Investigation of Molecular Beacon Aptamer-Based Bioassay for Platelet-Derived Growth Factor Detection

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This report describes studies on the use of a molecular-beacon aptamer (MBA) as a synthetic high-affinity DNA probe that exhibits fluorescence resonance energy transfer (FRET) in response to a specific protein biomarker, platelet-derived growth factor (PDGF). As a step toward the application of the MBA in a fluorescence-based assay for biological specimens, we examined the influence of certain physical and chemical parameters of incubation that would affect DNA conformation and DNA-backbone modification, and thus improve nuclease resistance. This bioassay is compatible with pH, temperature, and monovalent cation levels typically encountered in biological samples, and phosphorothioate backbone-modified MBA is able to exhibit specific FRET. With minimal sample processing and without assay optimization, the MBA is able to detect as little as 10 ng PDGF per μg of serum proteins from cell-culture media. We also show that different sets of known fluorophore–quencher pairs can be successfully used in the MBA for sensitive detection of the PDGF target. It should, therefore, be possible to develop multiplex bioassays that monitor either quenching or enhancement for the simultaneous detection of several biomarkers by using MBAs created from high-affinity DNA ligands for the desired protein targets. Interestingly, we observed that, with a DNA ligand with multiple binding sites for a standard multimeric protein target, the FRET bioassay could be accomplished by using a mixture of two individually labeled DNAs—one carrying the fluorophore and the other with the matching quencher. This observation has significant implications in the future design of more selective DNA-based FRET bioassays that use more than one ligand for the same protein target.

Introduction

Synthetic oligonucleotide aptamers that display high-affinity binding with selectivity for protein molecules hold significant promise in the design of ultrasensitive assays for protein biomarkers with potential advantages over antibodies. The enormous diversity of random oligonucleotide libraries can exceed the diversity of antibodies in the mammalian genome by several orders of magnitude. Oligonucleotides can be easily produced in pure form by chemical synthesis, modified to incorporate signal-transduction moieties, immobilized to solid surfaces through various linkers, and derivatized to enhance their stability in biological fluids. Although shorter DNA aptamers might display low affinities for their targets, longer DNAs have been selected with affinities comparable to those of antibodies for protein biomarkers vascular endothelial growth factor (VEGF)/platelet-derived growth factor (PDGF)).[1,2] Available methods for characterizing and quantitating protein biomarkers include gel electrophoresis,[3] isoelectric focusing, affinity chromatography, proximity ligation,[4] immunological assays such as ELISA,[5] and cell-based bioactivity assays.[6] Many of these methods are time consuming, labor intensive and require multiple steps, such as immobilization, repetitive incubations and washings, as well as additional reagents to amplify the signal.

We have previously reported the creation of a "molecular-beacon aptamer (MBA)" for intact dimeric PDGF[7] using minor modifications of a synthetic DNA aptamer with high-affinity binding for the B subunit of PDGF selected by Green et al.[1] Binding of the specific target protein to the MBA is readily detectable by fluorescence resonance energy transfer (FRET)[8] between a fluorophore and a quencher that are attached to two different locations on the oligonucleotide aptamer with specific high-affinity binding to PDGF. While the free MBA presumably exists in an open conformation and elicits a fluorescence signal, the target-bound MBA assumes a close conformation, thus reducing the distance between the fluorophore and the quencher. Fluorescence enhancement assays with molecular beacons for DNA and RNA targets have been extensively studied and are based on the hybridization of the loop sequence to the target sequence that results in the melting of a double-stranded stem formed by palindromic sequences that flank the loop sequence.[9] In contrast, the use of MBAs for protein targets is in its infancy and the basis for differences in secondary structures between the free and bound forms of MBAs for protein targets is not well understood. However, interactions with

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different proteins are likely to result in significantly different consequences to the secondary structure of the DNA that might result in different FRET signals. In prior studies, we observed that fluorescence quenching is dependent on the PDGF-to-MBA ratio and that the dose dependence of quenching had distinctly different patterns with the three known major molecular variants of naturally occurring PDGF dimers, namely AA, AB, and BB.[7] These observations led us to hypothesize that synthetic DNA sequences can discriminate between closely related but nonidentical members of a protein family or different functional or conformational states of the same protein. MBAs thus have the potential to be novel tools for structure–function analysis of molecular variants of proteins and detection of these biomarkers in biological samples.

In order to advance the use of MBA-based FRET for protein biomarker targets into practical bioassays, it is necessary to further analyze the effects of assay parameters and MBA modifications. We now report the results of a systematic study of various physical and chemical parameters of incubation and selected modifications of the MBA on a fluorescence quenching FRET assay as a step toward the development of an MBA-based bioassay for protein biomarkers in real biological samples. Our results show that the MBA bioassay is reliable over a range of pH and temperature and monovalent cation concentration that might be encountered in biological specimens. Divalent cations show interference with the assay by causing quenching in the absence of the PDGF target and will need to be removed from biological samples. This standard one-step assay can detect as little as 2.5 ng of PDGF in the presence of a nearly 1000-fold excess of serum-derived proteins commonly present in cell culture media. Alternative fluorophore–quencher pairs, fluorescence-enhancement assay formats and MBAs with nuclease-resistant backbone modification are also effective in this novel assay method that is based on specific conformational features of synthetic, high-affinity DNA aptamers in the presence of the specific protein targets to which these aptamers bind. The results offer us new insights into, and justify the need for, future development and optimization of this novel, one-step MBA-based FRET assay into a bioassay method applicable to protein biomarkers relevant in cancer and other important diseases.

Results and Discussion

Selectivity of the MBA-based fluorescence-quenching assay

A FRET assay with MBAs is novel in that the conformational difference between the free and the protein-bound MBA molecules determines the distance between the fluorophore and the quencher and hence changes in the signal. If the conformation of the protein-bound MBA is influenced by the nature of the interaction with a protein, the pattern of signal change as a function of protein-to-MBA ratio will be different for different proteins. The selectivity of the fluorescence quenching FRET assay with the standard MBA for PDGF can, therefore, be effectively evaluated by monitoring signal transduction at different molar ratios of the MBA to PDGF as compared to other biomolecules.

We tested a selected group of natural biomolecules including growth factors (epidermial growth factor (EGF)), common proteins that are abundant in biological specimens (thrombin, ovalbumin, and serum albumin) and glycogen (an abundant natural polysaccharide in tissues that is not known to bind to or cause any conformational change in DNA or protein molecules) individually in the fluorescence-quenching assay. Dose–response curves were obtained by incubating increasing amounts of each biomolecule in the standard fluorescence-quenching assay. The results are presented in Figure 1 A and B.

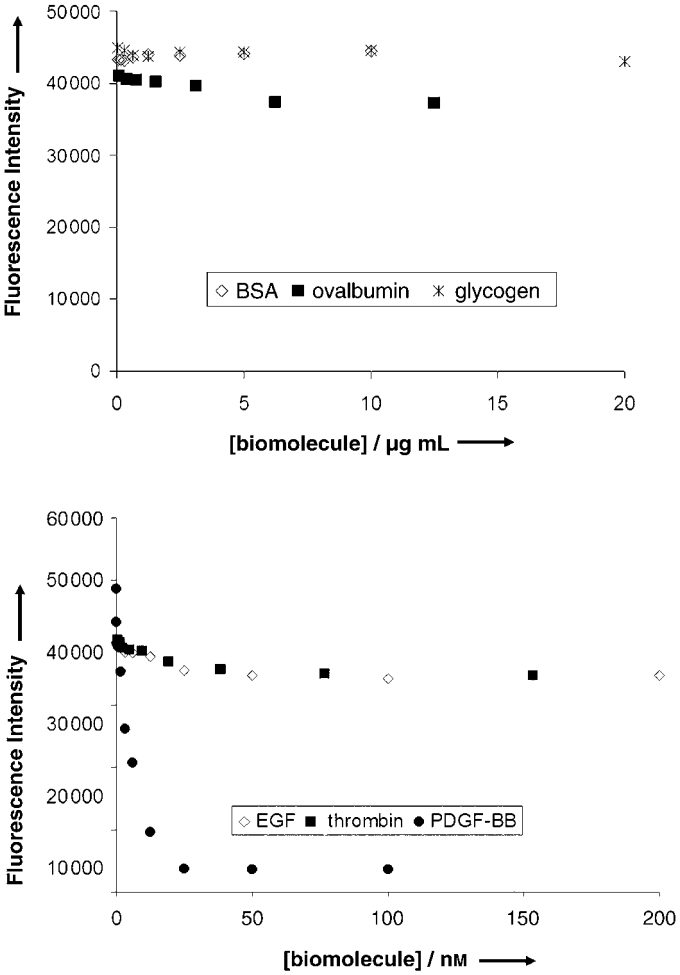

Figure 1. *Fluorescence quenching displayed by the MBA in response to increasing concentrations of PDGF and other biomolecules. In a typical fluorescence-quenching assay containing MBA (75 nM) in Tris buffer (pH 7.0, 20 nM) and NaCl (20 nM) as described in the Experimental Section, the fluorescence signal was measured following the addition of increasing concentrations of various biomolecules: A) PDGF–BB, epidermal growth factor and thrombin; and B) BSA, ovalbumin and glycogen. The marks for each protein are shown in the figures.*

As reported previously,[7] the two molecular variants of PDGF (AA and BB) showed different patterns of fluorescence quenching as a function of protein concentration. The small dose-dependent increase in quenching indicates that thrombin and EGF might interact slightly with MBA, although the final extent

of quenching even at a significant molar excess is substantially lower than the quenching observed with either of the molecular variants of PDGF. Insignificant changes in fluorescence intensity, even at a significant molar excess (up to 20 $\mu g\,mL^{-1}$) of the different unrelated commonly occurring biomolecules (bovine serum albumin, ovalbumin, and glycogen) tested, indicated that the assay is indeed selective for PDGF. Since only PDGF is capable of producing a large change in fluorescence signal, the assay should be selective and sensitive for PDGF detection even in the presence of other proteins commonly found in biological specimens. When using biological specimens, nonspecific DNA–protein binding might interfere with many traditional methods for the detection of proteins with DNA probes. The advantage of FRET signal transduction with an MBA is that the dose-response curve for the fluorescence signal as a function of target concentration is not only dependent on the selective binding of the MBA to its target but also on the specific conformation of the MBA in its target-bound form.

Modifications of incubation conditions and DNA backbone—Bioassay development

Effects of temperature. A critical factor in the MBA-based bioassay is the difference in distance between the quencher and the fluorophore in the free MBA (open conformation) and the target-bound MBA (closed conformation). Since FRET is dependent on the distance between the fluorophore and the quencher, physical factors that affect DNA conformation are expected to influence the dynamic range of the assay. If higher temperature increased the fluorescence signal of the free MBA by allowing it to assume a more open conformation, then it might be possible to increase the extent of quenching observed in response to PDGF. In order to assess the effect of temperature, we measured the fluorescence intensity of the MBA at different temperatures under conditions used in the standard fluorescence-quenching assay (see Experimental Section). The results presented in Figure 2 show that, as the temperature increases, the fluorescence intensity increases presumably resulting from a larger separation of the arms of a predicted three-way helix junction where the six-base-pair double-stranded region would be formed. Although these data suggest that it might be possible to perform the fluorescence-quenching assay at higher temperatures than that used in the standard fluorescence-quenching assay (30 °C), initial experiments do not show any significant increase in the dynamic range. Additional experiments to evaluate the effect of temperature on PDGF–MBA association are currently in progress.

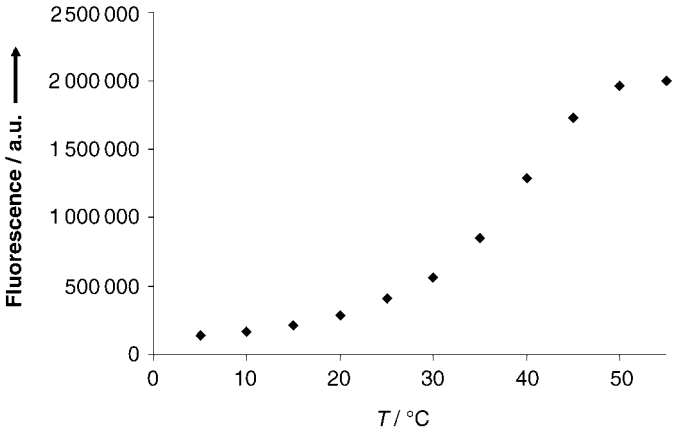

Figure 2. *The effect of increasing temperature on the fluorescence signal of free MBA in solution. A solution of MBA (75 nM) in standard fluorescence-quenching assay buffer as described in the Experimental Section was subjected to a gradual increase in temperature (5 °C min^{-1}), held at the indicated temperatures for 5 min and the fluorescence signal was measured.*

Effects of pH. We next measured the fluorescence intensity of a 75 nM MBA solution in the buffer used in the standard fluorescence-quenching assay except that the pH was varied between 6.0 and 10 (data not shown). The scope of this experiment is limited by the pH dependence of the fluorescence intensity of fluorescein. The results show that, within a pH range of 6.5 to 9.5, the fluorescence intensity of the MBA is within $\pm$10% of that observed at pH 7.0. Our data indicate that our goal to monitor PDGF in biological specimens at or near physiological pH is achievable with the MBA.

Effects of ionic strength. Certain monovalent and divalent cations commonly encountered in biological specimens are known to affect DNA conformation. We, therefore, performed a series of experiments to study the effects of varying concentrations of two divalent cations (Mg^{2+} and Ca^{2+}) and two monovalent cations (Na^{+} and K^{+}) on the fluorescence intensity of the free MBA in solution. The results are presented in Figure 3. Each of the divalent cations caused a concentration-dependent decrease in fluorescence intensity (Figure 3 A), and, at $>$1 mM concentration, the extent of quenching was comparable to that obtained with an excess of PDGF–BB (see Figure 1). These results were expected since divalent cations frequently stabilize secondary structures in DNA molecules and would thus favor a closed conformation of the MBA. The concentrations of Mg^{2+} and Ca^{2+} in a typical cell culture medium (Dulbecco's Modified Eagles Medium (DMEM) from Life Technologies, MD) are approximately 2 and 1.5 mM, respectively. Although the chelating agent EDTA is commonly used to counter the effects of divalent cations such as Mg^{+2} and Ca^{+2}, we discovered that, at a concentration between 2 to 3 mM, EDTA caused approximately 10% quenching in fluorescence intensity of the MBA as compared to approximately 90% quenching caused by comparable concentrations of either Mg^{2+} or Ca^{2+}. The effects of EDTA on the PDGF–MBA interaction, the fluorophore or the quencher used in the MBA are unclear at the present time. Additional experiments in progress are aimed at sample-processing methods to reduce the levels of divalent cations and the use of lower amounts of EDTA to abrogate the interference by Mg^{2+} and Ca^{2+}.

Na^{+} and K^{+} caused only a small initial decrease in fluorescence intensity (Figure 3 B) and, even at concentrations of $>$200 mM, the extent of quenching by these ions was less than 5% as compared to $>$90% quenching obtained with a fourfold molar excess (300 nM) of PDGF–BB in a standard fluorescence-quenching assay. The typical combined concentration of Na^{+} and K^{+} in cell-culture medium is between 150 and 160 mM, significantly below the concentration of these mono-

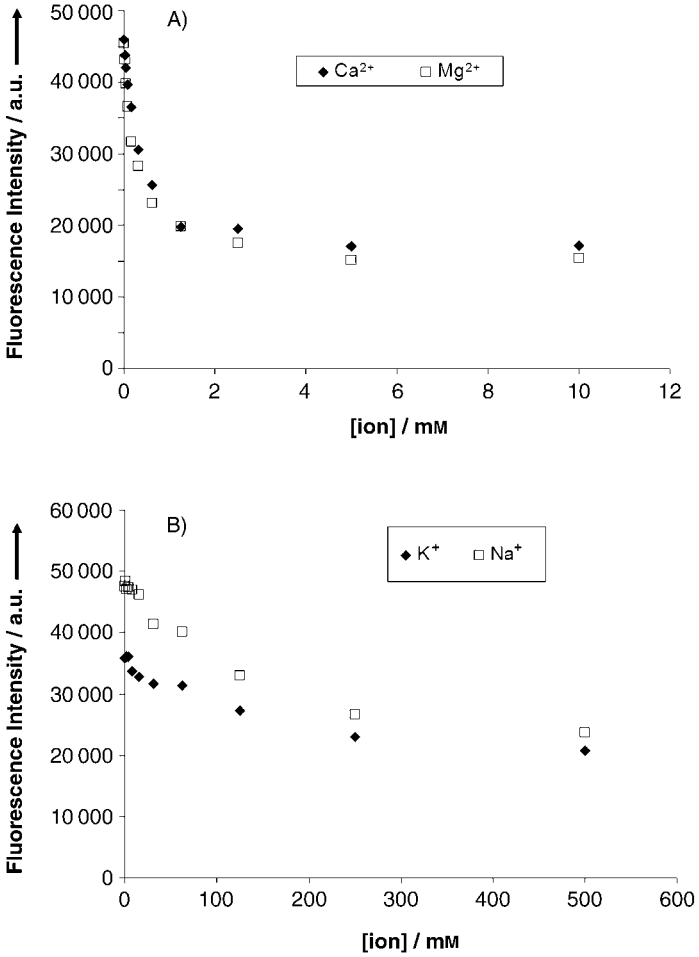

Figure 3. *The effect of cation concentration on the fluorescence signal of free MBA in solution. Increasing concentrations of A) divalent cations (Ca^{2+} and Mg^{2+}) and B) monovalent cations (Na^{+} and K^{+}) were added in form of the respective chloride salt to a solution of MBA (75 nM) in the standard fluorescence-quenching assay buffer and the fluorescence signals were measured. The absolute fluorescence intensities for each experimental set of a selected cation might vary based on the instrument setting.*

valent cations that would result in an unacceptable level of background quenching.

The results obtained with various incubation conditions described above support the notion that MBA-based FRET may be used in bioassays for protein detection in biological specimens since the FRET assay is effective at physiological pH and temperature, there is minimal interference by monovalent cations even at concentrations that are higher than their intracellular levels, and minimal sample processing can be used to remove possible interfering divalent cations commonly encountered in biological samples.

Effects of DNA-backbone modification. Since the normal phosphodiester backbone of synthetic DNAs is susceptible to nucleases present in biological specimens, we investigated whether the MBA with a backbone-modified DNA is still functional in mediating FRET in response to PDGF binding. A phosphorothioate backbone-modified DNA was synthesized by using the standard sulfurizing Beaucage reagent,[10] during DNA synthesis. The backbone-modified DNA synthesized by using a 4-(4-(dimethylamino)phenylazo)benzoic acid (Dabcyl) moiety at the 3′-end was modified with fluorescein, and the resulting MBA was purified by using standard protocols. The backbone-modified MBA showed detectable quenching, approximately 75% as compared to 90% observed with unmodified MBA, when incubated with a fourfold molar excess of PDGF–BB in a standard fluorescence-quenching assay. These results indicate that DNA-backbone modification to achieve nuclease resistance did not significantly inhibit the binding and the conformational change of the MBA in response to PDGF. Since the backbone-modified DNA-based MBA is more stable than the control MBA to nucleases encountered in biological specimens, the use of the modified MBA is promising. We are further exploring a modification of the synthesis and purification method for the backbone-modified MBA to avoid exposure of the quencher or the fluorophore to the sulfurizing reagent. We are also investigating changes in incubation conditions that might affect the interaction between the modified MBA and PDGF–BB.

PDGF detection in biological specimens

We have previously reported that our fluorescence-quenching assay with MBA is able to detect PDGF in the presence of unrelated proteins and growth factors.[7] As a step toward the development of an effective MBA-based bioassay it is important to investigate in greater detail the potential interference that might be caused by cell-derived proteins and the relative abundance of PDGF in biological specimens that might be detectable in the presence of such interference. A simulated specimen was used to evaluate and develop sample-processing steps that would be required for the use of an MBA-based FRET assay for the detection of PDGF produced by cells in culture or in serum and tissue specimens. The simulated biological specimens used in these studies were prepared to mimic conditioned media collected from tissue culture cells that are commonly used to study the expression of proteins by normal and deceased cells. The control specimen contained DMEM supplemented with 0.5% fetal calf serum. A parallel specimen that would mimic conditioned media from tissue culture cells that have been induced or treated to overproduce PDGF was prepared by adding pure PDGF–BB to the control specimen at 0.2 $\mu g\,mL^{-1}$. Each simulated biological specimen was processed and protein fractions from a gel filtration column were collected as described in the Experimental Section. In order to estimate the relative recovery efficiency of PDGF and its relative abundance compared to other common biological proteins in the processed samples, we tested equivalent portions of the column fractions (representing different size classes of proteins) in the standard fluorescence-quenching assay. The results for the simulated control specimen without added PDGF–BB (Figure 4A) show that fractions 6–12, which contain significant amounts ($>16.5\ \mu g\,mL^{-1}$) of serum protein, caused detectable quenching even though PDGF–BB had not been added to the specimen. Equivalent fractions from the specimen to which PDGF–BB had been added did not show any additional quenching above that observed with the control specimen (data not shown). The slope of the dose-dependent

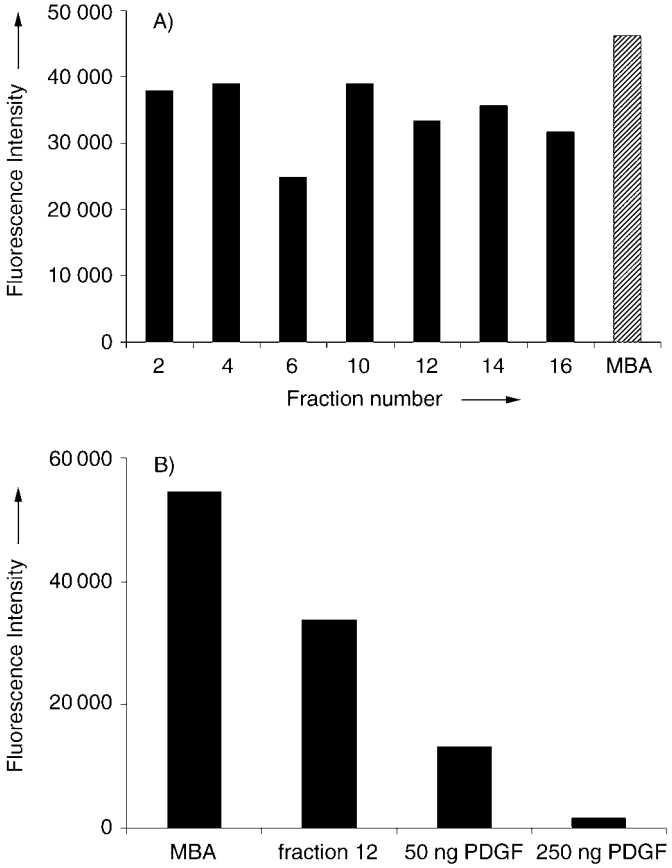

Figure 4. *Fluorescence quenching caused by PDGF in the presence of proteins in a simulated biological specimen. Protein fractions were obtained from a Sephadex G-100 gel filtration of acetic acid soluble materials in a simulated biological specimen as described in the Experimental Section and each fraction was resuspended in Tris stock buffer (130 μL, pH 7.5, 10 mM) with NaCl (20 mM). A) 65 μL of each fraction indicated on the x-axis was incubated in a standard fluorescence-quenching assay (final volume 100 μL) with the MBA (75 nM) and the resulting fluorescence was measured. B) Indicated amounts of PDGF–BB were added to a dilution of Fraction 12 with approximately 3 μg mL^{-1} of serum-derived proteins and incubated in the standard fluorescence-quenching assay.*

quenching obtained with these fractions was different from the slope when pure PDGF–BB was used in the assay. At a total serum-derived protein concentration of $\leq 3\ \mu g\,mL^{-1}$, the extent of quenching with the serum proteins was less than 10% of quenching caused by a comparable amount of control PDGF–BB (data not shown). One reason for the failure to observe a detectably higher quenching with fractions from the specimen with added PDGF–BB is that the abundance of PDGF relative to total serum proteins was lower than the effective range of the assay when applied to a complex biological specimen. To investigate this possibility and establish the limit of detection of our current assay for PDGF in the presence of serum proteins, we added varying amounts of PDGF–BB to the fraction from the gel filtration column where PDGF–BB is expected to be eluted and performed the standard fluorescence-quenching assay. The results (Figure 4B) show a readily detectable increase in quenching upon the addition of as little as 50 ng of PDGF–BB in the presence of a nearly 1000-fold excess of similar serum proteins. Work in progress to optimize the MBA-based FRET bioassay is focused on sample-preparation steps that reduce the level of background quenching, achievement of a quantitative recovery of PDGF from biological specimens, and modifications in assay conditions that improve the dynamic range of PDGF detection.

Evaluation of additional fluorophore quencher pairs—Potential development of multiplex assays

One of the important advantages of this MBA bioassay is that the use of FRET, which is based on a specific DNA conformation in its target-bound form, allows for a single-step detection of the target without having to isolate the MBA–protein complex from the solution. The versatility of the bioassay would be significantly improved if various known fluorophore–quencher pairs were found to be effective in MBAs. We, therefore, evaluated MBA molecules carrying the following known FRET pairs and compared these to the standard MBA with fluorescein–Dabcyl pair: Texas Red–Black Hole Quencher 2 (BHQ2), Cy5–Black Hole Quencher 2 and fluorescein–tetramethylrhodamine (TMR). MBAs with these FRET pairs were synthesized and purified as described in the Experimental Section and tested for decrease in fluorescence intensity upon addition of a fourfold molar excess of PDGF–BB in the standard fluorescence-quenching assay. As shown in Figure 5, all the selected FRET

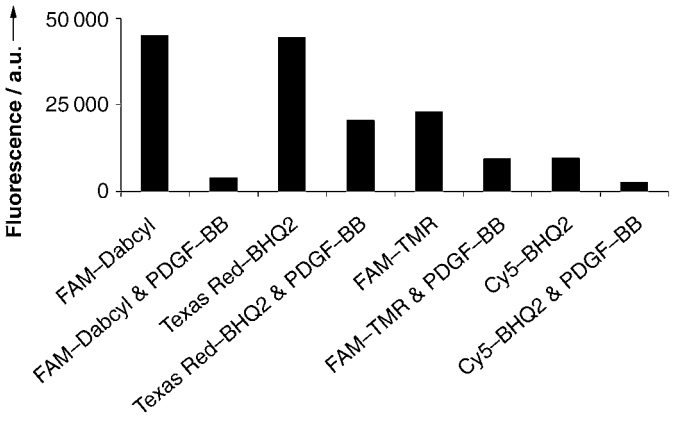

Figure 5. *Evaluation of fluorescence quenching caused by PDGF–BB with MBAs containing different fluorophore–quencher combinations. PDGF–MBA molecules labeled with different fluorophore–quencher pairs were incubated in the standard fluorescence-quenching assay buffer (final volume 100 μL) with a fourfold molar excess of PDGF–BB (300 nM) to the concentration of the MBA (75 nM). FAM = fluorescein.*

pairs showed specific quenching of the fluorescence signal when PDGF was added, albeit to different degrees. Since FRET is a distance-dependent phenomenon, it should be possible to improve the performance of other fluorophore–quencher pairs by changing the location of the label molecules in the MBA. The availability of choices in the selection of fluorophore and quencher is an important part of MBA-based bioassay development because various biological specimens might present po-

tential interference at certain excitation–emission spectra that might be effectively addressed by selecting the proper FRET pair. In addition, the development of a multiplex assay for the simultaneous detection of multiple proteins in a single homogeneous incubation might be possible by using two or more MBAs that are selective for different biomarkers and that are labeled with different fluorophore–quencher pairs.

Fluorescence enhancement assays with MBAs—Increase in sensitivity

Since the principle of the MBA-based assay relies on signal transduction resulting from differences in the structure of free versus protein-bound MBA, it should be possible to modify the assay from a quenching to an enhancement format by selecting appropriate pairs of fluorophores. It is generally believed that an enhancement assay format provides a higher degree of sensitivity than that achieved with a quenching format. We used the standard fluorescence-quenching assay to compare the response of our standard MBA to one that was labeled with a Cy3 dye at the 3′-end and a Cy5 dye at the 5′-end. The latter MBA is suitable for an enhancement format since it can be excited at the absorption maximum of the donor dye, Cy3 (546 nM), and the fluorescence signal can be measured at the emission maximum of the acceptor dye, Cy5 (662 nM), when the MBA is in its closed form upon binding to PDGF–BB. A dose-dependent increase in fluorescence was observed with the Cy3/Cy5-labeled MBA upon addition of increasing amounts of PDGF (data not shown). Photo-crosslinking experiments reported by Green et al.[1] suggested that the Phe84 residue in the B-chain of PDGF forms a point of contact with a specific nucleotide residue in the loop at the helix junction of the closed conformation of a PDGF-specific aptamer. We hypothesized that if the homodimer PDGF–BB could bind two MBA molecules with appropriate conformational characteristics, then FRET could occur between two aptamers each labeled with one of the two dyes of a FRET pair. This would allow the design of a novel fluorescence enhancement format for a FRET PDGF assay that uses two single fluorophore-labeled aptamers. Specifically, the aptamer sequence was labeled either with Cy3 (the donor) at the 3′-end or with Cy5 (the acceptor) at the 5′-end. A solution was then prepared by mixing an equimolar ratio of the 3′-Cy3- and the 5′-Cy5-labeled PDGF aptamer preparations. Once two aptamers carrying two different labels bound to the same PDGF molecule, the two fluorophores would be in close proximity. It should then be possible to excite the donor fluorophore, Cy3, at its absorption band and due to FRET its fluorescence will be quenched by Cy5. However, there will be a simultaneous increase in the fluorescence of Cy5 even when the MBA molecule is excited at the absorption band of Cy3. Figure 6B shows a dose-dependent increase in Cy5 fluorescence when increasing amounts of PDGF–BB were incubated with an equimolar mixture of the single fluorophore-labeled aptamers (combined concentration of 75 nM) as compared to the standard quenching observed with the standard double-labeled MBA (Figure 6A). These results demonstrate the capability of this mixed-aptamer solution in a sensitive enhancement assay, consistent with the hypothesis[1] that two or more aptamers bind to a single PDGF–BB molecule. This is the first demonstration of a fluorescence-enhancement assay in a single incubation in which the binding of two separate aptamer molecules carrying a donor and an acceptor dye to a target protein molecule presumably results in FRET because of the specific closed conformation of both aptamer molecules. This is a striking contrast to the opening up of a double-stranded stem of a molecular beacon to produce fluorescence enhancement in studies on nucleic acid targets. We also tested equimolar mixtures of the aptamer labeled with fluorescein and with either Dabcyl or tetramethylrhodamine as the quencher in parallel assays and observed dose-dependent specific quenching with PDGF–BB (data not shown). The ability to perform a FRET bioassay with singly labeled DNA molecules has important future implications in the development of novel fluorescence assays for multimeric proteins, protein modifications and protein aggregates by using high-affinity DNA ap-

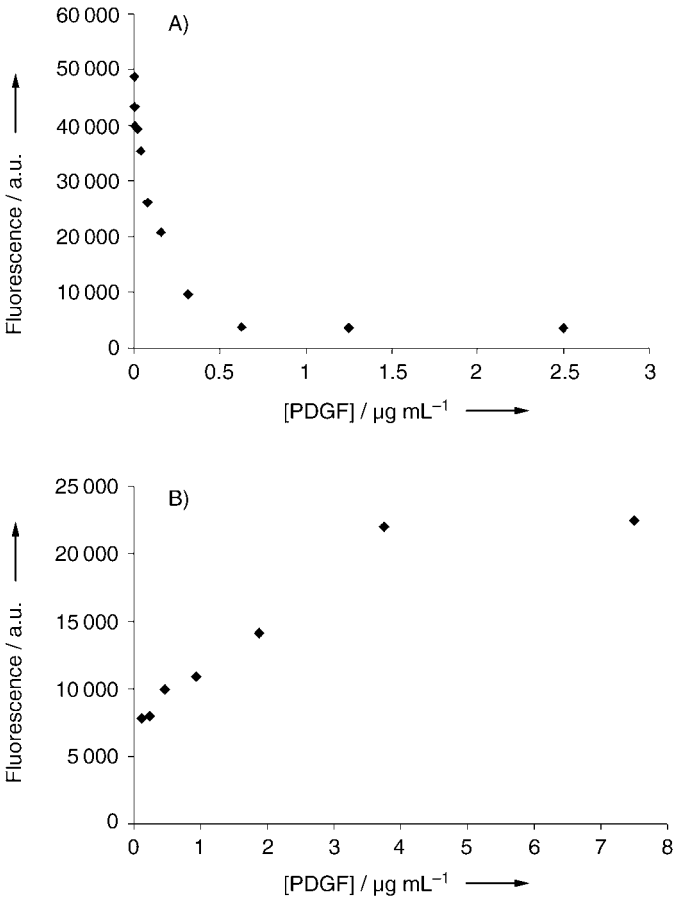

Figure 6. *A comparison of quenching and enhancement formats of the MBA-based FRET assay. Increasing concentrations of PDGF–BB were incubated in the standard fluorescence-quenching assay buffer (final volume 100 μL) with A) an MBA labeled with Dabcyl quencher at the 3′-end and fluorescein at the 5′-end (fluorescence measurements were made at the excitation and emission maxima for fluorescein) or B) with an equimolar mixture of the PDGF aptamer labeled with Cy3 at the 3′-end and a PDGF aptamer labeled with Cy5 at the 5′-end (fluorescence measurements were made at excitation maximum of Cy3 and emission maximum of Cy5).*

tamers that recognize different domains of these proteins. We are currently evaluating the usefulness of mixed aptamers as compared to MBAs in the detection of PDGF by enhancement-based FRET as compared to the standard fluorescence-quenching assay.

Conclusion

In this study, we have demonstrated how a synthetic DNA sequence can be modified to report the binding of a target protein biomarker, PDGF, in a single step. The selectivity of this assay method arises from the fact that the signal generation requires not only the binding to the target protein but also specific conformational characteristic of the bound DNA. Our results show that the conditions required for successfully using MBAs to perform the novel single-step FRET bioassay are compatible with physiological pH and temperature range and the presence of common monovalent cations. The results are promising for future applications of this assay for the detection of PDGF in real biological samples although processing methods to remove divalent cations and major protein contaminants still need to be optimized. The successful results reported here with several modifications—including DNA-backbone modification to create a nuclease-resistant MBA, the use of different known fluorophore–quencher pairs, and a dye–dye pair for signal enhancement—demonstrate that an MBA can be readily modified to satisfy experimental requirements for bioassays for different biological samples. Optimization of the assay might be achieved by using other combination of dye–quencher or dye–dye FRET pairs for higher signal and lower background and also by adjusting the distance between the FRET signaling moieties based on the conformation of the closed form of the bound MBA as predicted by DNA-structure modeling software. The ability to achieve signal transduction by using several known FRET pairs not only indicates the versatility of the MBA-based assay method but also strongly supports the notion that multiple MBAs for selected biomarkers can be created with distinct FRET pairs and used in a multiplex bioassay. There is a need to develop multiplex assays to simultaneously monitor disease biomarkers in a single incubation in homogeneous solutions. The design of a successful MBA for protein biomarkers will require a careful choice of FRET pairs, an analysis of their relative positions in the open versus closed conformations of the aptamer and studies on incubation conditions that will give the largest signal change upon binding. Additional development of sample processing methods for selected biomarkers in specific biological specimens of choice and further modifications of MBA molecules should lead to the use of MBA-based bioassays for successful detection of protein biomarkers in real biological specimens.

Experimental Section

Synthesis and purification of MBA: DNA molecules were synthesized by using the standard phosphoramidite chemistry in Expedite 8909 automated DNA synthesizers on controlled pore glass beads, deprotected in ammonium hydroxide and purified by gel filtration. Unmodified DNA aptamers were purified by ion-exchange HPLC followed by gel filtration to remove salts. The standard MBA was synthesized by using Dabcyl immobilized on controlled pore glass beads (BioSearch Technologies, Novato, CA) and fluorescein was added at the 5′-end by using 6-carboxyfluorescein phosphoramidite (BioSearch Technologies, Novato, CA). The dually modified DNA was deprotected at 55 °C for 6 h in 50 % ammonium hydroxide in methanol. The deprotected DNA was first subjected to gel filtration followed by two cycles of reversed-phase HPLC by using two different hydrophobic resins in triehylammonium acetate buffer (0.05 M) at pH 6.0 and eluted with an acetonitrile gradient. The final purified material was subjected to gel filtration to remove solvents, dried under vacuum and stored dry at −20 °C until use. MBAs with tetramethylrhodamine(TMR)-fluorescein pair was synthesized and purified as described above by using TMR immobilized on controlled pore glass (BioSearch Technologies, Novato, CA), except that deprotection was carried out in potassium carbonate (0.1 M) in 80 % methanol for 4 h at 55 °C before processing the deprotected dual-labeled DNA. For the synthesis of MBA with Black Hole Quencher 2 (BHQ2)—Texas Red pair, BHQ2 immobilized on controlled pore glass (BioSearch Technologies, Novato, CA) and a 5′-modification with a functional amine group and a C3 spacer was used. Following deprotection at 55 °C with 20 % ammonium hydroxide in methanol and gel filtration, the DNA was treated with the *N*-hydroxysuccinimidyl ester of Texas Red (Molecular Probes, Eugene, OR) in sodium borate buffer (0.1 M) at pH 8.2 and the labeled material was again subjected to gel filtration prior to two cycles of reversed-phase gel filtration as described above. For the synthesis of MBA labeled with Cy3 and Cy5, the DNA was synthesized with a functional amine group immobilized on controlled pore glass beads and Cy5 was added to the 5′-end by using Cy5 phosphoramidite (Amersham Bioproducts, Piscataway, NJ). Following deprotection with potassium carbonate/methanol (0.1 M) and gel filtration, the DNA was treated with *N*-hydroxysuccinimidyl ester of Cy3 dissolved in dimethyl sulfoxide at 5 mg per 100 μL and mixed with DNA (at 400 $\mu g\,mL^{-1}$ in 0.1 sodium borate buffer) at a molar ratio of 50:1 (Amersham Bioproducts, Piscataway, NJ), and the dual-labeled material was purified as described above for Texas Red labeling.

MBA stock solutions (100 μM) were prepared in Tris/HCl buffer (pH 7.5, 20 mM) with NaCl (20 mM), and stored in aliquots at −20 °C. The DNA aptamer sequence in the standard MBA used in all studies described here is the one designated 36t by Green et al:[1]

5′-CACAGGCTACGGCACGTAGAGCATCACCATGATCCTGTG-3′

and contains a six-base complementary sequence at the 5′- and 3′-ends that can form a double-stranded stem in a closed conformation.

Materials: Recombinant human PDGF and Epidermal Growth Factor (EGF) were purchased from R&D Systems (Minneapolis, MN). Other biomolecules used include: bovine serum albumin (BSA; New Englands Biolabs, MA), thrombin (Haematologic, Inc, VT), ovalbumin (US Biological, Sawmpscott, MA), and glycogen (Fisher Biotech, USA).

Standard fluorescence-quenching assay: The fluorescence-quenching assay (except where indicated) was performed by incubating a solution of the MBA (75 nM) carrying a fluorescein moiety at the 5′-end and a Dabcyl quencher at the 3′-end in Tris/HCl buffer (pH 7.5, 20 mM) with NaCl (20 mM) with varying amounts of biomolecules at 30 °C in a Nunc microtiter plate (Fisher Biotech, USA) in a final volume of 100 μL. Fluorescence was measured with

a Saphire Microplate reader (Tecan Instruments, NC). The samples were excited at the excitation maximum (480 nm) and the fluorescence intensity was measured at the emission maximum (520 nm).

The effect of temperature on MBA fluorescence was monitored by using a Fluorolog-Tau-3 spectrofluorometer (Jobin Yvon Inc, NY) equipped with a thermostat accurate to 0.1 °C. The sample cell was a 100 μL cuvette (Starna cells). The fluorescence intensity of an MBA solution (75 nM) in the incubation buffer described above was measured as the temperature was increased from 5 to 80 °C in steps of 5 °C. The solution was maintained at each temperature for 10 min before recording the fluorescence intensity.

Simulated biological specimen: A simulated specimen that mimics the complexity of the protein mixture characteristic of cell culture conditioned media, a typical biological sample, was prepared by adding Fetal Bovine Serum (final concentration 0.5% (*v*/*v*); Invitrogen, CA) to DMEM. The serum-supplemented medium (60 mL) was lyophilized, resuspended in acetic acid (0.1 M, 5 mL), and separated in a centrifuge at 12000 *g* for 5 min, then the supernatant was subjected to gel filtration through a Sephadex G-100 column (55 mL packed volume) equilibrated with acetic acid (0.1 M). Fractions (5 mL) were collected and lyophilized; each fraction was resuspended in Tris/HCl buffer (130 μL, pH 7.5, 10 mM). The protein solution (65 μL) was used in a standard fluorescence-quenching assay as described above (final volume 100 μL).

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Keywords: aptamers · bioassays · fluorescence · molecular beacons · proteins

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