwalk, CT) at 25°C. Typically, 100 μM of 1 μM MAB in physiological buffer (20 mM Tris–HCl (pH 7.4), 140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5% (v/v) glycerol) was put into a 100 μl cuvette. The binding reaction was started by adding 1 μl of 300 nM human alpha-thrombin (Haematologic Technologies, Essex J. CT., VT; without specification, all thrombin used in this paper refer to human alpha-thrombin) solution with a quick manual mixing for about 5 seconds. Before use, human α-thrombin (provided with 50% (v/v) glycerol) was dialyzed against physiological buffer for 2 h at 4°C. The fluorescence intensity of the MAB solution was recorded before and after the addition of thrombin. For the quenching-type MAB, the wavelengths for excitation and emission were 490 and 520 nm, respectively. For the FRET-type MAB, the wavelengths for excitation and emission were 355 and 520 nm, respectively. Two control experiments for the quenching type MAB were done. In the first control experiment, the single labeled aptamer was used to replace the MABs when all other experimental conditions were the same as those for the quenching type MAB. In the second control experiment, the reaction buffer contained 0.1% of a protein denaturant, sodium dodecyl sulfate (SDS), and all other experimental conditions were the same. To examine the specificity of the MABs, several other proteins were tested for their binding to the MAB, including human γ-thrombin (Haematologic Technologies, Essex J. CT., VT), bovine serum albumin (BSA, Promega, Madison, WI),...
porcine lactate dehydrogenase V (LDH-V, Sigma, St. Louis, MO), E. coli RecA (New England Biolabs, MA) and E. coli single-stranded DNA binding protein (SSB, Promega). Before use, all proteins were dialyzed against physiological buffer for 12 h at 4°C. From the time course curves, quenching percentage was calculated for each protein. To examine buffer effect on thrombin binding, time course measurements were conducted in three buffers: 20 mM Tris-HCl buffer (pH 7.4); 20 mM Tris-HCl buffer (pH 7.4), 200 mM NaCl; physiological buffer. The measurement procedures were the same as above. Only quenching-type MAB was tested.

Gel electrophoresis. Gel electrophoresis was used to confirm the binding between thrombin and the quenching type MAB. Seven samples (Nos. 1 to 7) were prepared and analyzed. The volume for each sample was 20 μl. Physiological buffer (as defined above) was used for all samples. Samples 1 to 6 contained 5 μM MAB, while No. 7 had no MAB. From samples 1 to 7, the thrombin concentration was 0, 1, 2, 5, 10, 25 and 5 in μM, respectively. From samples 1 to 7, the molar ratio of MAB to thrombin was thus 5/0, 5/1, 5/2, 5/5, 5/10, 5/25 and 0/5, respectively. All seven samples were left at room temperature for 5 min to complete binding reaction, and were then supplemented with 5 μl 50% glycerol solution. Reactions were analyzed by electrophoresis through 15% native polyacrylamide gel (19:1 Laemmli). Electrophoresis was performed at room temperature – 7.4); 20 mM Tris-HCl buffer (pH 7.4), 200 mM NaCl; physiological buffer. The measurement procedures were the same as above. Only quenching-type MAB was tested.

RESULTS AND DISCUSSION

Construction of Thrombin-Binding Molecular Aptamer Beacon

The basic concept of MBs can be adopted and broadened for the construction of MABs for proteins. First, both direct quenching (between fluorophore and quencher) and FRET (between donor and acceptor) [15] can be used for signal transduction. Second, any conformational change, both opening and closing, may change the quenching efficiency or FRET efficiency. Finally, both signal increase and decrease can be used to report protein-binding events. Combining all these points, a general working principle for MABs can be similarly summarized in two points. Binding of target proteins forces the MABs to undergo a conformational change; the conformational change rearranges the relative positions between the fluorophore and the quencher, leading to an increase or a decrease in fluorescent signal. Apparently, whether there is a conformational change in an aptamer is the basic requirement for the MAB. It is known that aptamers usually experience a conformational change upon target binding [16–18]. In addition, conformational change as large as that by a MB can be created in an aptamer by changing the aptamer’s structure, as demonstrated by previous reports [13, 14]. Therefore, our general strategy for designing MABs can be briefly stated as how to transform conformational change to fluorescent signal change. Since energy transfer is highly dependent on distance between the two moieties, this problem can then be simplified as where and how to conjugate the moiety pair on the MAB in such a way to generate the largest possible fluorescent signal change upon protein binding.

A conformational change in the thrombin MAB occurs when thrombin binds to aptamer. The thrombin-binding aptamer has a 15-nucleotide consensus sequence, as shown in Fig. 1a. Both NMR and X-ray diffraction studies showed that the aptamer, no matter in free or in complex forms, could adopt a similar compact unimolecular quadruplex structure with two
FIG. 1. (a) Molecular structure of the fluorophore–quencher-labeled molecular aptamer beacon (MAB) for thrombin binding. Letters G₁ to G₁₅ represent the sequence of the 15-mer aptamer. One thymidine nucleotide is added to each end of the aptamer. 6-FAM (6-carboxyfluorescein) and DABCYL (4-(4′-dimethylaminophenylazo)benzoic acid) are conjugated through linkers to the ends of the thrombin aptamer as fluorophore and quencher, respectively. The structure of donor–acceptor-labeled MAB is the same except coumarin is used as a donor and FAM is used as an acceptor. (b) Working mechanism of the fluorophore–quencher-labeled MAB. The MAB exists at equilibrium between a nonstructured random coil and a compact intramolecular quadruplex. Addition of thrombin (gray ellipse) shifts the equilibrium in favor of the quadruplex structure, which draws the fluorophore (F) and quencher (Q) closer, leading to fluorescence quenching. The working mechanism of the donor–acceptor-labeled MAB is similar to the quench-type MAB. The arrows of the backbone of the oligonucleotides indicate the 5′-3′ polarity of DNA.
G-quartets (shown as the middle portion of the folded structure in Fig. 1b) [19]. However, high concentration of ions, especially potassium, and low temperature were needed to keep the free aptamer at quadruplex state [19]. In the absence of salt and at room temperature (25°C), the aptamer was basically at random coil state. Our recent observation indicated that, in its free form, thrombin aptamer was at equilibrium between random coil state and quadruplex state. Thrombin binding could shift the equilibrium in favor of the quadruplex state. This conformational change lays the basis for the construction of the thrombin MAB.

The labeling of signal transduction moieties needs to make full use of the conformational change so as to produce high fluorescent signal. As shown in Fig. 1a, residues 1 and 15 seem to be the best labeling sites, since they are close to each other at quadruplex state, and are the farthest apart at random coil state. The larger the change in distance between the two moieties, the larger the change in fluorescence signal will be.

Special attention should be paid to prevent the labeled moieties from severely interfering with the interaction between MAB and protein. According to a model reported for the solution structure of thrombin-aptamer complex, the aptamer binds to the anion-binding exosite of thrombin using residues 4 through 12, while residues 1, 2, 3, 13, 14, and 15 locate at the exposed side of the aptamer [19]. Therefore labeling at residues 1 and 15 should not directly interfere with the thrombin-aptamer interaction. Yet, to reduce any possible negative effect, we have further extended the length of the aptamer from a 15-mer to a 17-mer by adding one thymidine nucleotide at each end as labeling sites (Fig. 1a). This extension, on one hand, will not change the binding affinity and specificity between thrombin and the MAB [19], and, on the other hand, may alleviate potential impact on the labeled aptamer.

We constructed two MABs through conjugation of different moieties. The first MAB is of quenching type. A fluorophore (6-FAM) and a quencher (DABCYL) are conjugated to the two ends of the 17-mer (Fig. 1a). We expect the conformational change caused by thrombin binding to result in a decrease in fluorescence intensity of the MAB (Fig. 1b). The second MAB is FRET type. A donor (coumarin) and an acceptor (6-FAM) are conjugated to the ends of the 17-mer. Contrary to the quenching type MAB, the FRET type MAB is expected to give a signal increase when it binds to thrombin.

Molecular Aptamer Beacons Reporting Thrombin Binding in Real Time

The quenching-type MAB experienced a significant fluorescence signal decrease when binding with thrombin in a physiological buffer. As shown in Fig. 2a, when excess amount of thrombin was added into the MAB solution, the fluorescence intensity of MAB decreased by about 60%. Under the experimental conditions we used, signal change was quick, taking less than 10 s. We next confirmed, using gel electrophoresis, that the fluorescent signal change was indeed caused by binding between thrombin and the MAB. As shown in Fig. 2b, the binding was revealed by MAB–thrombin complex that appeared as slower migration bands. In the presence of 5-fold excess of thrombin, all MABs were changed into bound form, as there was no band indicating free MAB in the gel electrophoresis. The fluorescence of the complex was weaker than that of free MAB, which was consistent with the fluorescence drop in Fig. 2a. In a control experiment, fluorescence decrease disappeared when thrombin binding was disabled by the addition of SDS (the inset of Fig. 2a). All these results indicated that the fluorescence signal was decreased only when a binding between thrombin and MAB occurred.

The fluorescence signal change resulted from the decrease in fluorophore-quencher separation, which in turn was caused by a conformational change in MAB upon thrombin binding. To show that the above signal decrease was due to distance variation between the fluorophore and the quencher after thrombin binding, we conducted two more control experiments. First, we excluded the influence of thrombin to the fluorescence of the free fluorophore (data not shown). Second, we synthesized a MAB for thrombin with just the fluorophore (single labeled), no quencher. Whether there was a distance variation between the fluorophore and the quencher after thrombin binding could be determined by comparing the fluorescence signal change of the dual labeled MAB with that of the single labeled one. With the former, there was a significant decrease in fluorescence upon binding with thrombin, while the latter gave rise to a slight fluorescence increase after binding with thrombin (the minute fluorescence increase might be caused by the change of the local environment of the fluorophore after binding). Since the only difference between the two labeled MABs is the quencher, it is therefore reasonable to conclude that the fluorescence decrease must have been caused by the fluorophore-quencher interaction. It is known that the quenching efficiency increases with the decrease in fluorophore-quencher distance; therefore, the signal decrease shown in Fig. 2 reflects a distance decrease between the two labeled moieties in the MAB. This indicated that the two ends of the aptamer were drawn closer to each other after thrombin binding, demonstrating the MAB changing from a random coil to a quadruplex structure (Fig. 1).

The FRET-type MAB was also tested for its fluorescent response to the addition of thrombin. As expected, the RRET MAB resulted in an increased fluorescent signal upon binding to thrombin (Fig. 2c).
Binding Specificity

The MAB shows both target specificity and aptamer sequence specificity. Figure 3 shows the specificity of the quenching type MAB. Human α-thrombin, the active form of human thrombin, brought about 60% fluorescence quenching. In contrast, human γ-thrombin, an inactive form of thrombin which contains three cleavages to produce four noncovalently associated fragments [20], did not cause apparent signal change. As expected, BSA, which shows neither specific binding toward the aptamer nor nonspecific binding toward DNA, did not present apparent effect on the MAB. Surprisingly, porcine LDH-5, which has high affinity toward single-stranded DNA and can open molecular beacons [8], did not cause significant fluorescence increase. This may be due to the fact that the MABs largely form quadruplex structure in physiological buffer, which may decrease the affinity between LDH and the MAB. E. coli RecA, which is well known for its single stranded DNA binding property in the presence of ATP, did not cause significant fluorescence increase. This can be explained by its low affinity toward DNA oligonucleotides in the absence of ATP, especially when the DNA oligonucleotides are short and have second-order fluorescence response before and after the addition of 3-fold thrombin into the solution of the FRET-type MAB. The emission intensity of 6-FAM was monitored when coumarin was excited.
ary structures. In contrast, E. coli SSA, which has very high affinity towards single-stranded DNA and is capable of binding short oligonucleotides and opening secondary structures [8], caused about 5-fold fluorescence signal change. Adding excess amount of oligonucleotides of random sequences into the MAB solution can greatly reduce the influence of SSB. In summary, the MAB has high target selectivity after nonspecific DNA binding proteins are inhibited.

The binding selectivity between thrombin and the MAB is also reflected by the fact that the MAB sequence cannot be changed without losing affinity to its target. When the sequence in MAB was replaced by a scrambled sequence that cannot form a quadruplex, no fluorescence signal increase was observed. This suggests that the unique quadruplex structure formed by the aptamer sequence is required for thrombin binding, which has been revealed by previous NMR and X-ray diffraction studies [19]. Both specificities have been observed for the FRET MAB (data not shown).

Binding Affinity and Detection Limit

Titration of the MABs by thrombin was carried out to show the MABs' capability for homogeneous quantitation of target proteins. The titration was conducted in physiological buffer at 25°C. Figure 4 shows the titration curve for the quenching-type MAB. The fluorescence intensity was decreased smoothly with the increase of thrombin concentration, suggesting only one binding site was involved in the thrombin-MAB binding. The fluorescence of the MAB was decreased almost linearly at the initial stage, indicating that the added thrombin was largely bound to the MAB. When excess amount of thrombin was added, the fluorescence intensity reached a plateau. The stoichiometric binding ratio of thrombin to MAB, obtained from the titration curve, was 1:1, which confirmed previous presumptions [19].

Dissociation constant, $K_d$, for the complex between thrombin and quenching-type MAB can be calculated from the titration curve. The $K_d$ for thrombin-aptamer has been reported in the range from 2.68 to 450 nM [19, 21]. The relatively large discrepancy in reported $K_d$ values may be attributed to different experimental methods. In this study, $K_d$ has been determined to be $5.20 \pm 0.49$ nM in physiological buffer at 25°C. The low $K_d$ value indicates high affinity of MAB to its target. This confirmed our expectation that the labeling did not interfere with the interaction between the MAB and thrombin. FRET type MAB was also titrated with thrombin. The $K_d$ for FRET type MAB was calculated to be about $4.87 \pm 0.55$ nM, which is consistent with the $K_d$ for the quenching type MAB.

Using the quenching type MAB, we have achieved a detection limit at $373 \pm 30$ pM of thrombin (based on signal/noise ratio > 3), while that for the FRET type MAB was $429 \pm 63$ pM of thrombin. The subnanomolar detection limit shows MAB's excellent capability to quantitate trace amounts of thrombin in homogeneous solution.

Ratiometric Imaging and Better Protein Quantitation Using FRET-Type MAB

While the quenching-type MAB is suitable for homogeneous quantitation of target proteins in real time, it will be difficult for monitoring proteins in living specimen. The decrease in fluorescent signal upon binding makes it hard to detect minute amounts of protein and to trace a target protein in a living cell. To solve this problem, a protein probe needs to produce a signal increase when it recognizes target proteins. As shown in Figs. 2c and 5a, the FRET-type MAB can give an increased signal upon protein binding. Here the fluorescent signal increase refers to the increase in fluorescence intensity of the acceptor. The signal enhancement after binding may be significantly improved by using ratiometric imaging [22]. Figure 5b shows how ratiometric imaging has improved the signal enhancement factor. In the FRET-type MAB, the emission spectrum of coumarin overlaps well with the excitation spectrum of 6-FAM, and the largest possible separation between the two fluorophores would be approximately 58 Å (presuming the DNA conformation is in B-form) which is within the space range in which FRET can occur [15]. Thus, FRET should occur be-
between the acceptor and donor. Since thrombin-binding bring the donor and acceptor closer, an increased FRET efficiency is expected in the presence of thrombin, which should result in an increase in acceptor intensity (as shown in Fig. 2c) and a simultaneous decrease in donor intensity. Such changes indeed oc-
curred, as shown by the fluorescence spectra in Fig. 5a. After binding, the emission intensity of 6-FAM increased from 87.3 to 168.4 arbitrary units, which was consistent to the observation in Fig. 2c. At the same time, the emission intensity of coumarin decreased from 40.3 to 5.4 arbitrary units. To improve signal enhancement factor, we compared the ratio of acceptor intensity to donor intensity before and after thrombin binding instead of (see Materials and Methods). By doing this, we increased the signal enhancement factor by about 14-fold, as shown by the insert of Fig. 5a.

In Fig. 5b, both direct intensity imaging and ratiometric imaging are conducted and compared. Under the same experimental conditions, the ratiometric imaging showed significant signal enhancement of about 12, while there was only a slight signal enhancement of 0.7 when analysis was performed with direct intensity imaging. The two enhancement factors obtained in these imaging experiments are slightly smaller than the corresponding values obtained by spectrofluorometer as shown in the inset of Fig. 5a. This is most likely due to the different optical instrumentations used in the two experiments. Clearly, ratiometric imaging gives a much improved signal to background ratio and, therefore, much higher sensitivity in protein imaging. When the ratiometric measurement was applied to protein in solution using a spectrofluorometer, a much lower detection limit, \(112 \pm 9\) pM, was obtained for thrombin quantitation.

There is no easy way to monitor proteins in real time and with living specimen. Our MAB approach has the potential to accomplish these challenging and significant tasks. Here the signal transduction is based on the conformational change of the aptamer. Because conformational change is usually accompanied by protein binding, this approach should be generally useful for the construction of other MBAs based on other protein binding aptamers. As molecular beacons, these MAB probes can be used in situations where it is either not possible or desirable to isolate the probe-target complex from an excess of the probes. The usefulness of “detection without separation” for protein studies cannot be overemphasized in diagnostic imaging and in living specimen studies. MABs are expected to function in real-time as ideal in vivo protein probes with high sensitivity and excellent selectivity. The development of MAB represents one important and promising step for efficient protein determination in homogeneous solutions.

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