atom. The $[Ti_2Cl_9]$ units are present about 90% of the time, so that the net stoichiometry is $Tl_{4.87(16)}Ti_{7.81(3)}Nb_{18}Cl_{51.51(16)}O_{12.40(4)}$. The highest residual electron-density peak (1.724 e Å^{-3}) is located between the two titanium atoms that form the dimers. Anisotropic refinement of all atoms except for O7 converged to $R_1 = 0.064$, $wR_2 = 0.102$. Further details of the crystal structure investigation may be obtained from the Fachinformationszentrum Karlsruhe, 76344 Eggenstein-Leopoldshafen, Germany (fax: (+49) 7247-808-666; e-mail: crysdata@fiz-karlsruhe.de) on quoting the depository number CSD-410776.

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Molecular Beacons: A Novel Approach to Detect Protein – DNA Interactions**

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Protein – DNA interactions are known to play an important role in a wide variety of biological processes. Binding of proteins to genomic DNA is involved in various biological processes including DNA replication, transcription, recombination, and repair.[1] To study these protein – DNA binding events, a variety of techniques such as band shift and footprinting assays, affinity chromatography, fluorescence analysis, and microscopy visualization have been developed.[2–5] While all of these techniques provide insight into one specific aspect of protein – DNA binding, optical and fluorescence methods offer significant advantages in analytical sensitivity and simplicity. Here we describe a new approach to study protein – DNA binding and to quantify proteins using a recently developed oligonucleotide probe that serves as a molecular beacon (MB).[6–11]

The molecular beacon is designed in such a way that it can report the presence of a specific complementary nucleic acid in homogeneous solutions.[6] The molecular beacon is labeled with a fluorophore at one end and a nonfluorescent quencher at the opposite end. In solution, the molecular beacon forms a hairpin loop which brings the fluorophore and quencher close enough to allow quenching to occur by fluorescence resonance energy transfer. Upon hybridization to a complementary sequence, the hairpin loop is broken and the fluorophore and quencher are separated, resulting in the restoration of fluorescence. The molecular beacon approach has great advantages over other DNA probes, including the ability to detect single-base mismatches, the capability of studying biological processes in real time, and the possibility for in vivo analysis. There have been several applications of molecular beacons, such as sensitive monitoring of the polymerase chain reaction,[7] real-time detection of DNA – RNA hybridization in living cells,[8] DNA mutation analysis,[9] and even detection of pathogenic retroviruses.[10] Recently, a biotinylated single-stranded DNA (ssDNA) molecular beacon was designed and used for DNA-hybridization studies at a liquid – solid interface and for the development of ultrasensitive DNA sensors.[11] Molecular beacons hold great promise in studies in genetics and disease mechanisms as well as in disease diagnostics. So far, however, all applications have been based on the hybridization between a molecular beacon and DNA or RNA molecules. The use of a molecular beacon for studying protein – DNA interactions could be of great benefit for understanding the many important biological processes that require ultrasensitive and specific protein detection. Herein, we report for the first time the interactions between proteins and a molecular beacon.

The molecular beacon synthesized for this study is shown in Figure 1. It was designed with tetramethylrhodamine (TAMRA; Molecular Probes, Eugene, OR) as the fluorophore and 4-(4′-dimethylaminophenylazo)benzoic acid (DABCYL; Molecular Probes) as the quencher. The molecular beacon was synthesized by using DABCYL-derivatized controlled pore glass (CPG) as the starting material. The synthesis was started from the 3′ end. The nucleotides were added sequentially by using standard cyanoethylphosphoramidite chemistry. The 5′ end nucleotide is conjugated to a $(CH_2)_6$-NH linker arm, producing a primary amine group at the 5′-end. This amino group was used to attach tetramethylrhodamine. The product was purified by gel filtration chromatography and reverse-phase high-pressure liquid chromatography (RP-HPLC). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was used to confirm the synthesis of the designed molecular beacon (data not shown). In the absence

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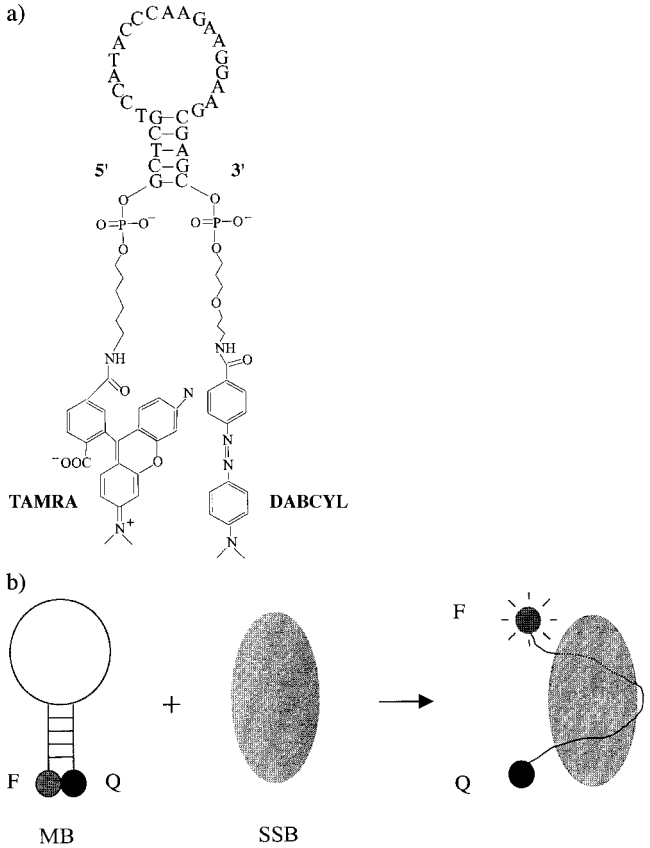

Figure 1. a) The structure of the molecular beacon used in this study. The MB was designed as a 19-mer loop sequence flanked on the 5′- and 3′- ends with 5-mer long complementary sequences. The oligonucleotide has a stem-and-loop structure, stabilized by a five base-pair duplex formed by intramolecular hybridization of the complementary ends. The fluorophore (TAMRA) and quencher (DABCYL) are attached to the 3′- and 5′- ends of the oligonucleotide by $(CH_2)_6$-NH and $(CH_2)_3$-O-$(CH_2)_3$-NH linker arms, respectively. b) Schematic representation of the light-generating mechanism for the molecular beacon (MB) that occurs upon binding to proteins (SSB). See text for details. F = fluorophore; Q = quencher; the ellipsoid represents a protein molecule.

of the target sequence, the molecular beacon adopts a hairpin structure, including a stem of five base pairs and a loop of 19 bases. The stem keeps the fluorophore (TAMRA) and quencher (DABCYL) in close proximity (Figure 1 a), causing the fluorophore to be quenched by energy transfer. Any conformational reorganization that opens the stem should lead to the separation of the fluorophore and the quencher, and therefore to the restoration of fluorescence. The conformational state of a molecular beacon is thus directly reported by its fluorescence: In the closed state, the molecular beacon is not fluorescent; in the open state, when the fluorophore and the quencher molecules are apart, it emits intense fluorescence. It has been reported that fluorescence from molecular beacons can be restored by different approaches: hybridization to their complementary DNA (cDNA) and denaturation by either heat or high pH.[6–10] In addition, it is known that the binding of proteins to DNA or RNA molecules can disturb and rearrange their conformation.[12] Therefore we expect that the interactions between protein and MB may result in the spatial separation of the quencher and the fluorophore part of the molecular beacon, which would restore the MB fluorescence (Figure 1 b).

To test this principle, a well-characterized *E. coli* protein, single-stranded DNA binding protein (SSB; Promega, Madison, WI), was chosen to study the interactions between proteins and molecular beacons. SSB consists of four identical 18.9 kDa subunits, binds with high affinity to ssDNA, and plays an essential role as an accessory protein in DNA replication, recombination, and repair.[13] As controls, histone (HIS, type II-AS; Sigma, St. Louis, MO), a protein which mainly binds double-stranded DNA (dsDNA), and bovine serum albumin (BSA, non-acetylated; Sigma), a protein which does not bind DNA, were tested for their effect on the molecular beacon. Perfect and mismatched complementary sequences were used for hybridization experiments with the MB in order to compare the effect of DNA hybridization and protein binding on the fluorescence restoration of the molecular beacon. The titration of the molecular beacon with proteins and complementary DNAs was carried out on a spectrophotometer (F-112A, SPEX Industries). Samples were excited at 558 nm and detected at 580 nm. The fluorescence was measured at room temperature (25 °C) after mixing the samples with a pipette for about five seconds in a 100 μL cuvette cell. The buffer (10 mM Mg^{2+}, 50 mM NaCl, 20 mM Tris-HCl, pH 7.8) was used for both protein binding and hybridization experiments.

We observed that in solution the interaction between the molecular beacon and SSB is rapid and tight. Fluorescence detection was used to probe the interaction between the protein and the molecular beacon. SSB was added to the MB solution, and the intensity of the fluorescence of the mixture was monitored. The time scans showed that SSB quickly interacts with MB, resulting in significant fluorescence enhancement (Figure 2). A control experiment with free

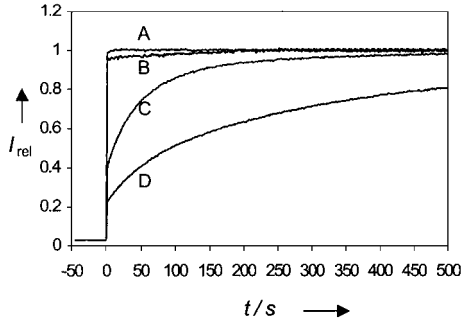

Figure 2. Relative fluorescence intensity time scans of the binding of the MB to SSB and cDNA, respectively, at two different molar ratios: A, SSB:MB = 4:1; B, SSB:MB = 1:1; C, cDNA/MB = 4:1; D, cDNA:MB = 1:1. Before $t = 0$ s, the fluorescence of the MB solution was detected; from $t = 0$ s on, the fluorescence of the mixture of the MB with the binding reagent (either SSB or cDNA) was monitored. The cDNA sequence is 5′-CTT CCT TCT TGG GTA TGG A -3′.

TAMRA in the same buffer solution revealed that the fluorescence intensity was not influenced on addition of SSB (data not shown). This demonstrates that the enhancement in fluorescence intensity is indeed due to the conformational change of the molecular beacon: The binding of SSB to the MB causes the separation between the fluorophore and

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the quencher, and consequently leads to the restoration of MB fluorescence (Figure 1b). The reaction, as monitored by the changes in fluorescence intensity, reaches a stable plateau within ten seconds. This indicates that the binding reaction is fast and that the equilibrium can be reached quickly. We have also performed similar time scan experiments by using a variety of concentrations of complementary DNA under optimal binding conditions. These analyses reveal that the interaction between the SSB and the MB occurs much faster than that between the cDNA and the molecular beacon. Also, it is worth noting that there is no obvious change in the binding rate when the molar ratio of SSB to MB decreases from 4:1 to 1:1, while the same change in molar ratio for cDNA to MB results in a significant binding rate difference. This rapid protein binding reaction could provide the basis for a quick protein assay.

We titrated MB with SSB solutions, and the resulting fluorescence titration curve is shown in Figure 3. At low SSB concentrations, there is a linear relationship between the fluorescence intensity of the solution and the concentration of

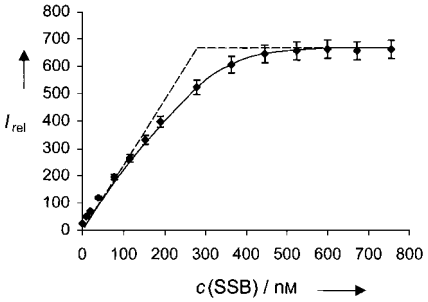

Figure 3. Continuous fluorescence titration of the molecular beacon with SSB. Each fluorescence value (◆) at a certain SSB concentration is the average of ten readings of the fluorescence after the binding has reached equilibrium (corresponding to the plateau part of the time-scan curve shown in Figure 2). The dilution effect has been considered in data processing. The concentration of the MB was 250 nM. The stoichiometric ratio of MB to SSB was determined by the intersection point of two straight lines (——) extended, respectively, from the initial linear part and the plateau part of the titration curve (– – –). The results indicate that one SSB molecule binds to one MB molecule.

SSB. When the SSB concentration exceeds 40 $\mu g\,mL^{-1}$, the intensity reaches a plateau. The stoichiometry for the binding of SSB to MB can be estimated based on the saturation concentration data shown in Figure 3. By extending the linear and the plateau parts of the curve, an intersection point is obtained. There is no additional fluorescence enhancement when more SSB is added beyond this point. Therefore, this point should give the stoichiometry of the binding reaction, which is estimated to be 1:1 (MB:SSB tetramer). Based on sedimentation experiments, one SSB tetramer can bind either four $d(pT)_8$ or two $d(pT)_{16}$ deoxyoligonucleotides, or one $d(pT)_{30-40}$ oligonucleotide.[14] Among all polynucleotides, SSB has the highest affinity to poly(dT).[14–16] It is thus not surprising that one SSB can only bind one molecule of the MB which has 29 mixed bases. We have determined the binding constant K_b of SSB–MB based on the stoichiometry information and the data presented in Figure 3. Under our experimental conditions, K_b is determined to be $2.0 \times 10^7\,M^{-1}$. This binding constant is within the range of previously reported results ($6 \times 10^5 - 5 \times 10^8\,M^{-1}$).[13, 14] That the binding constant of the molecular beacon fits well in this range suggests that the stem structure as well as the fluorophore and the quencher at the two ends of the molecular beacon do not significantly influence the binding affinity between SSB and MB. This clearly demonstrates that the MB described here is a suitable oligonucleotide probe for the study of protein–DNA interactions.

Our results further show that the MB can be used to detect SSB sensitively under optimal conditions. The concentration detection limit for SSB under our experimental conditions is calculated to be $2 \times 10^{-10}\,M$. It is worth noting that all the experiments described herein were carried out with a spectrometer equipped with a mercury lamp. It is known that the use of a laser usually results in an enhancement in detection limit of three orders of magnitude.[17] We thus expect that for protein analysis a sufficiently low detection limit will be achieved with better optical detection and laser excitation systems.

Our results also indicate significant differences in enhancement of fluorescence intensity of the molecular beacon by three different proteins and two DNA molecules. Figure 4 shows that the cDNA and SSB cause a similar enhancement in fluorescence intensities upon interaction with the molecular

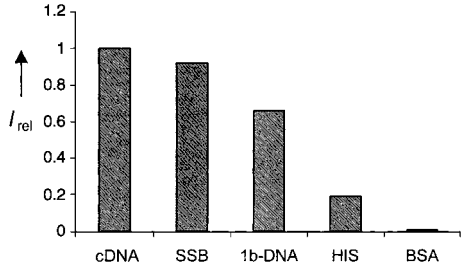

Figure 4. Relative fluorescence enhancement occurring upon binding of the molecular beacon to various DNA and protein molecules. The concentrations used were: cDNA, 1 μM; SSB, 40 $\mu g\,mL^{-1}$; 1b-DNA, 4 μM; HIS, 200 $\mu g\,mL^{-1}$; BSA, 400 $\mu g\,mL^{-1}$; MB, 250 nM. The concentrations of the proteins and DNA molecules are high enough to saturate the molecular beacon in all binding studies. The perfect cDNA sequence is shown in Figure 2. The one base-pair mismatch complementary sequence is: 5′-CTTCCTTCTTGGGTATGGA-3′ (the underlined base represents the mismatch).

beacon. This indicates that the conformational change induced by SSB binding is comparable to that observed when the cDNA hybridizes to the MB. The enhancement brought about by the SSB is higher than that caused by binding of a cDNA molecule containing one base mismatch (1b-DNA). The reason that the fluorescence enhancement by SSB is slightly lower than that caused by interaction with cDNA might lie in the conformational difference of the "stretched" MB molecule in the two types of complexes. The molecular beacon is straightened by the formation of a dsDNA structure on the loop when cDNA is used. As a result, the spatial separation between the fluorophore and the quencher is the largest, while that for the SSB–MB complex will be slightly smaller since the ssDNA may still coil around the SSB.[13] We also noticed that interaction of the molecular beacon with histone caused a much smaller fluorescence enhancement,

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whereas for BSA, no apparent enhancement was detected. This is because SSB specifically binds to ssDNA with high affinity, while histone mainly binds to dsDNA. There has been no report suggesting any significant DNA binding to BSA. By intelligent molecular beacon design, this difference in fluorescence enhancement, which can be converted into a difference in bioassay sensitivity, might serve as the basis for the use of molecular beacons in specific and sensitive protein bioanalysis.

Our results demonstrate that there is a direct relationship between the restoration of molecular beacon fluorescence intensity and the DNA binding capability of the protein. Even though nonspecific DNA binding proteins are used here, the fact that different proteins do have different binding efficiencies with the molecular beacon in this first study is very encouraging. The molecular beacon used in this report lacks sequence specificity with regard to binding to particular proteins. Thus the next step is to develop MBs which can recognize certain proteins with high selectivity. There have been many recent studies aimed at designing functionalized single-stranded DNA or RNA molecules (aptamers) for selective protein binding studies and for enzymatic applications.[18–20] Therefore, it is likely that aptamer-based molecular beacons can be developed for specific protein binding studies and for protein analysis with excellent selectivity.

In summary, we have opened a new way for studying protein–DNA interactions and for protein bioanalysis by using molecular beacons. The interaction between single-stranded DNA binding protein and molecular beacon results in significant restoration of the fluorescence of the molecular beacon. The fluorescence enhancement brought by SSB and by complementary DNA is very comparable. The molar ratio of the binding between SSB and MB is 1:1 with a SSB-MB binding constant of around $2.0 \times 10^7\,\text{M}^{-1}$. The detection limit of SSB is $2.0 \times 10^{-10}\,\text{M}$. Preliminary results also show that there are significant differences in MB binding affinity by different proteins, which will constitute the basis for highly selective bioassay of a variety of proteins. The new approach is potentially useful for the study of protein–DNA/RNA interactions because of its high selectivity, speed, and convenience. The result also opens the possibility of using easily obtainable and designer DNA molecules for genomics and proteomics and for new drug development.

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Novel Zinc Fluorescent Probes Excitable with Visible Light for Biological Applications**

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Zinc is an essential component of many enzymes and transcription factors (for example, Zn^{II} in carbonic anhydrase and zinc finger proteins).[1] Synaptic vesicles in excitatory nerve terminals contain high concentrations of chelatable Zn^{II},[2] which is released by neuronal activity[3] and may modulate the *N*-methyl-D-aspartate (NMDA) receptor.[4]

Thus, Zn^{II} plays an important role in various biological systems. However, little is known about the cellular regulation of Zn^{II} in comparison with that of Ca^{II}. Chemical tools for measuring Zn^{II} in living cells (like fura-2 for Ca^{II}) are required. *N*-(6-Methoxy-8-quinolyl)-*P*-toluenesulfonamide (TSQ) was the first fluorescent probe to be developed for Zn^{II}: Its fluorescent intensity is increased by Zn^{II}, but not by Ca^{II} or Mg^{II}.[5] TSQ is used for histochemical staining of Zn^{II} in tissue sections.[6] Zalewski et al. developed a water-soluble TSQ derivative, Zinquin, which can, as an ethyl ester, be used to stain Zn^{II} in living cells.[7] However, TSQ and derivatives are excited at UV wavelengths, which may cause cell damage, and is subject to interference by autofluorescence from pyridine nucleotides, among others.[8]

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