Biosensors and Bioelectronics 26 (2010) 850–853

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Short communication

Double recognition of oligonucleotide and protein in the detection of DNA methylation with surface plasmon resonance biosensors

Shiyang Pan[a,*], Jian Xu[a], Yongqian Shu[b], Fang Wang[a], Wenying Xia[a], Qingqing Ding[b], Ting Xu[a], Chun Zhao[a], Meijuan Zhang[a], Peijun Huang[a], Shan Lu[c]

[a] Department of Laboratory Medicine, the First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, China
[b] Department of Oncology, the First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, China
[c] Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605, United States

ARTICLE INFO

Article history:
Received 26 May 2010
Received in revised form 31 July 2010
Accepted 2 August 2010
Available online 8 August 2010

Keywords:
DNA methylation
Surface plasmon resonance
Biosensor
MBD
APC

ABSTRACT

DNA methylation plays an essential role in maintenance of cellular function. A growing number of human diseases have been found to be associated with aberrant DNA methylation, especially cancer. However, current technologies used in DNA methylation detection are complicated and time consuming. A promotor of the Adenomatous polyposis coli (APC) gene, a well-studied tumor suppressor gene, was used as the detection target DNA sequence. The double recognition mechanism was realized with oligonucleotide probe hybridization and specific protein binding. First, complementary target DNA was captured by the probe immobilized onto a surface plasmon resonance (SPR) sensor chip. Then, the recombinant methyl-CpG binding domain (MBD) protein was passed over the surface to recognize and bind to methylated CpG sites. Binding resulted in an increase in the refractive index, and a detectable optical signal was generated. Five picomoles of methylated APC promotor DNA could be easily detected with this method. The entire detection could be completed within 1 h. This work represents the first SPR based biosensor technology, which achieves simple and specific DNA methylation detection and avoids complicated bisulfite treatment and methylation-sensitive restriction digestion. It will improve our ability to detect DNA methylation specifically and rapidly, and promote our understanding of the role of DNA methylation in gene regulation and diseases.

1. Introduction

DNA methylation at the 5′-position of cytosine in CpG dinucleotides plays an essential role in the cellular processes of transcriptional regulation, chromatin compaction, imprinting and X-chromosome inactivation (Bernstein et al., 2007). In normal cells, most promotor-associated CpG islands are almost always methylation free, but in cancer cells, promotor region CpG islands are likely to be methylated. Aberrant hypermethylation of CpG islands in the promotor regions of tumor suppressor genes is one of the earliest and most common genome alterations in many different human cancers (Jones and Baylin, 2007). Therefore, DNA methylation analysis is crucial to life science and cancer research.

To date, a variety of methods have been developed to detect DNA methylation, including methylation-sensitive restriction digestion, bisulfite sequencing, and methylation-specific PCR. Unfortunately each of these methods is laborious and time consuming (Dahl and Guldberg, 2003). Development of a new strategy that overcomes these obstacles could have a significant impact upon early detection and prevention of cancer. Surface plasmon resonance (SPR) biosensor technology is a highly sensitive technique for label-free analysis of biomolecular interactions (Malmqvist, 1999). Here, to the best of our knowledge, we present the first study using SPR to determine methylation status that does not require complicated bisulfate treatment or methylation-sensitive restriction digestion. Detection was achieved by applying a double recognition process, using both oligonucleotide probe hybridization and specific protein binding.

Adenomatous polyposis coli (APC) is a tumor suppressor gene, and its gene product indirectly regulates transcription of a number of genes that are crucial for cell proliferation (Fearnhead et al., 2001). There are 31 CpG sites in promotor 1A of the APC gene (Pan et al., 2009). Hypermethylation of CpG sites in APC promotor 1A has been confirmed in several cancers (Esteller et al., 2000; Virmani et al., 2001; Worm et al., 2004). The APC gene promotor 1A with comparatively small CpG sites was selected as the target of this detection model.

* Corresponding author. Tel.: +86 25 83674121.
E-mail address: sypan@njmu.edu.cn (S. Pan).

0956-5663/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.bios.2010.08.007

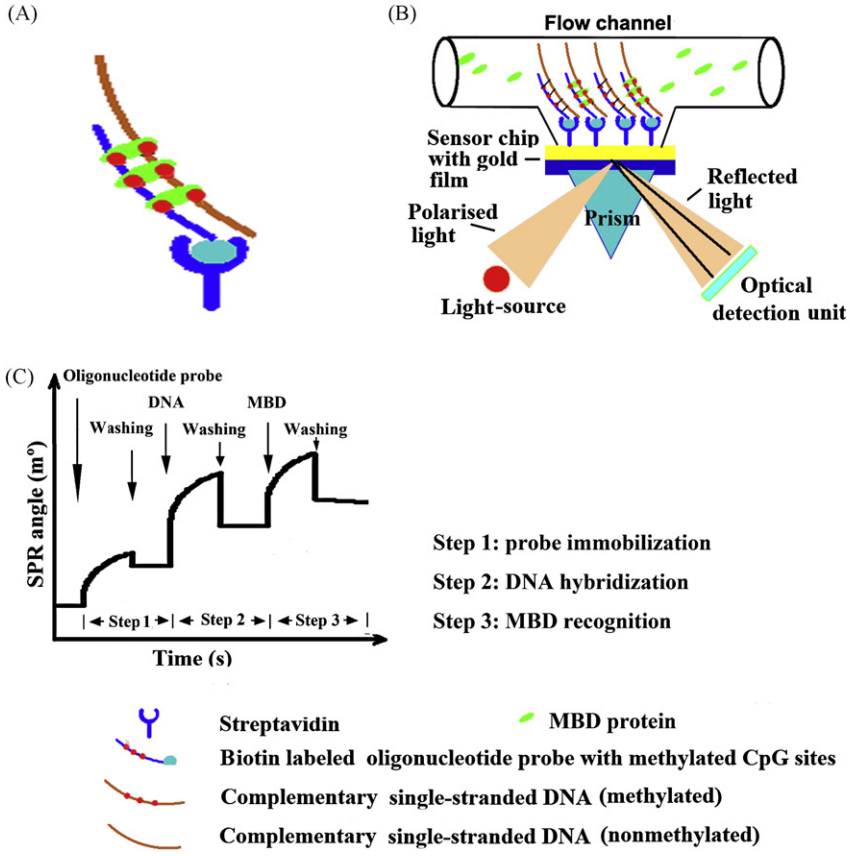

Fig. 1. Schematic diagram for double recognition of oligonucleotide and protein. (A) Double recognition strategy; (B) DNA methylation detection with SPR biosensor; (C) SPR curve of three steps.

2. Materials and methods

2.1. Overview of the detection mechanism

An overview of the detection mechanism is shown in Fig. 1. Initially, a biotin-labeled sequence specific oligonucleotide probe that contained methylated CpG sites, was immobilized on an SPR sensor chip coated with streptavidin. Then, single-stranded (ss) genomic DNA was introduced into the flow channel. After washing, non-specific binding DNA was removed. Methyl-CpG binding domain (MBD) protein, which binds exclusively to DNA that contains one or more symmetrically methylated CpGs (Bogdanovic and Veenstra, 2009), was introduced to the chip. This recognized and bound to symmetrically methylated CpG sites on hybridization products. Interaction between the methylated DNA and the MBD protein on the chip surface resulted in an increase in the refractive index, which generated a detectable optimal signal.

The SPR biosensor (UMPHO A450) was obtained from CytoTrend Biotech Engineering (Beijing, China). It had four channels that allowed four simultaneous measurements on a sensor chip. The SPR angle resolution was <0.5 m° and the minimum assay limit was 1 pg/mm^2. The sensor chip (CT415) was coated with streptavidin and used to immobilize biotin-labeled oligonucleotides upon. Experiments were performed at 37 °C with a running buffer of HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20, pH 7.40) and a flow rate of 5 μl/min. In each experiment, the sensor surface was regenerated by a 1 min treatment with 1 mM HCl, which allowed multiple use of the chip. For kinetic studies, the data were analyzed with SPRViewer software (version 1.0) provided by the manufacturer. A 1:1 Langmuir model was globally fitted to the sensorgram data to obtain k_a and k_d values for the interaction. The equilibrium dissociation constant K_D was subsequently calculated as the ratio k_d/k_a.

2.2. Oligonucleotide probe and target DNA

A 5′-end biotin-labeled 25-base oligonucleotide probe (mAPC25) was designed, that contained four methylated CpG sites. The sequence was located within the CpG island of the APC promotor 1A. A 100 bp double-stranded DNA sequence with four corresponding methylated CpG sites, which presented the promotor of the APC gene, was used as target DNA (mAPC100). The mAPC25 and the mAPC100 had a 25-base complementary sequence. After hybridization, products included four symmetrically methylated CpG sites. Sequences of the probe and target DNA are listed in Table 1. Control unmethylated DNA, named APC100, had the same sequence as mAPC100. All these oligonucleotides were synthesized by Shanghai Shengon Technology (Shanghai, China).

2.3. Identification of the interaction between MBD and methylated DNA

The MBD portion of MeCP2 protein (Lewis et al., 1992) was expressed in *Escherichia coli* and purified by Ni affinity column chromatography. SPR analysis was used to identify whether the expressed recombinant MBD protein had the biological function of specific binding to methylated DNA.

First, 200 μl of 0.4 M biotin-labeled poly($_m$CGA) diluted in the HBS-EP buffer was immobilized on the CT415 sensor chip. Simultaneously, poly(CGA) at the same concentration was injected to a parallel channel. Then, 50 μl of 50 μg/ml MBD protein or bovine

Table 1
Sequences of synthetic oligonucleotides.

Oligonucleotide	Sequence[a]
mAPC25	5′-biotin-CTG$_m$CGGAGTG$_m$CGGGT$_m$CGGGAAG$_m$CGG-3′
mAPC100[b,c] (sense strand)	5′-GATTACACAGCTGCTTCTCTCTC_mGCTTCC$_m$CGACC$_m$CGCACTC$_m$CGCAGTGGGGCACAGGGCCCCGCCCAA CCGCACAGCCTGCCTAGCCCTAGCCCCGTAC-3′
poly(GAM)[b] (sense strand)	5′-GATC$_m$CGA$_m$CGA$_m$CGA$_m$CGA$_m$CGA$_m$CGA$_m$CGA$_m$CGA$_m$CGA$_m$CGA$_m$CGA$_m$CGA$_m$CGATC-3′

[a] $_m$C denotes 5-methylcytosine.
[b] Complementary strand has symmetrical 5-methylcytosines.
[c] Underlined section is complementary to the mAPC25 sequence.

serum albumin (BSA) as a control in HBS-EP buffer was introduced. Unmethylated DNA poly(CGA) and methylated DNA poly($_m$CGA) are ligated polymers of a synthetic 42-bp sequence that contained 12 CGA repeats, which in poly($_m$CGA), had cytosine substituted by 5-methylcytosine (Table 1) (Nan et al., 1993).

2.4. Double recognition detection of DNA methylation with SPR biosensor

At first, 200 μl of 0.4 M mAPC25 diluted in HBS-EP buffer was introduced onto the CT415 sensor chip coated with streptavidin. After probe immobilization, 5 pmol DNA of target mAPC100, or control APC100, in hybridization buffer (3× SSPE: 0.54 M NaCl, 30 mM NaH_2PO_4, 3 mM EDTA, pH 7.4, with 10% PEG 6000) was subjected to a denaturation step (5 min incubation at 95 °C followed by 1 min on ice) to obtain ssDNA. Hybridization was conducted by injecting 100 μl of ssDNA solution onto the chip. The sensor chip was then washed with running buffer to remove unbound DNA molecules. Finally, 100 μl of 50 μg/ml MBD in MBD buffer (20 mM HEPES, pH 7.9, 5% glycerol, 0.1% Triton X-100, 0.1 M NaCl) was flowed across the hybridization products. The reaction was monitored for 20 min, and then unbound material was flushed away. The analytical signal derived from the difference between the refractive index before (baseline) and after MBD capture.

3. Results and discussion

3.1. Interaction between MBD protein and methylated DNA

The MBD protein can specifically bind symmetrically methylated CpG sites with high affinity. (Roloff et al., 2003; Nan et al., 1993). MBD protein was expressed in bacteria and purified (Supplementary information, Fig. S1).

Specific binding responses of recombinant MBD to methylated DNA were observed by SPR analysis. MBD was capable of binding to poly($_m$CGA) with a 75 m° increase in the SPR curve. However, after adding of MBD, there was no increase in the SPR curve of poly(CGA) channel, which indicated that no binding occurred. Also, no increase was observed in the curve of the poly($_m$CGA) channel or the poly(CGA) channel after BSA injection (Supplementary information, Fig. S2). This indicated that the recombinant MBD protein bound specifically to methylated DNA.

Recently, MBD proteins have been used in some newly developed methylation detection assays, such as MIRA, MECP2 column based assay and COMPARE-MS (Rauch and Pfeifer, 2005; Yegnasubramanian et al., 2006; Zou et al., 2007). In these previous studies, MBD proteins were used to capture and enrich methylated DNA, and methylation status was analyzed by subsequent bisulfite modification and PCR amplification, or methylation-sensitive restriction digestion. However, this present study demonstrates that, using an SPR biosensor, methylation can be directly detected by measuring MBD recognition.

3.2. Oligonucleotide and protein double recognition of methylated target DNA on SPR biosensor

After probe mAPC25 was immobilized on the chip, equal amounts of denatured mAPC100 and APC100 target DNA were injected into two parallel channels. In both cases, SPR curves rose in similar fashion, which indicated that equal amounts of target DNA, either methylated or non-methylated were captured by the probes. However, after adding MBD, the curves diverged. As MBD recognizes and binds to symmetrically methylated CpG sites, the curve of mAPC25-mAPC100 channel rose rapidly following MBD addition, and after rinse, still maintained a value of 60 m° higher than that before injection. The SPR curve of the mAPC25-APC100 channel rose slightly and slowly, and then declined to the baseline after non-specific binding was removed by washing (Fig. 2). From the curves, it is possible to distinguish methylated DNA sample from non-methylated clearly. This experiment was repeated four times and similar sensorgrams were obtained. The mean value of the final SPR angle rise was 56.4 m° with the standard deviation (SD) of 14.1 m°.

At present, two main strategies are used for gene specific methylation analysis: bisulfite treatment of DNA and methylation-sensitive restriction digestion. Most of the current DNA methylation detection methods are based on sodium bisulfite treatment. Methylation-specific PCR (Herman et al., 1996) uses PCR primers that target bisulfite-induced sequence changes to specifically amplify either methylated or unmethylated alleles. Quantitative variations of this technique, such as MethyLight (Eads et al., 2000), HeavyMethyl (Cottrell et al., 2004) and MethylQuant (Thomassin et al., 2004), employ methylation-specific oligonucleotides in conjunction with Taqman probes or SYBR Green-based real-time PCR amplification to quantitate alleles with a specific pattern of methylation. These techniques are sensitive and specific for detection of DNA methylation. However, all bisulfite-based

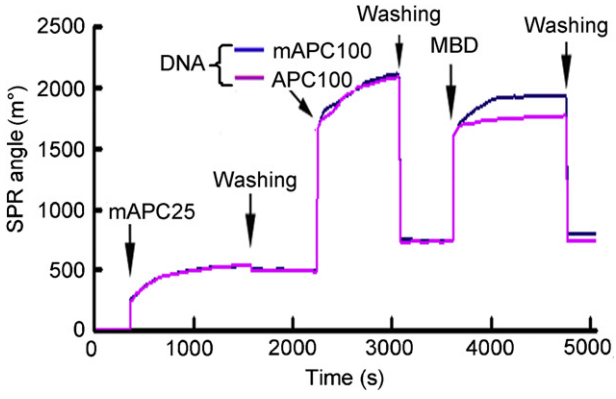

Fig. 2. Oligonucleotide and protein double recognition of methylated target DNA with SPR biosensors. The blue and the red lines represent SPR sensorgrams for addition of mAPC100 and APC100, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

techniques are quite cumbersome, involving time and labor intensive chemical treatments. Furthermore, chemical treatment has some inevitable pitfalls, including a decrease in specificity and sensitivity of methylation detection due to incomplete deamination of DNA, especially as >90% of DNA degradation is due to acid-catalyzed depurination (Tanaka and Okamoto, 2007). Additionally, PCR primer design becomes difficult due to the reduction in sequence complexity following bisulfite treatment, which leads to inability to investigate the methylation pattern at some CpG sites of interest.

Other DNA methylation detection assays use methylation-sensitive restriction enzymes to digest unmethylated DNA, while leaving methylated DNA intact for detection by Southern blot analysis (Singer et al., 1979), PCR (Singer-Sam et al., 1990) or real-time PCR (Bastian et al., 2005). The use of methylation-sensitive restriction enzymes, however, has several limitations. Apart from the fact that incomplete digestion may complicate analysis, the greatest disadvantage is that methylation-sensitive restriction digestion only provides information about the methylation status at the enzyme recognition sites, but not elsewhere.

With the advent of genomics, DNA methylation microarray technology has developed rapidly. However, neither methylation-specific microarray (Gitan et al., 2002) nor differential methylation hybridization (Huang et al., 1999) can overcome the drawbacks of bisulfite treatment or methylation-sensitive restriction digestion.

The novel strategy reported here overcomes the disadvantages associated with existing methods, and realizes the rapid and automatic detection of methylation in a simple manner. DNA methylation detection can be completed automatically on an SPR biosensor chip within 1 h.

Recently, Maki has developed a nanowire transistor based DNA methylation detection method (Maki et al., 2008), which also avoids bisulfite treatment or methylation-sensitive restriction digestion. However, this method is much more complicated than the one that we have described. The Maki method requires “off chip” procedures to capture complementary target DNA with the probe, whereas DNA hybridization was accomplished on the chip with our method. One additional notable difference is that we used MBD for recognition, whereas Maki used anti-5-methylcytosine antibodies. Anti-5-methylcytosine antibodies only bind to methylated ssDNA, and for this reason, can only determine if the DNA is methylated, but cannot confirm which CpG sites are methylated (Weber et al., 2005). MBD is a better candidate for recognition, because it recognizes symmetrically methylated CpG sites. Our method could detect the exact location by designing appropriate methylated CpG sites in the oligonucleotide probe.

In this study, we only used the synthesized both methylated and unmethylated APC gene promotor DNA samples for the purpose of standardization, and did not include genomic DNA samples. Nevertheless, SPR technology has developed in the direction of high-throughput systems and multi-analyte measurements, and there are several commercial high-throughput SPR instruments available. This method, coupled with high-throughput SPR sensors, gives the promising prospect of genome-wide methylation detection in the future.

4. Conclusion

In summary, a novel SPR biosensor based DNA methylation detection strategy that was simple, automatic and specific was developed. Methylated CpG sites on specific DNA sequences can be easily detected by our oligonucleotide and protein double recognition method. It avoids the complicated traditional process such as bisulfite treatment or methylation-sensitive restriction digestion. It will improve our ability to detect DNA methylation specifically and rapidly and promote our understanding of the role of DNA methylation in gene regulation and diseases.

Acknowledgements

We are grateful for the financial support of the National Natural Science Foundation of China (30972821 and 30901262), the Natural Science Foundation of Jiangsu Province (BK2009440), Key Laboratory for Laboratory Medicine of Jiangsu Province (XK200731), Project of Health Administration of Jiangsu Province (H200707) and Project of Jiangsu Science and Technology Department (BS2007073).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.08.007.

References

Bastian, P.J., Palapattu, G.S., Lin, X., Yegnasubramanian, S., Mangold, L.A., Trock, B., Eisenberger, M.A., Partin, A.W., Nelson, W.G., 2005. Clin. Cancer Res. 11, 4037–4043.
Bernstein, B.E., Meissner, A., Lander, E.S., 2007. Cell 128, 669–681.
Bogdanovic, O., Veenstra, G.J., 2009. Chromosoma 118, 549–565.
Cottrell, S.E., Distler, J., Goodman, N.S., Mooney, S.H., Kluth, A., Olek, A., Schwope, I., Tetzner, R., Ziebarth, H., Berlin, K., 2004. Nucleic Acids Res. 32, e10.
Dahl, C., Guldberg, P., 2003. Biogerontology 4, 233–250.
Eads, C.A., Danenberg, K.D., Kawakami, K., Saltz, L.B., Blake, C., Shibata, D., Danenberg, P.V., Laird, P.W., 2000. Nucleic Acids Res. 28, e32.
Esteller, M., Sparks, A., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M.A., Gonzalez, S., Tarafa, G., Sidransky, D., Meltzer, S.J., Baylin, S.B., Herman, J.G., 2000. Cancer Res. 60, 4366–4371.
Fearnhead, N.S., Britton, M.P., Bodmer, W.F., 2001. Hum. Mol. Genet. 10, 721–733.
Gitan, R.S., Shi, H., Chen, C.M., Yan, P.S., Huang, T.H., 2002. Genome Res. 12, 158–164.
Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D., Baylin, S.B., 1996. Proc. Natl. Acad. Sci. U.S.A. 93, 9821–9826.
Huang, T.H., Perry, M.R., Laux, D.E., 1999. Hum. Mol. Genet. 8, 459–470.
Jones, P.A., Baylin, S.B., 2007. Cell 128, 683–692.
Lewis, J.D., Meehan, R.R., Henzel, W.J., Maurer-Fogy, I., Jeppesen, P., Klein, F., Bird, A., 1992. Cell 69, 905–914.
Maki, W.C., Mishra, N.N., Cameron, E.G., Filanoski, B., Rastogi, S.K., Maki, G.K., 2008. Biosens. Bioelectron. 23, 780–787.
Malmqvist, M., 1999. Biochem. Soc. Trans. 27, 335–340.
Nan, X., Meehan, R.R., Bird, A., 1993. Nucleic Acids Res. 21, 4886–4892.
Pan, S.Y., Zhang, L.X., Gao, L., Gu, B., Wang, F., Xu, J., Shu, Y.Q., Yang, D., Chen, Z., 2009. Biomed. Pharmacother. 63, 463–468.
Rauch, T., Pfeifer, G.P., 2005. Lab. Invest. 85, 1172–1180.
Roloff, T.C., Ropers, H.H., Nuber, U.A., 2003. BMC Genomics 4, 1.
Singer-Sam, J., LeBon, J.M., Tanguay, R.L., Riggs, A.D., 1990. Nucleic Acids Res. 18, 687.
Singer, J., Roberts-Ems, J., Riggs, A.D., 1979. Science 203, 1019–1021.
Tanaka, K., Okamoto, A., 2007. Bioorg. Med. Chem. Lett. 17, 1912–1915.
Thomassin, H., Kress, C., Grange, T., 2004. Nucleic Acids Res. 32, e168.
Virmani, A.K., Rathi, A., Sathyanarayana, U.G., Padar, A., Huang, C.X., Cunnigham, H.T., Farinas, A.J., Milchgrub, S., Euhus, D.M., Gilcrease, M., Herman, J., Minna, J.D., Gazdar, A.F., 2001. Clin. Cancer Res. 7, 1998–2004.
Weber, M., Davies, J.J., Wittig, D., Oakeley, E.J., Haase, M., Lam, W.L., Schubeler, D., 2005. Nat. Genet. 37, 853–862.
Worm, J., Christensen, C., Grønbaek, K., Tulchinsky, E., Guldberg, P., 2004. Oncogene 23, 5215–5226.
Yegnasubramanian, S., Lin, X., Haffner, M.C., DeMarzo, A.M., Nelson, W.G., 2006. Nucleic Acids Res. 34, e19.
Zou, H., Harrington, J., Rego, R.L., Ahlquist, D.A., 2007. Clin. Chem. 53, 1646–1651.