

Screening for the breast cancer gene (*BRCA1*) using a biochip system and molecular beacon probes immobilized on solid surfaces

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Abstract. We describe the use of a biochip based on complementary metal oxide semiconductor (CMOS) technology for detection of specific genetic sequences using molecular beacons (MB) immobilized on solid surfaces as probes. The applicability of this miniature detection system for screening for the *BRCA1* gene is evaluated using MB probes, designed especially for the *BRCA1* gene. MB probes are immobilized on a zeta-probe membrane by biotin-streptavidin immobilization. Two immobilization strategies are investigated to obtain optimal assay sensitivity. The MB is immobilized by manual spotting on zeta-probe membrane surfaces with the use of a custom-made stamping system. The detection of the *BRCA1* gene using an MB probe is successfully demonstrated and expands the use of the CMOS biochip for medical applications. © 2004 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.1691025]

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1 Introduction

The cancer susceptibility genes (*BRCA1* and *BRCA2*) are associated with a genetic predisposition to breast and ovarian cancer. Intense research has been conducted to identify their physiological mode of action and to detect them through biochemical assays.^{1,2} Women carrying a mutation (abnormality) in these genes are at an increased risk of breast or ovarian cancer. At least 10% of observed breast cancer cases in the general population are related to a genetic predisposition. Today breast cancer remains a worldwide public health concern and about 180,000 women are diagnosed with the disease yearly in the United States.³ The detection of *BRCA1* offers an opportunity to characterize the function of genetic features in breast and ovarian cancer, and to screen these cancer patients for the presence of germline mutations. Discovery of a mutation in patients can greatly affect the prediction of cancer risk and help doctors and patients take the appropriate steps for treatment.

Hybridization of a nucleic acid to its complementary target is one of the most well-studied and understood biomolecular recognition events. A molecular beacon (MB), a short oligonucleotide with a loop and stem structure, takes advantage of this recognition feature. The stem part contains 5 to 7 base pairs that are complementary to each other but unrelated to the target oligonucleotide. The loop section of an MB is complementary to its target oligonucleotide. Fluorescing and quenching chemical moieties are covalently attached to the end of each stem. Because the stem keeps these two moieties in close proximity, the fluorogenic probe is unable to fluoresce. This is due to fluorescence quenching that is caused by

the proximity between the quencher and fluorophore.⁴ When an MB probe is hybridized with its complementary target, the stem is forced apart, thus resulting in the restoration of fluorescence.

Since Tyagi and Kramer⁵ introduced MB probes in 1996, they have been used in numerous applications: quantitative polymerase chain reactions (PCR),^{6,7} detection of single-nucleotide polymorphism (SNP) and mutations,^{8,9} detection of pathogenic organisms, and detection of RNA in a single cell.^{7,10}

In most sensor applications, it is necessary to immobilize MBs on surfaces. When an MB is immobilized on a surface, there are at least two factors that must be carefully considered that can affect the fluorescence intensity: incomplete quenching caused by the proximity of the MB to the surface, and the difference in the ionic environment at the interface and in the solution. These effects may result in loss of sensitivity by decreasing the signal-to-noise ratio. However, by following a careful immobilization strategy and appropriate design of experimental conditions, these effects can be minimized.

Several researchers recently reported the immobilization of MBs on surfaces for sensor applications. Fang et al.¹¹ reported a simple procedure for immobilization of MB on a silica surface through a biotin-avidin link. Later Liu and Tan¹² reported a similar immobilization procedure on a fiber optic surface. Li et al.¹³ evaluated two different immobilization strategies. They immobilized MB on a glass surface through bovine serum albumin (BSA)-biotin-streptavidin-biotin and streptavidin-biotin links. They found that the first strategy produced a higher fluorescence signal, owing to the greater distance of the MB from the surface. In addition, Wang

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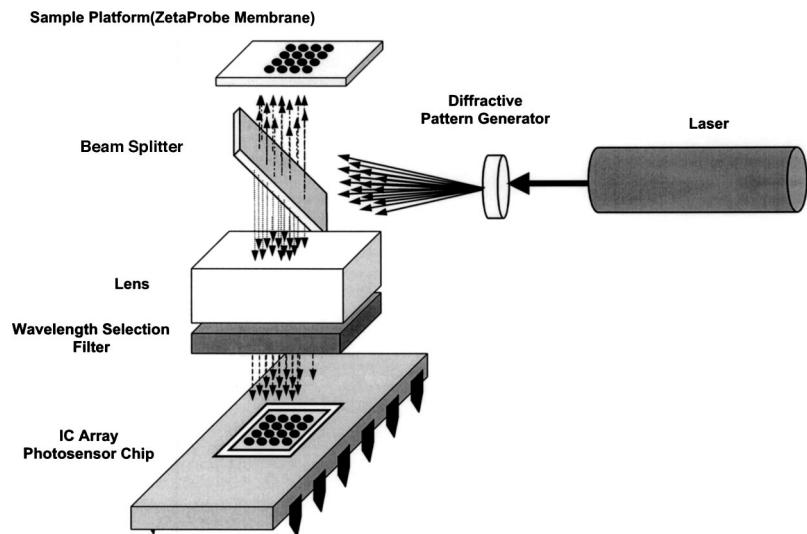


Fig. 1 Schematic diagram of the miniature biochip detection system.

et al.¹⁴ studied MBs immobilized on an agarose film-coated and glutaraldehyde-derivitized glass slide.

Earlier we developed an integrated circuit chip known as the multifunctional biochip (MFB), which has demonstrated great potential for a variety of applications. The MFB has a number of distinct advantages over other biosensing technologies.^{15–18} These include a fabrication process based on complementary metal oxide semiconductor (CMOS) technology and multianalyte detection. The CMOS fabrication process allows application-specific circuitry (i.e., signal amplification and filtering) to be integrated into the chip, thereby significantly reducing the size and power requirements of the system. Another important consideration is that the CMOS process is very cost-effective, which is ideal when mass-producing portable detection devices. Furthermore, the chip is composed of an array of individual detector elements, each of which could be devoted to the detection of a different analyte for multiplexed detection. Thus, in this work a 4×4 array of photosensors was used, which could be capable of performing sixteen simultaneous bioanalyses in a single, compact unit. The biochip has recently been used for the detection of biological pathogens.¹⁹

In this study we investigated the use of MB probes immobilized on a zeta-probe membrane substrate along with the miniaturized biochip detection system for detection of the *BRCA1* gene. The successful use of such a system will result in fast, cost-effective, and multiplex detection of diseases on one substrate in the physician's office. A variation of the biotin-streptavidin immobilization strategies reported by others was explored for our system. We have found that the spotting of an MB solution on a prepared glass surface is troublesome, owing to the spreading of the spotted solution. Thus, a zeta-probe membrane instead of a glass surface was used to immobilize the MBs. It has been found that the membrane helps to keep the solution localized in discrete spots.

2 Materials and Methods

2.1 Molecular Beacon and Target Gene

An MB probe consisting of (3'-DABCYL-GGA T (biotin dT) CG GTG TTG TTT CTC GTA TGT ATC CCC GAT CC-Cy5-

5') and its complementary single-stranded DNA target (*BRCA1*) (5'-AAA TTT GAT TTT TAA AAA AAA TCA CAG GTA ACC TTA ATG CAT TGT CTT AAC ACA ACA AAG AGC ATA CAT AGG GTT TCT CTT GGT TTC TTT GAT TAT AAT TCA TAC ATT TTT CTC TAA CTG CAA-3') (the sequence complementary to the MB sequence is underlined) were synthesized by Gene link Inc. (Hawthorne, New York) and used without further purification. In the MB, Cy5 was used as the fluorophore and (4-dimethylaminophenylazo)benzoyl (DABCYL) as the quencher.

2.2 Biochip Detection System

The integrated circuit (IC) biochip described here was designed and developed at Oak Ridge National Laboratory (ORNL), Oak Ridge, Tennessee. This detection system features an IC-based 4×4 array detector containing individual photodiode elements that operate independently. The individual photodiodes of the 4×4 array are square, with 900-μm edges, and each of them is arranged with 1-mm center-to-center spacing. They are integrated along with amplifiers, discriminators, and logic circuitry on a single platform.

Figure 1 is a drawing of the biochip detection system. The system consists of an excitation source, excitation and collection optics, and an IC photosensing array chip. A diode laser with 5 mW of output power and a 635-nm wavelength (model VHK 4.9 mW, Edmund Scientific, Barrington, New Jersey) was selected for excitation of the Cy5 labels. The laser beam was launched through a diffractive pattern generator, which produced a 4×4 array of laser beamlets of equal intensity. The intensity of a single laser spot was estimated as ~0.2 mW. A small piece of zeta-probe membrane was marked with a metal mold to produce a pattern with a size corresponding that of the 4×4 array of photosensors and the array of beamlets. The image of the laser spot array was projected from the membrane onto the corresponding 4×4 array of photosensors of the IC detector via a 1-in.-diameter, f/2 lens and an emission bandpass filter (HQ 700/75 nm, Chroma Technology Corp.). The output from the IC biochip was recorded as voltage by a digital multimeter (model 506, Protek).

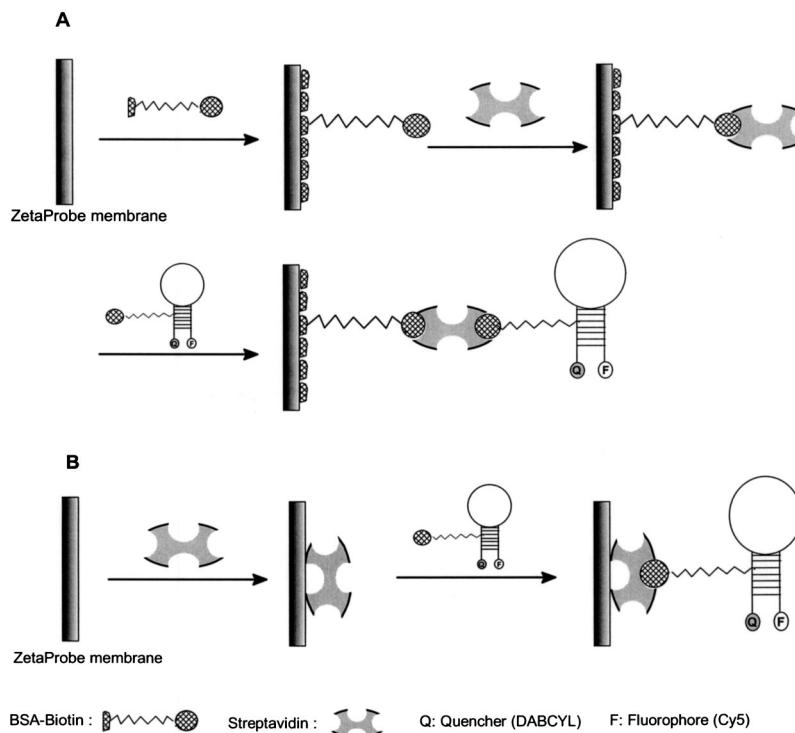


Fig. 2 Illustration of the immobilization strategies of the MB on a zeta-probe membrane. (a) BSA-biotin-streptavidin-MB strategy, (b) streptavidin-MB strategy.

2.3 Immobilization of the MB on a Zeta-Probe Membrane

Figure 2 depicts the two immobilization strategies used on zeta-probe membranes. Each membrane was first physically marked by applying slight pressure with a custom-made mold that was compatible with the size of the miniature biochip detector. In the first strategy [Fig. 2(a)], BSA-biotin was adsorbed on the membrane surface by soaking a square-cut membrane in 1 mg/ml BSA-biotin solution in 20 mM phosphate buffer at pH 7.0 overnight. To remove the unbound BSA-biotin, the membrane was rinsed with distilled water. Next, a 1-mg/ml streptavidin solution in 20 mM phosphate buffer at pH 7.0 was used to treat the BSA-biotin-bound membrane and left to incubate for 3 h. Then the membrane was rinsed with distilled water several times to remove excess streptavidin. A 0.5- μ l solution of 1 μ M MB in 20 mM phosphate buffer at pH 7.0 was carefully spotted on the marked membrane using a micropipette. In order to complete the biotin-streptavidin reaction, the sample was left at 4°C overnight. After carefully rinsing the MB-immobilized membrane with hybridization solution, hybridization was performed by soaking the membrane in hybridization buffer (20 mM Tris-HCl and 100 mM MgCl₂ at pH 7.5) containing the *BRCA1* gene sequence at 5 mM.

The second strategy is illustrated in Fig. 2(b). The BSA-biotin absorption step was eliminated and streptavidin was absorbed directly onto the membrane. A 1-mg/ml streptavidin solution in 20 mM phosphate buffer at pH 7.0 was used to incubate the membrane overnight. The remainder of the procedure was the same as in the first strategy. Herring sperm (HS) DNA was used as a negative control for both strategies.

An aliquot of HS DNA solution was taken from boiling solution and was spotted on the marked membrane.

3 Results and Discussion

Several factors might influence the signal-to-noise ratio when MBs are immobilized on a surface. That is why specifically applicable molecular design and immobilization strategies are important. It may not be straightforward to overcome the current limitations such as background noise caused by incomplete quenching, and the electrostatic difference between the liquid–solid interface and the liquid solution. However, some of these limitations can be minimized with an understanding of molecular interactions and appropriate experimental design.

Tsourkas et al.²⁰ demonstrated that the performance of an MB could be very sensitive to its structural characteristics, such as probe and stem lengths. They reported that a stem of at least 4 base pairs in length was required for lower background noise, and shorter probe domains (22 to 25 bases) were required for higher selectivity. In addition, Marras et al.⁴ studied several dyes and molecules as fluorophores and quenchers. These two studies were taken as reference points in designing the MB used in this study. The stem was composed of 7 base pairs and the probe domain was composed of 22 bases. In order to achieve a full hybridization with the MB, a *BRCA1* gene fragment composed of 123 bases was used. The MB probe was complementary to the 22 bases in the middle of the *BRCA1* gene fragment. When the MB probe was designed, the requirements for instrumentation were also taken into account. Because a diode laser with a 635-nm

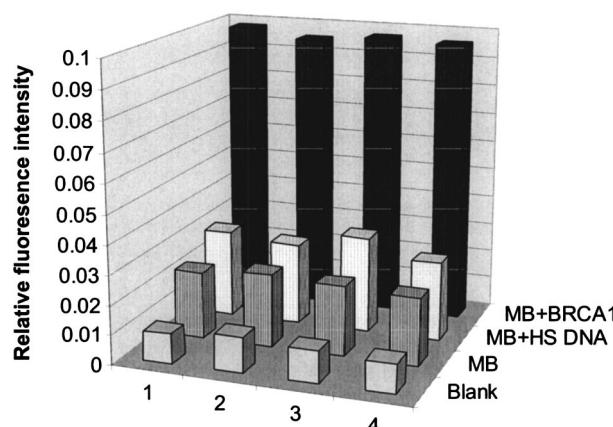


Fig. 3 Biochip readout of the MB immobilized using strategy 1 for the detection of *BRCA1*. The numbers 1, 2, 3, and 4 represent each 4-element row of the 4×4 IC photodetector array. The results of four measurements are represented by each row, including a blank, MB microdot, MB microdot exposed to *BRCA1* gene (MB+*BRCA1*), and a microdot exposed to herring sperm DNA (MB+HS DNA).

wavelength was used, a Cy5 label, which absorbs at the laser excitation wavelength, was chosen.

The two immobilization strategies were evaluated with our CMOS biochip detection system. As indicated, in the first immobilization strategy, BSA-biotin was first adsorbed on a nylon membrane, followed by streptavidin. This procedure creates a double layer of proteins, which is intended to help reduce the undesired interaction of the MB with the surface, resulting in incomplete quenching. In the second strategy, the BSA-biotin was omitted. It can be seen from Fig. 3 and Fig. 4 (see row designated MB) that the background noise is higher with the second immobilization strategy. Again Fig. 3 and Fig. 4 show that hybridization with noncomplementary DNA has little additional effect on the increase in fluorescence intensity compared with the MB alone. Even though the first strategy is superior to the second one, both are useful for screening purposes.

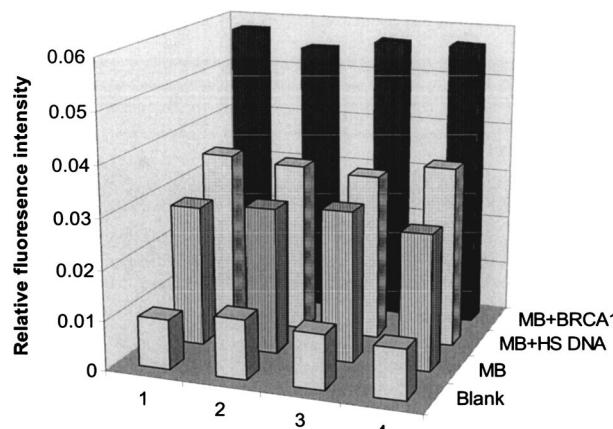


Fig. 4 Biochip readout of MB immobilized using strategy 2 for the detection of *BRCA1*. All conditions and representations are the same as in Fig. 3 except its immobilization strategy.

It is interesting to note that the fluorescence intensity associated with the negative control (i.e., herring sperm DNA) is only slightly higher than the fluorescence intensity observed with the molecular beacon alone, regardless of the immobilization strategy. This suggests that the interactions of the probe domain of the MB are very specific for its target DNA sequence, which is not surprising since a probe domain 22 bases in length was chosen for the MB. The use of even more stringent hybridization conditions would be expected to improve the difference between the negative control and the positive experimental target even more. We cannot at this time, however, rule out the possibility that the relatively small differences between the negative control and the MB alone may be an artifact of some significant loss of secondary structure of the MB that is due to its close proximity to the membrane surface. That is, the fluorescence intensity of the MB alone may be higher than is optimally achievable, owing to partial unfolding of the secondary structure of the stem portion under our experimental conditions. We have not made an exhaustive study of linkage possibilities for tethering the MB to the surface. It is apparent from even these preliminary results, however, that careful consideration of the immobilization strategy for MBs is probably critical for efficient use of the sensor. The emerging field of nanoscience-nanotechnology may give us tools to more systematically investigate the structural interactions of these complex biomolecules with solid surfaces.

4 Conclusion

Rapid, simple, cost-effective medical devices for screening for multiple medical diseases and infectious pathogens are essential for early diagnosis and improved treatments of many illnesses. An important factor in medical diagnostics is rapid, selective, and sensitive detection of genes and gene mutations. In this work, detection of the *BRCA1* cancer susceptibility gene using MB probes and a miniature biochip detection system was demonstrated. The MB was designed for use with the biochip system. Herring sperm DNA was used as a control to evaluate the degree of nonspecific binding. It was demonstrated that two immobilization strategies could be used with our biochip detection system.

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