Synthetic DNA Aptamers to Detect Protein Molecular Variants in a High-Throughput Fluorescence Quenching Assay

Xiaohong Fang,[a] Arup Sen,[b] Marie Vicens,[a] and Weihong Tan*[a]

Real-time protein detection in homogeneous solutions is necessary in many biotechnology and biomedical studies. The recent development of molecular aptamers, combined with fluorescence techniques, may provide an easy and efficient approach to protein elucidation. This report describes the development of a fluorescence-based assay with synthetic DNA aptamers that can detect and distinguish molecular variants of proteins in biological samples in a high-throughput process. We used an aptamer with high affinity for the B chain of platelet-derived growth factor (PDGF), labeled it with a fluorophore and a quencher at the two termini, and measured fluorescence quenching by PDGF. The specific quenching can be used to detect PDGF at picomolar concentrations even in the presence of serum and other cell-derived proteins in cell culture media. This is the first successful application of a synthetic aptamer for the detection of tumor-related proteins directly from the tumor cells. We also show that three highly related molecular variants of PDGF (AA, AB, and BB dimers) can be distinguished from one another in this single-step assay, which can be readily adapted to a microtiter plate assay for high-throughput analysis. The use of fluorescence quenching as a measure of binding between the DNA probe and the target protein eliminates potential false signals that may arise in traditional fluorescence enhancement assays as a result of degradation of the DNA aptamer by contaminating nucleases in biological specimens. This assay is applicable to proteins that are not naturally DNA binding. The excellent specificity, ultrahigh sensitivity, and simplicity of this one-step assay addresses a growing need for high-throughput methods that detect changes in the expression of gene products and their variants in cell cultures and biological specimens.

KEYWORDS:

aptamers · fluorescence · molecular beacon · proteins · real-time assay

Introduction

Proteins play very important roles in almost all functions of life. Assays for specific and sensitive detection of proteins and their molecular variants are necessary in many biotechnology applications and biomedical studies. Monoclonal antibody-based competition immunoassays and specific bioassays have been used during the past decades for these analyses. There have been recent advances in the use of DNA aptamers for the detection and analysis of proteins.[1–9] Translation of these observations into practical, high-throughput assays to analyze biological specimens will greatly facilitate molecular biology studies on cells, drug discovery research, and disease diagnosis. In this report, we describe a single-step assay for the detection of protein variants by using high-affinity synthetic DNA aptamers. There are several key features of our approach and these are described in the sections below.

A single-step homogeneous solution assay for high-throughput analyses

Most immunological methods and many nucleic-acid-based methods involve multiple steps to achieve amplification of the specific signal produced by a target protein. By attaching a fluorophore and a quencher to an aptamer with high affinity for a target protein, we have combined the high degree of sensitivity afforded by fluorescent signals (down to single fluorophores)[9] with the specificity of binding of the DNA to the target protein in its natural conformation in a homogeneous solution. The result is a single-step assay that is suitable for high-

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throughput analyses and can be performed at different molar ratios of the probe and the target for optimal results. The specificity of the assay can be readily confirmed by the simple addition of related proteins or DNA sequences without the need for additional steps.

Fluorescence quenching reduces false signals in biological samples

Fluorescence resonance energy transfer (FRET) has been an effective tool for many biomedical mechanism studies and biomedical diagnosis. One of the most recent successful examples is a DNA probe molecular beacon.[8] The use of fluorophores and quenchers to construct nucleic-acid-based molecular beacons has to date been applied in measuring fluorescence enhancement upon target binding.[8] Although this approach provides sensitivity, nucleases that are frequently present in biological specimens can result in nonspecific enhancement by cleaving the DNA probe. Fluorescence quenching was used in our probe design in the development of an aptamer probe for platelet-derived growth factor (PDGF) protein studies. The decrease in fluorescence signal can only be caused by specific structural changes that result from the interaction of the DNA molecule with protein molecules and bring the fluorophore and the quencher into close proximity. This is especially important for target monitoring where a biological specimen is concerned.

Ease of multiplexing

The assay relies on specific binding between a synthetic DNA molecule and the protein target used to detect the ability of the DNA molecule to bind and undergo conformational changes resulting in close proximity of the two termini of the DNA molecule. Distinct DNA molecules with desirable specificities for chosen target proteins can be synthesized and coupled to different fluorophore – quencher combinations to allow simultaneous detection of multiple target proteins in the same solution at different excitation/emission wavelengths. In addition, the multiple-well microtiter plates can be used for many DNA probes in multiple protein monitoring. It is thus possible to use either spectral differences or spatial location of the wells to differentiate multiple target analytes without extensive instrumentation or difficulties.

PDGF is an important biological marker with several natural molecular variants

PDGF is a dimeric protein for which several natural molecular variants are known, and at least two natural variants of cell-membrane receptors have been described with different specificity for the PDGF variants.[10] The expression of variants of PDGF and the PDGF receptors have been implicated in malignancy and developmental abnormalities.[11, 12] Our fluorescence quenching assay is able to distinguish between various molar ratios of AA and BB dimeric forms of PDGF in solution in a 96-well microtiter plate assay, which makes possible high-throughput and ultrasensitive determinations of protein variants in real biological samples by a single synthetic DNA probe with a single-step homogeneous assay.

Dual labeling of a synthetic nucleic acid molecule with a fluorophore and a quencher has been used to create molecular beacon (MB) DNA probes as sensitive molecular tools to detect specific nucleic acid sequences[8–9] and study protein – DNA interactions.[6–7] The signal transduction mechanism for MBs is based on fluorescence resonance energy transfer. The MB acts like a switch that is normally closed, which brings the fluorophore/quencher pair together to turn fluorescence "off". Upon binding to the target molecule, the MB undergoes a conformational change that opens the stem structure and separates the fluorophore and the quencher, thus turning the fluorescence "on". MBs have been designed for various molecular biology applications, such as to detect single nucleotide polymorphisms and real-time PCR processes, and for the detection of mRNA in living cells.[8–9] Using a principle similar to that used in the creation of MBs for nucleic acid sequences, a few recent reports describe the use of FRET that presumably results from conformational changes in dually labeled DNA molecules upon binding to selected proteins.[6–8] The results indicated that less than nanomolar concentrations of specific proteins produce measurable changes in fluorescence signals upon binding to specific dually labeled synthetic DNAs.

Green et al.[13] described the selection of a series of DNA aptamer molecules from a synthetic library that display high binding affinity for PDGF BB homodimers, and also showed by using a binding assay that the affinities of these DNA molecules for the three different homodimeric forms of PDGF are distinguishable. The aptamer shown in Figure 1 was used to construct a molecular beacon aptamer (MBA) for PDGF by labeling it with a fluorophore and a quencher at opposite termini. When the MBA is in a physiological buffer, the fluorophore and the quencher are far apart and a fluorescence signal is generated. The efficiency of FRET is dependent on the

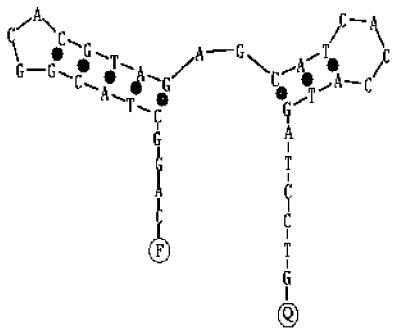

Figure 1. Structure of the PDGF aptamer: the stable conformation of the aptamer under physiological conditions in the absence of PDGF. Upon PDGF protein binding, the aptamer forms a close-packed tight structure, which reduces the distance between the two termini of the aptamer and causes fluorescence quenching. Different molecular variants of PDGF dimers will bind with the aptamer with differences not only in binding affinity, but also in binding avidity. The avidity can be easily determined in the signaling step based on FRET between the fluorophore (F) and the quencher (Q).

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distance between the fluorophore and the quencher. We hypothesized that binding of PDGF to the dually labeled aptamer would reduce the distance between the two termini of the aptamer and cause fluorescence quenching. We also hypothesize that the differences in the affinity and the *avidity* of the aptamer for the different molecular variants of PDGF dimers (AA, AB, and BB) might be reflected in differences in the quenching of the MBA fluorescence as a function of the concentration of the PDGF variant in the test solution. The avidity can be easily determined from signaling step based on FRET between the fluorophore and the quencher. Therefore, a single MBA might be able to detect and distinguish molecular variants of a protein of interest in a simple single-step assay.

We used PDGF and a single DNA aptamer to build the fluorescence quenching assay method platform and show herein that conformational changes of a PDGF aptamer selected for specific high-affinity binding to the target protein can be used to design a high-throughput binding assay to not only specifically detect the target protein but also distinguish between its molecular variants. Fluorescent-signal-based detection offers high sensitivity (down to single molecules[9]), while the specific high-affinity binding by molecular aptamers forms the basis of a system to detect subtle molecular changes in the protein that affect the binding of molecular variants with the synthetic DNA.

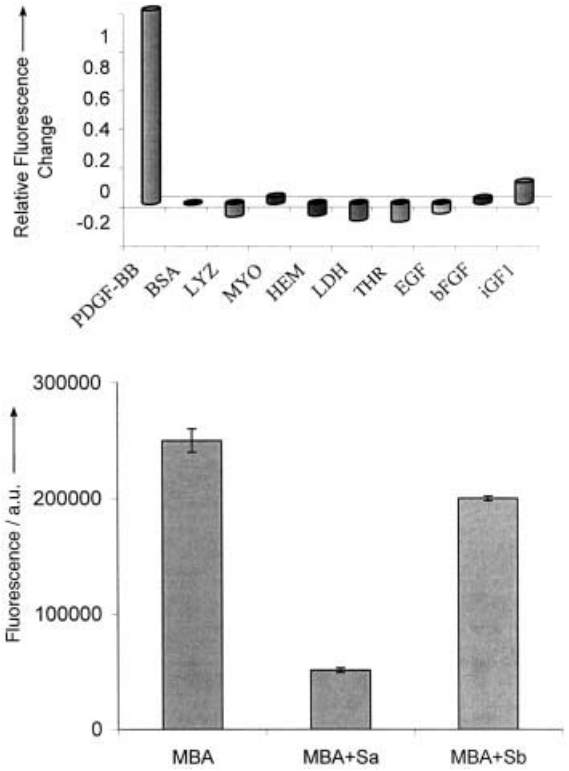

Figure 2. *Binding specificity of the MBA. Top: fluorescence signals of incubation mixtures containing MBA (20 nM) and one of the following proteins (100 nM): bovine serum albumin (BSA), hemoglobin (HEM), lactate dehydrogenase (LDH), lysozyme (LYZ), myoglobin (MYO), thrombin (THR), or one of the growth factors (20 nM), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (iGF1), or PDGF-BB. Bottom: fluorescence signals of incubation mixtures of MBA (50 nM) with calf serum proteins without (Sb) or supplemented with (Sa) PDGF-BB, as compared to the MBA alone.*

Results and Discussion

Fluorescence quenching for specific and sensitive detection of PDGF

We have created an MBA for PDGF from a 35-nucleotide-long DNA sequence (shown in Figure 1) based on aptamer sequences with a high affinity for PDGF-BB, with the labels DABCYL at the 3′- and fluorescein at the 5′-end. The aptamer is reported to have about 700-fold higher affinity for PDGF than that observed with other random DNA sequences.[13] We first examined the specificity of the MBA. A fixed amount of the MBA was incubated with either PDGF-BB or one of several extracellular proteins (serum albumin, hemoglobin, lactate dehydrogenase, lysozyme, myoglobin, and thrombin) or unrelated growth factors (such as epidermal growth factor and insulin-like growth factor-1). The results shown in Figure 2 A indicate that only pure human PDGF-BB causes a marked reduction in fluorescence signal while all the other proteins tested fail to cause any significant change in fluorescence, even at 10-fold higher concentrations than that used for PDGF-BB. The binding reaction is quick; it takes less than 20 seconds (including the mixing time) to reach equilibrium. We achieved a detection limit of 110 pM PDGF without optimization (data not shown). To further test the specificity of binding between PDGF-BB and the MBA, we used an unlabeled scrambled sequence designed to possess a predicted helix structure essentially identical to that of the DNA sequence of the MBA.[13] In a competition binding assay, increasing amounts of the scrambled sequence was added to a mixture containing 50 nM MBA and 50 nM PDGF-BB. No measurable reduction in quenching was observed even when 250 nmol scrambled sequence was added. However, in a parallel experiment, a synthetic DNA sequence identical to the one used in the MBA effectively competed with the MBA and fluorescence quenching was inhibited in a dose-dependent manner.

Fluorescense quenching allows specific detection of PDGF in biological samples

We examined the specificity of the MBA and evaluated the potential application of this one-step fluorescence-quenching assay for PDGF-BB detection in biological samples. We used a simulated biological specimen made by adding PDGF-BB to Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.5 % fetal bovine serum (FBS). The sample was lyophilized and split in two equal portions, one of which (Sa) was additionally supplemented with 100 nmol recombinant human PDGF-BB. Each portion (Sa and Sb) was resuspended and the protein components were collected through Sephedax G-10 gel filtration. Fluorescence quenching activity of the two preparations was compared by adding 50 nM MBA. As shown in Figure 2 bottom, Sa caused a marked reduction in fluorescence, comparable to that observed with pure human PDGF-BB, but Sb did not cause a significant change. Analytical denaturing and reducing polyacrylamide gel electrophoresis of Sa and Sb showed that the protein compositions of these two samples

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were indistinguishable from each other. The results show that this rapid one-step fluorescence-quenching assay can specifically detect nanomolar quantities of PDGF in complex biological specimens, which is the basis for the application of this method in real tumor sample PDGF detection.

Fluorescence quenching assay detects PDGF in human tumor cell culture media

We next tested the application of this fluorescence quenching assay to detect the expression of human PDGF-BB in cultured tumor cells. A human breast carcinoma cell line, HTB-26, has been reported to secrete PDGF-BB in culture medium.[12] Serum-free conditioned media were collected from human HTB cells and normal murine BALB/3T3 fibroblast cells in culture. Serial dilutions of protein preparations from each cell line were incubated with a fixed amount of the MBA in a 96-well microtiter plate to obtain a dose – response curve. Parallel sets of dose – response curves were obtained by using different amounts of the MBA for each such set. For each set, a control dose – response curve for fluorescence quenching was obtained with serial dilutions of a solution of pure human PDGF-BB. The results obtained with 50 nmol MBA are presented in Figure 3. The assay

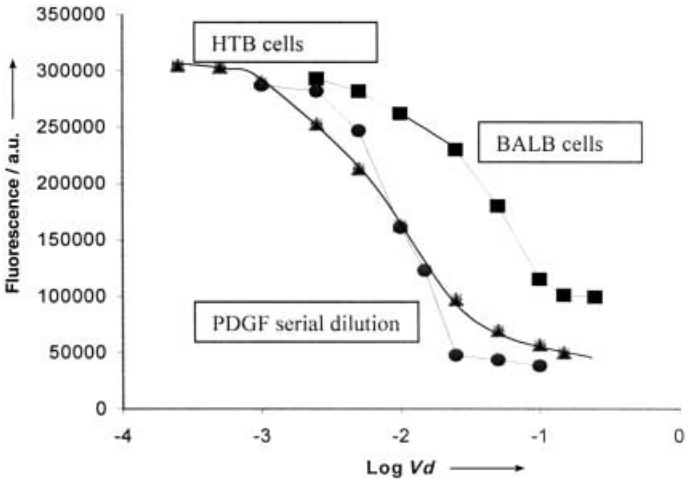

Figure 3. Cancer cell sample dose – response curve: fluorescence signals of mixtures of MBA (10 nM) with serial dilutions of protein preparations of conditioned cell media from HTB cells (triangles) or BALB cells (squares). Serial dilutions of a 500 nM solution of PDGF-BB (circles) were used as a control. Vd *is the dilution factor.*

indeed detects the presence of human PDGF-BB in HTB-conditioned media, as indicated by the similarity of the slope and final extent of quenching to that obtained with pure PDGF-BB. In contrast, the BALB/3T3-conditioned media appears to contain one or more related protein(s), as indicated by the different slope and less than maximal quenching. This experiment is the first successful application of a synthetic aptamer for the detection of tumor-related proteins directly from the tumor cells. In view of the high specificity of the MBA to PDGF-BB,[13] the difference between the dose curves of the HTB and BALB cell culture media samples and that of the pure PDBF-BB indicated that, when used for detection in a biological sample, the MBA probe also has the capability to respond differently to isoforms of PDGF-BB or other possible structure/function-related proteins or molecules. These results demonstrate a new application of labeling aptamers for protein variant detection in a simple assay with a single DNA probe.

Fluorescence quenching assay distinguishes molecular variants of PDGF

Green et al. reported that the PDGF aptamers selected for binding to PDGF-BB by using isotropic labeling bound to all three molecular variants of PDGF, namely BB, AB, and AA, albeit with different affinities,[13] presumably because of the amino acid sequence homology (60 %) between the A and the B chains. We tested the three common PDGF variants for their effects on the fluorescence quenching assay. Serial dilutions of each protein were incubated with 10 nM MBA in triplicate in 96-well microtiter plates and the dose – response curves for fluorescence quenching were compared. Parallel sets of experiments were conducted with varying amounts of the MBA. The dose – response curves obtained with 10 nM MBA are shown in Figure 4. The results

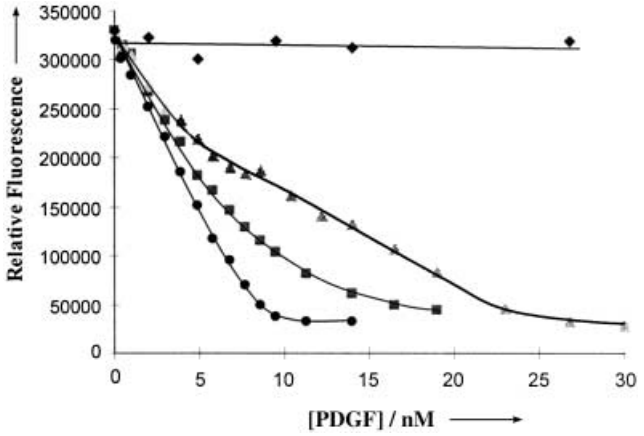

Figure 4. Dose – response curves of PDGF variants: fluorescence signals of MBA for PDGF-AA (triangles), PDGF-AB (squares), PDGF-BB (circles), and denatured PDGF-BB (diamonds). The concentration of the MBA was 10 nM.

show that the slopes and the concentration of protein required to attain 50 % of the maximum quenching are distinct for the three molecular variants of PDGF. Although the response for PDGF-AB is more similar to that for PDGF-BB than to that for PDGF-AA, the difference between PDGF-AB and PDGF-BB is much more easily distinguishable than that reported by the isotropic method.[13] When PDGF-BB is reduced by dithiothreitol and denatured with sodium dodecyl sulfate, the resulting protein fails to cause any fluorescence quenching. These results indicate that the fluorescence quenching assay is not merely dependent on the primary sequence of the protein chains, but is capable of distinguishing conformational characteristics or the folding of highly related protein molecules. Therefore, it may be possible to use synthetic DNA molecules selected for high-affinity binding to proteins in the design of rapid, high-

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throughput, and sensitive single-step homogeneous assays for proteins and their functional variants in complex biological materials.

High-throughput assay for protein molecular variants

Based on the above observations, we evaluated the potential of adapting this fluorescence quenching assay to a high-throughput format. We prepared PDGF samples that contained varying molar ratios of PDGF-AA and PDGF-BB and determined the extent of fluorescence quenching with three dilutions of each mixture with a fixed amount of the MBA in a 96-well microtiter plate. Since the Spectrafluor Plus instrument uses filters for excitation (485 nm with a bandwidth of 20 nm) and emission (535 nm with a bandwidth of 25 nm), the sensitivity of the assay is considerably lower than the readings obtained with our Fluorotag Tau-3 spectrofluorometer, which uses monochromatic excitation (470 nm) and emission (520 nm) wavelengths. The results obtained with 75 nM MBA are shown in Figure 5. The data

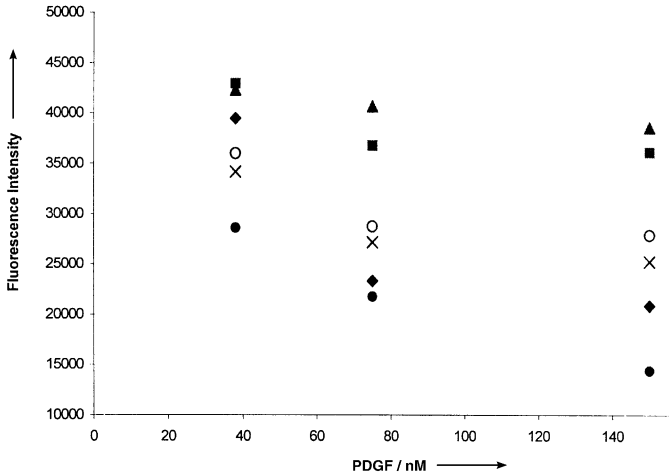

Figure 5. Dose–response curves for a mixture of PDGF-AA (AA) and PDGF-BB (BB) with MBA (75 nM). 100% AA (■), 90% AA/10% BB (▲), 50% AA/50% BB (◆), 25% AA/75% BB (○), 10% AA/90% BB (×), and 100% BB (●).

show that at each of three concentrations of the PDGF preparations, which ranged from a 0.5:1 to 2:1 molar ratio of proteins to MBA, the extent of fluorescence quenching was directly related to the relative abundance of PDGF-BB. Experiments done with 40 nM and 100 nM concentrations of MBA and an MBA created by labeling the same aptamer with another fluorophore/quencher pair (black hole quencher and fluorescein) yielded similar results (data not shown).

Although the specific structural features of protein molecules that affect fluorescence quenching of the MBA are not known at this time, it may be possible to analyze the dose–response curves obtained with mixtures of PDGF variants and to develop an algorithm that enables the simultaneous quantitation of the variants in a mixture. The fluorescence quenching assay can be modified to fluorescence enhancement by using two fluorophores to label the two termini of the synthetic DNA. As has been demonstrated with MBs in nucleic acid detection,[14] FRET resulting from the conformational changes in an MBA with two fluorophores enables ratiometric measurements and provides enhanced sensitivity and larger dynamic range. The rapidly growing options for fluorophores and quenchers will enable the use of multiple MBAs in a single assay to monitor multiple gene products and their variants. Backbone modifications and unusual base substitutions in the synthetic DNA may be used to improve the stability[5] of the MBAs in biological fluids and to alter the affinity of a DNA molecule for its protein target. Although we have only tested the assay system in 96-well microtiter plates, it should be possible to adapt the assay to various higher-throughput formats or fluorescence readers.

Conclusions

We have developed a single-step assay that can distinguish molecular variants of proteins in biological samples by using a molecular beacon aptamer. A fluorescence assay has been designed to use the aptamer for ultrasensitive analysis of PDGF protein in biological samples. The aptamer can be used to detect PDGF at picomolar concentrations, even in the presence of serum and other cell-derived proteins in cell culture media. This aptamer probe has also been used with tumor cells in PDGF determination. This is the first successful application of a synthetic aptamer for the detection of tumor-related proteins directly from the tumor cells. We also show that three highly related molecular variants of PDGF (AA, AB, and BB dimers) can be distinguished from one another in a single-step assay that can be readily adapted to a microtiter plate assay for high-throughput analysis. The use of fluorescence quenching as a measure of binding between the aptamer probe and the target protein eliminates the potential false signals that may arise in traditional fluorescence enhancement assays as a result of degradation of the aptamer by contaminating nucleases in biological specimens. The method of taking advantage of the conformational change of the dual-labeled aptamer itself to signal protein binding affinity and avidity may be applied to essentially all proteins, especially those that are not naturally DNA binding.

Experimental Section

Reagents and Materials: The PDGF aptamer and the control scrambled sequence were synthesized by standard phosphoramidite chemistry on controlled pore glass matrix.[6] Labeling at the 3′ end used the modifying moiety coupled to the controlled pore glass matrix as the first residue. For 5′-labeling, the modifying moiety was coupled by using its phosphoramidite derivative. The synthetic oligonucleotides were released from the controlled pore glass by mild deprotonation and then purified by reverse-phase high-

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performance liquid chromatography. The sequences used are as follows:

PDGF aptamer:5′-CAG GCT ACG GCA CGT AGA GCA TCA CCA TGATCC TG-3′

Controls: 5′-CAG CGT ACG GCA CGT ACC GAT TCA CCA TGA AGC TG – 3′

Recombinant human PDGF-BB, PDGF-AB, and PDGF-AA were purchased from R&D#38;D Systems (Minneapolis, MN). The proteins were dissolved in HCl (4 mM) and then diluted in tris(hydroxymethyl)aminomethane (Tris) buffer (40 mM, pH 7.4) at the final experimental concentrations before use. Other recombinant human growth factors, epidermal growth factor (EGF), insulin-like growth factor-I (IGF1), were bought from Roche (Indianapolis, IN). Human bovine serum albumin (BSA), human hemoglobin (HEM), porcine lactic dehydrogenase (LDH), horse myoglobin (MYO), chicken lysozyme (LYS), and human gamma-thrombin (THR), and other chemicals were purchased from Sigma (St. Louis, MO). Physiological buffer, which simulated the ionic strength under physiological conditions, was used in the experiments (20 mM Tris-HCl (pH 7.1), 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$.

Cell culture and preparation of conditioned media: The cell lines used in these studies, human breast carcinoma HTB-26, and murine BALB/c-3T3 fibroblasts (American Type Culture Collection) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Cellgro) supplemented with 1% Gentamicin (Sigma) and either 10% fetal calf serum (Gibco) for HTB-26 or 10% calf serum (Gibco) for BALB/3T3. The cells were incubated in humidified air containing 5% CO_2 at 37 °C. For the collection of serum-free conditioned media (CM), the culture medium was replaced with serum-free DMEM when the cell monolayers reached 80–90% confluence. After approximately 60 minutes, the medium was replaced with fresh serum-free DMEM and the cells were incubated for 24 h. The serum-free CM was collected and clarified by centrifugation. Acetic acid was added to the supernatant to a final concentration of 1 M and the material was lyophilized. The lyophilized powder was resuspended in acetic acid (1 M), clarified by centrifugation at high speed in a microfuge for 5 minutes, and the supernatant containing the soluble proteins was subjected to Sephadex G-25 gel filtration in acetic acid (1 M). The excluded volume fractions with the highest absorbance at 280 nM were collected and lyophilized again. The protein preparation was resuspended in physiological buffer and used as the stock solution from which dilutions were made for incubation in the fluorescence assay.

Fluorescence assay development: Initial fluorescence measurements were performed on a Fluorolog-Tau-3 spectrofluorometer (Jobin Yvon Inc, NY) equipped with a thermostat accurate to 0.1 °C. All experiments were carried out at 37 °C. The sample cell was a 100-μL cuvette. The fluorescence intensity of incubation mixtures was monitored by exciting the fluorophore (6-amino fluorescein) at 470 nm and measuring the emission at 520 nm. The relative standard deviation is less than 2% for all measurements. In order to adapt the assay method to a high-throughput system, a Tecan Spectrafluor Plus (Tecan Instruments, North Carolina) was used with 96-well flat-bottom microtiter plates (Nalge Nunc International). All incubations were carried out in a final volume of 100 μL and fluorescence was measured by using filters for excitation (485 nm with 20 nm bandwidth) and emission (535 nm with 25 nm bandwidth) provided by the manufacturer.

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