J|A|C|S COMMUNICATIONS

Published on Web 01/29/2009

Highly Specific and Sensitive Electrochemical Genotyping via Gap Ligation Reaction and Surface Hybridization Detection

Yong Huang,[†] Yan-Li Zhang,[†] Xiangmin Xu,[‡] Jian-Hui Jiang,*[,†] Guo-Li Shen,[†] and Ru-Qin Yu[†]

State Key Laboratory of Chemo/Bio-Sensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, 410082, P.R. China, Department of Medical Genetics, School of Basic Medical Sciences, South Medical University, Guangzhou 510515, P.R. China

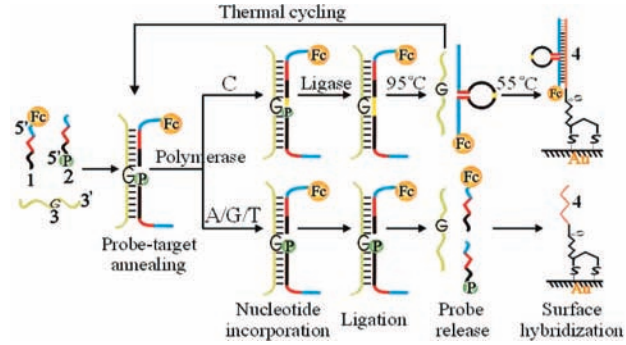
Received November 5, 2008; E-mail: jianhuijiang@hnu.cn

Single nucleotide polymorphisms (SNPs) are the most abundant and stable type of genetic variations found in human genome. Genotyping of such sequence variations is often diagnostic of particular genetic disorders and drug responses owing to their direct connections with transcriptional regulation or biological functions of many proteins.[1] SNP genotyping is generally performed through a certain allele discrimination mechanism followed by detection of the allele-specific products.[2] Typical allele discrimination mechanisms include allele-specific hybridization,[3] allele-specific nucleotide incorporation,[4] allele-specific cleavage,[5] and allele-specific oligonucleotide ligation.[6] Allele-specific hybridization requires double-stranded DNA hybrids resulting from single-base variations to show detectable differences in hybridization efficiency or thermal stability. Such a mechanism normally involves stringent control of the assay conditions, posing challenges in sequence-dependent probe design, and hybridization condition optimization.[1a,2a] Enzyme-aided allele discrimination, such as allele-specific nucleotide incorporation, cleavage and ligation, provides a more selective and flexible arsenal for SNP genotyping.[1a,2a] These techniques, though widely adapted to varying detection strategies from optical readouts[7] to mass spectrometry[8] and electronic measurements,[9−11] still have not prevailed owing to relatively high cost, limited specificity, or inadequate sensitivity.[1]

Electrochemistry holds potential as a next-generation molecular detection strategy for genotyping because of its high sensitivity, low cost, and excellent compatibility with miniaturization technologies. Along this direction, numerous attempts have been demonstrated via electrochemical probing of enzyme conjugates,[9] nanoparticle tags,[10] and redox labels[11] presented by allele-specific reactions. However, a major concern with most electrochemical genotyping is the implementation of multistep enzymatic discrimination reactions on the surface, which possibly leads to steric hindrance and thus influences enzymatic efficiency and reproducibility. Also, for those strategies using redox labels, current assay configurations fail to render electroactive reporters in close proximity to the electrode interface, limiting the sensitivity of the detection strategies. Motivated by our previous observations on surface hybridization of ferrocene (Fc)-tagged probe in sensitivity enhancement,[12] we report here the proof-of-principle of a novel electrochemical genotyping technique based on gap ligation reaction[13] with surface hybridization detection. This technique affords a robust, specific, and sensitive platform for enzymatic SNP typing.

The SNP typing strategy comprises a unique biphasic architecture with specific enzyme-aided allele discrimination reactions in a homogeneous solution followed by ultrasensitive surface hybridization detection of the allele-specific products with redox tags close to the electrode, as shown in Scheme 1. The gold electrode is modified by self-assembly with capture probe **4** via the thioctic acid label at 3′ terminal (~1.9×10^{12} strand/cm^2). Each detection probe, **1** or **2**, is designed with a short barcode tail sequence complementary to a half-segment of the capture probe. In SNP typing, probes **1** and **2** are annealed on the DNA template flanking the SNP site with polymorphic nucleotide left as a gap. Nucleotide complementary to the base gap allows allele-specific incorporation at 3′ end of probe **1** by DNA polymerase (AmpliTaq, Stoffel fragment). The nucleotide-incorporated probe **1** can then be covalently joined with probe **2** by DNA ligase. This two-step gap ligation reaction[13] involves joint use of DNA polymerase and ligase, furnishing the genotyping strategy with highly specific biochemistry in allele discrimination. On heating, the ligated product is released from the corresponding hybrid and folded into a molecular beacon structure[14] because of intramolecular hybridization of two complementary sequences in **1** and **2**. This intramolecular hybridization is known to exhibit a much higher T_m than intermolecular annealing of these two separate probes.[7a,15] In the ligated products two tail sequences of probes **1** and **2** are kept into close proximity, which promotes cooperative annealing of the ligated products to capture probes. Thus, Fc tags are drawn in adjacence to the electrode, resulting in a substantial current with the maximized sensitivity. Nucleotide noncomplementary to the gapped base disables the gap ligation reaction, thus the disjoined probes failing to anneal capture probes with no remarkable redox signal triggered (melting tem-

Scheme 1. Electrochemical Genotyping Strategy Based on Gap Ligation Reaction with Surface Hybridization Detection[a]



[a] Two probes, **1** and **2**, flank the SNP site with polymorphic nucleotide left as a gap on DNA target **3**. **2** has a arm sequence (red) complementary to the target-specific sequence (red) of **1**. Each probe has a tail sequence (blue) complementary to capture probe **4**. Nucleotide matching the gap can be incorporated at the 3′ end of **1** by polymerase followed by covalent ligation with **2** by ligase. Ligated product forms molecular beacon structure, promoting the proximate tail sequences to cooperative annealing on **4** with the Fc tag close to the electrode and redox current triggered. Mismatched nucleotides disable the gap ligation reaction with disjoined probes that cannot be annealed on **4** due to predesigned low melting temperature.

[†] Hunan University.
[‡] South Medical University.

10.1021/ja808700d CCC: $40.75

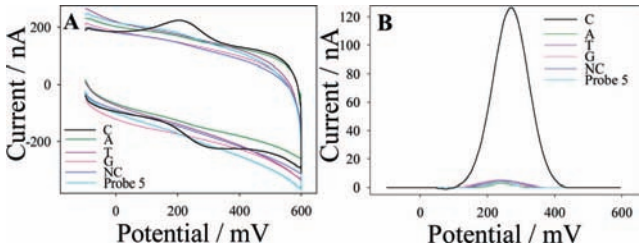


Figure 1. (A) CVs obtained via surface hybridization at 55 °C with varying nucleotides for genotyping 2 nM mutant **3** (defaut) and noncomplementary (NC) **6** targets using probe **2** (defaut) or **5** (specified). Potential scanning rate is 100 mV/s. (B) Corresponding DPVs. Scanning was performed in 0.1 M $KClO_4$ from −100 to 600 mV, amplitude of 50 mV. All potentials were referred to SCE.

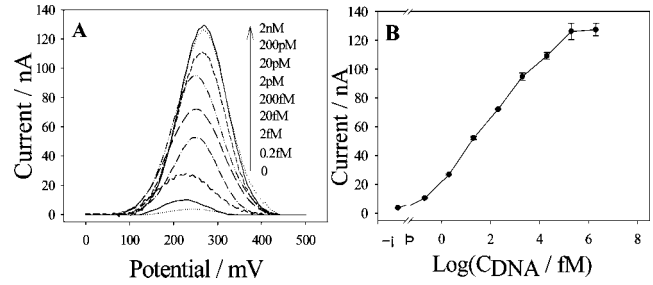


Figure 2. (A) Typical DPV curves obtained via surface hybridization at 55 °C in response to mutant target **3** of varying concentration. (B) Corresponding DPV peak currents versus mutant target **3** concentrations. DPV was recorded in 0.1 M $KClO_4$ from 0 to 500 mV (vs SCE). The error bars represented SD across four repetitive experiments.

perature for probes **1** and **4** is estimated to be 27.8 °C). Thermal cycling allows the reactions to proceed repeatedly, offering a route for amplifying the ligated products and further enhancing the detection sensitivity.

There are indeed several advantageous elements intrinsic in the genotyping strategy. First, the biphasic architecture with homogeneous enzymatic allele discrimination reactions followed by surface detection of allele-specific products circumvents most biochemical reactions on the electrode interface except the surface hybridization step. This not only ensures high efficiency and fidelity in enzymatic discrimination, but also makes the technique easily automated, highly reproducible, and readily accomplished with minimal working steps. Second, a cyclic disulfide anchor of thioctic acid is used for the immobilization of capture probe **4**, which is observed to improve stability of the surface-tethered probes and prevents their displacement by thiol compounds in enzyme buffers and dissociation from the surface during heating processes.[16] Third, surface hybridization promoted by cooperative annealing of two tail sequences in a ligated DNA strand provides a unique mechanism in selective detection of allele-specific products with maximized sensitivity and minimized background, since cooperative annealing renders the redox tags in very close proximity to the electrode and imposes the stringent requirement that surface hybridization only occurs for DNA strands with both tail sequences complementary to capture probes, which eliminates possible interferences from coexisting Fc-tagged probes and nonligated strands. Fourth, each tail sequence of the detection probe can be designed as a specific barcode[17] for each mutation site. Then using different barcode sequences the genotyping strategy can be implemented for multiplex detection of multiple SNPs in densely packed array format.[18]

The genotyping technique was tested using a model DNA target of human β-globin gene around −28 position, a known biallelic (A > G) SNP highly associated with β-thalassemia. Figure 1 depicts typical voltammetric responses of this technique for 2 nM mutant target **3** with varying nucleotide added. In cases of adding dTTP, dATP, or dGTP, no appreciable current peaks appeared in cyclic voltammograms (CVs). In contrast, with dCTP added in the reaction mixture, a couple of well-defined redox peaks were obtained in CV curves at 0.202 and 0.277 V (vs SCE), a typical redox potential range of Fc (Figure 1A). Because in gap ligation reaction only the nucleotide complementary to the gap base could mediate the formation of ligated product, one reasoned that dCTP was complementary to the gap base, that is, the SNP site of the mutant target was G, coinciding with the genotype of the mutant target. A control experiment with a DNA sequence different from the mutant in the presence of dCTP also gave insignificant redox peaks in CVs (Figure 1A), which implied that the developed technique was highly specific for the target and coexisting mismatched DNA targets did not cause false signals. A further control experiment using another detection probe **5**, which only had the tail sequence different from probe **2**, in place of probe **2**, was performed for mutant target **3** in the presence of dCTP. This experiment was expected to produce a molecular beacon-structured ligated product, but one tail sequence near 3′ terminal was noncomplementary to capture probe **4**, which disabled cooperative annealing of both tail sequences of the ligated product to capture probes. It was observed that there was no remarkable redox peaks in CV curves (Figure 1A), indicating that cooperative annealing of both tail sequences was essential for surface hybridization detection of the ligated products, and thus proximity-dependent surface hybridization could offer high selectivity in detecting the allele-specific products with no interferences from coexisting Fc-tagged probes. Better differentiation between the genotyping responses was achieved in differential pulse voltammograms (DPVs), as shown in Figure 1B. Only very small DPV peaks (<10 nA with SD across four repetitive experiments = 3.8%) were observed around 0.244 V in cases when dTTP, dATP, or dGTP was included in the mutant target system, or dCTP was added in the DNA sequence different from the mutant, while a strong reduction peak was present at 0.268 V (SD across four repetitive experiments = 3.3%) for the mutant target system in the presence of dCTP, affording a signal gain of ∼12-fold with comparison to those for noncomplementary nucleotides and mismatched target. Similar observations were obtained with 2 nM wild-type target **7** (single nucleotide variation in sequence versus mutant target **3**), in which addition of dCTP, dATP, or dGTP did not yield significant current signals, while the introduction of dTTP gave remarkable current peaks in CV and DPV curves, suggesting that the SNP site of the wild-type target was A. Therefore, it was clear that the pattern of voltammetric signals for varying nucleotides was able to signify the single nucleotide variations in DNA target, evidencing that the developed strategy provided a specific platform for SNP genotyping.

Figure 2 gives typical DPV responses of the genotyping technique to mutant sequence **3** of varying concentrations with the addition of dCTP. Dynamically increased DPV peaks in response to mutant target **3** of increasing concentrations within the range from 0.2 fM to 2 nM were observed. A high dose−response sensitivity over 20 nA/decade was obtained in a six-decade concentration range from 0.2 fM to 0.2 nM with a readily achieved detection limit of 0.1 fM, amounting to ∼3000 DNA molecules in 50 μL samples. Such high sensitivity allowed the developed strategy to be implemented directly for SNP typing of genome samples without preliminary amplification steps. Further results from detecting mixtures of mutant sequence **3** and wild-type target **7** as well as samples merely containing wild-type target **7** in the presence of dCTP are shown in Supporting Information, Figure S1. It was clear that up to 10^6-

fold wild-type target had little effect on scoring the mutant sequence, and in the absence of the mutant sequence no significant DPV signals (~5 nA) were obtained even with 20 nM wild-type target. The findings demonstrated that the technique afforded extremely high specificity, which might hold potential as an effective mutation detection technology for solid tumor-based cancer research.[2]

A close inspection of the CV currents versus different scan rates in genotyping the mutant sequence **3**, as shown in Figure S2, revealed that the peak current increased in linear correlation to the scan rate, a typical feature of surface-bound electrochemical processes, confirming that Fc was confined to the electrode surface. Impedance analysis of the surface hybridization reactions, as depicted in Figure S3, demonstrated that cooperative annealing of ligated products with a 5′ Fc tag resulted in dramatically decreased Faraday impedance, indicators for surface confinement of Fc labels that exhibited facilitated electron transfer kinetics. With the 5′ Fc tag replaced by the amino moiety in probe **1** in the gap ligation reaction, the electrochemical impedance was observed to increase substantially on surface hybridization of the ligated products. This gave immediate evidence for the surface binding events, since in this case the ligated products only had amino terminus with no redox labels in close contact to the electrode. The introduction of a noncomplementary nucleotide merely induced trivial variation of the electrochemical impedance, implying extremely low efficiency in annealing of unligated probes to the capture probes.

The electrochemical genotyping strategy was further validated by using human genomic DNA with SNP (A > G) at the −28 position in human β-globin gene. The 602-bp amplicons were generated by PCR from eight DNA samples, as comfirmed by the 1% agarose gel image shown in Figure S4. The genotypes of these samples were previously characterized by DNA sequence analysis. The amplicons were directly employed for SNP assay using the developed technique. Figure S5 depicts the characteristic DPV signals obtained for these amplicons in the presence of one of the four nucleotides. From these DPV patterns the genotypes of the samples could be unequivocally identified, that is, samples 1, 4, and 6 were in normal state (gene has no mutation at −28 site on both chromosomes), samples 2, 5, 7 and 8 were heterozygous (gene is mutant on one chromosome, and not on the other chromosome), and sample 3 was homozygous mutant (gene is mutant on both chromosomes). These results were in good agreement with the sequencing data, which clearly revealed that the genotyping strategy might become a promising technique for genomic research.

In conclusion, we developed a highly specific and sensitive electrochemical genotyping technique based on gap ligation reaction and surface hybridization detection. This technique utilized homogeneous enzymatic reactions to generate molecular beacon-structured allele-specific products that could be selectively and sensitively detected via surface hybridization with redox tags close to electrode surface. Such a unique biphasic architecture created a universal methodology for incorporating enzymatic discrimination reactions in electrochemical genotyping with desirable reproducibility, high efficiency, and no interferences from interficial steric hindrance. Also, multiplex detection of multiple SNPs could be implemented in densely packed array format with a specific barcode sequence designed for each SNP site. Because disulfide anchors of surface-immobilized capture probes were resistant to thiol compound displacement and heating treatment, the developed technique could be further adapted in a close-tube format using miniaturized three-electrode systems integrated in a thermal cycler, thereby allowing this technique to be easily automated and parallelly implemented for hundreds of samples. In view of these advantages, this new electrochemical genotyping strategy was expected to afford an intrinsically robust and specific genotyping platform for genetic diagnosis and association studies.

Acknowledgment. This work was supported by "973" National Key Basic Research Program (2007CB310500) and NSF (20875027, U0632005) of China.

Supporting Information Available: Detailed description of experimental procedures and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Syvänen, A.-C. *Nat. Rev. Genet.* **2001**, *2*, 930–942. (b) Michael, S.; Andreas, M. *Angew. Chem., Int. Ed.* **2005**, *44*, 7842–7849.
(2) (a) Kirk, B. W.; Feinsod, M.; Favis, R.; Kliman, R. M.; Barany, F. *Nucleic Acids Res.* **2002**, *30*, 3295. (b) Kwok, P.-Y. *Annu. Rev. Genomics Hum. Genet.* **2001**, *2*, 235–258. (c) Kim, S.; Misra, A. *Annu. Rev. Biomed. Eng.* **2007**, *9*, 289–320.
(3) (a) Yu, C. J.; Wan, Y.; Yowanto, H.; Li, J.; Tao, C.; James, M. D.; Tan, C. L.; Blackburn, G. F.; Meade, T. J. *J. Am. Chem. Soc.* **2001**, *123*, 11155–11161. (b) Xiao, Y.; Qu, X.; Plaxco, K. W.; Heeger, A. J. *J. Am. Chem. Soc.* **2007**, *129*, 11896–11897.
(4) (a) Hirschhorn, J. N.; Sklar, P.; Lindblad-Toh, K.; Lim, Y.-M.; Ruiz-Gutierrez, M.; Bolk, S.; Langhorst, B.; Schaffner, S.; Winchester, E.; Lander, E. S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 12164–12169. (b) Steemers, F. J.; Chang, W.; Lee, G.; Barker, D. L.; Shen, R.; Gunderson, K. L. *Nat. Methods* **2006**, *3*, 31–33.
(5) (a) Lyamichev, V.; Mast, A. L.; Hall, J. G.; Prudent, J. R.; Kaiser, M. W. *Nat. Biotechnol.* **1999**, *17*, 292–296. (b) Chen, Y.; Shortreed, M. R.; Peelen, D.; Lu, M.; Smith, L. M. *J. Am. Chem. Soc.* **2007**, *129*, 4154–4155.
(6) (a) Lizardi, P. M.; Huang, X.; Zhu, Z.; Bray-Ward, P.; Thomas, D. C.; Ward, D. C. *Nat. Genet.* **1998**, *19*, 225–232. (b) Ogasawara, S.; Fujimoto, K. *Angew. Chem., Int. Ed.* **2006**, *45*, 4512–4515.
(7) (a) Wabuyele, M. B.; Farquar, H.; Stryjewski, W.; Hammer, R. P.; Soper, S. A.; Cheng, Y.-W.; Barany, F. *J. Am. Chem. Soc.* **2003**, *125*, 6937–6945. (b) Duan, X.; Li, Z.; He, F.; Wang, S. *J. Am. Chem. Soc.* **2007**, *129*, 4154–4155.
(8) (a) Tost, J.; Gut, I. G. *Mass Spectrom. Rev.* **2002**, *21*, 388–418. (b) Birikh, K. R.; Korshun, V. A.; Bernad, P. L.; Malakhov, A. D.; Milner, N.; Khan, S.; Southern, E. M.; Shchepinov, M. S. *Anal. Chem.* **2008**, *80*, 2342–2350.
(9) Patolsky, F.; Lichtenstein, A.; Willner, I. *Nat. Biotechnol.* **2001**, *19*, 253–257.
(10) (a) Liu, G.; Lee, T. M. H.; Wang, J. *J. Am. Chem. Soc.* **2005**, *127*, 38–39. (b) Liu, G.; Lin, Y. *J. Am. Chem. Soc.* **2007**, *129*, 10394–10401.
(11) (a) King, G. C.; Giusto, D. A. D.; Wlassoff, W. A.; Giesebrecht, S.; Flening, E.; Tyrelle, G. D. *Hum. Mutat.* **2004**, *23*, 420–425. (b) Wakai, J.; Takagi, A.; Nakayama, M.; Miya, T.; Miyahara, T.; Iwanaga, T.; Takenaka, S.; Ikeda, Y.; Amano, M. *Nucleic Acids Res.* **2004**, *32*, e141. (c) Wu, Z.; Jiang, J.-H.; Shen, G. L.; Yu, R. Q. *Hum. Mutat.* **2007**, *28*, 630–637.
(12) Zhang, Y. L.; Huang, Y.; Jiang, J.-H.; Shen, G. L.; Yu, R. Q. *J. Am. Chem. Soc.* **2007**, *129*, 15448–15449.
(13) (a) Hardenbol, P.; Baner, J.; Jain, M.; Nilsson, M.; Namsaraev, E. A.; Karlin-Neumann, G. A.; Fakhrai-Rad, H.; Ronaghi, M.; Wiuis, T. D.; Lemdegren, U.; Davis, R. W. *Nat. Biotechnol.* **2003**, *21*, 673–678. (b) Stewart, J.; Kozlowski, P.; Sowden, M.; Messing, E.; Smith, H. C. *Nucleic Acids Res.* **1998**, *26*, 961–966.
(14) (a) Tyagi, S.; Kramer, F. R. *Nat. Biotechnol.* **1996**, *14*, 303–308. (b) Tan, W.; Wang, K. M.; Drake, T. J. *Curr. Opin. Chem. Biol.* **2004**, *8*, 547–553.
(15) Li, J. J.; Geyer, R.; Tan, W. *Nucleic Acids Res.* **2000**, *28*, e52.
(16) (a) Letsinger, R. L.; Elghanian, R.; Viswanadham, G.; Mirkin, C. A. *Bioconjugate Chem.* **2000**, *11*, 289–291. (b) Sharma, J.; Chhabra, R.; Andersen, C. S.; Gothelf, K. V.; Yan, H.; Liu, Y. *J. Am. Chem. Soc.* **2008**, *130*, 7820–7821.
(17) (a) Gerry, N. P.; Witowski, N. E.; Day, J.; Hammer, R. P.; Barany, G.; Barany, F. *J. Mol. Biol.* **1999**, *292*, 251–262. (b) Hill, H. D.; Mirkin, C. A. *Nat. Protoc.* **2006**, *1*, 324–336.
(18) Compared with ligase detection reaction, the described technique offers enhanced specificity and reduced sets of probes at a cost of requiring two to four pot reactions each using one of the four dNTPs. Both strategies have high multiplexing capability with properly designed probes.

JA808700D