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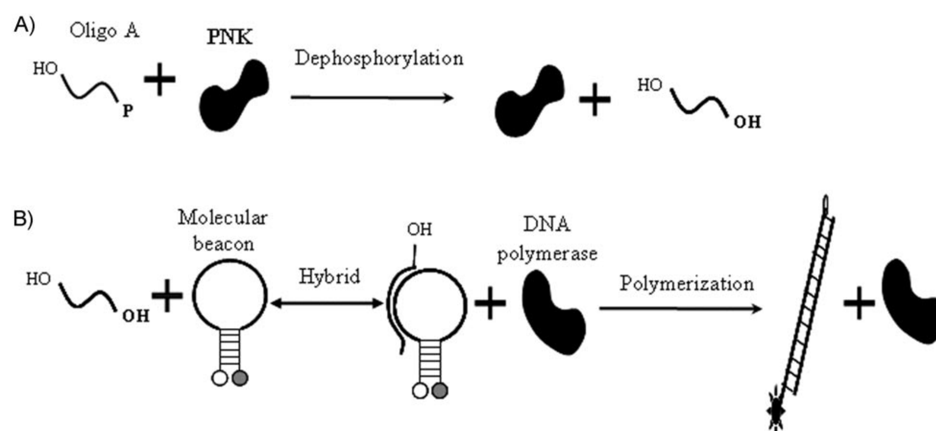
# Real-Time Monitoring of Nucleic Acid Dephosphorylation by Using Molecular Beacons

Changbei Ma, Zhiwen Tang, Kemin Wang,* Weihong Tan, Xiaohai Yang, Wei Li, Zhaohui Li, Huimin Li, and Xiaoyuan Lv[a]

Dephosphorylation of the 3′ termini of nucleic acids is important for cellular events, such as DNA replication, recombination, and repair of DNA damage induced by a variety of genotoxic agents, which include ionizing radiation,[1] certain antineoplastic alkylating agents,[2] bleomycin,[3] and topoisomerase inhibitors.[4] Dephosphorylation of the 3′ phosphate is usually catalyzed by various repair enzymes, which include the commonly used T4 polynucleotide kinase (T4 PNK). Since the identification of T4 PNK over 40 years ago,[5] T4 PNK has exemplified a family of bifunctional enzymes with 5′-kinase and 3′-phosphatase activities, and has become a molecular biology workhorse as well as an invaluable research tool in biology and bioengineering.[6,7] Its 3′-phosphatase activity allows T4 PNK to dephosphorylate the 3′ termini of DNA molecules. This converts the DNA to a 3′-hydroxyl substrate that is required for DNA polymerases and ligases, which play important roles in RNA and DNA repair. Traditionally, $^{32}$P radiolabeling, PAGE, and autoradiography[8–11] are used to assay the dephosphorylation of nucleic acids. These methods are discontinuous, time consuming, laborious, require radiolabeled substrates, and cannot be used to analyze the rapid continuous dephosphorylation processes. Recently, Dobson and Allinson used fluorescent probes in combination with denaturing PAGE to study human PNK,[12] but this method also suffers from being discontinuous. There are many advantages to using real-time assays instead of discontinuous ones, particularly in dissecting the molecular mechanisms of enzymes and their regulation. Continuous assays are relatively less laborious than discontinuous assays and they save time. Furthermore, they can be easily adjusted and optimized for high-throughput systems. Therefore, to investigate the dephosphorylation of nucleic acids in real time it is necessary to develop simple and sensitive methods that do not require isotopic labeling.

In this paper, a novel method is described for real-time monitoring of the dephosphorylation process by using molecular beacon (MB) DNA probes. Since they were first reported in 1996,[13] MBs have become a class of DNA probes that are widely used in chemistry, biology, biotechnology, and medical sciences for biomolecular recognition, due in part to their high sensitivity and excellent specificity.[14,15] Previously, we developed techniques for real-time monitoring of nucleic-acid ligation and phosphorylation using MBs;[16–18] these provided powerful systems for exploring the interactions between nucleic acids and proteins (enzymes). However, these methods are complex. Here, we describe the development of a new technique based on a polymerase-extension reaction to monitor nucleic acid dephosphorylation with MBs in real time. This approach is very simple, rapid, cost effective, and requires only one oligonucleotide. This method is schematically represented in Scheme 1.




**Scheme 1.** Schematic representation of the method used for monitoring nucleic-acid dephosphorylation with MBs. A) Oligo A with the 3′ phosphate (P) was dephosphorylated with T4 polynucleotide kinase (PNK). B) Dephosphorylated Oligo A was hybridized with the molecular beacon (MB), and then polymerized by DNA polymerase; this resulted in a fluorescent signal from the MB probe.

Oligonucleotide A (Oligo A) is dephosphorylated and hybridized with the MB to form a oligonucleotide–MB duplex. In the presence of DNA polymerase dephosphorylated Oligo A can be elongated by polymerization; this opens the MB and restores its fluorescence. In these procedures, the DNA polymerase plays a role as a converter that transforms the information of "oligonucleotide has been dephosphorylated" into restoration of fluorescence in the MB. Thus, dephosphorylation of an oligonucleotide can be monitored in real time by using this "dephosphorylation and polymerization" enzyme-coupled reaction. By utilizing the excellent selectivity and high sensitivity of MBs and the rapid polymerization feature of DNA poly-

[a] C. Ma, Dr. Z. Tang, Prof. K. Wang, Prof. W. Tan, Prof. X. Yang, W. Li, Z. Li, H. Li, X. Lv
State Key Laboratory of Chemo/Biosensing and Chemometrics
College of Chemistry and Chemical Engineering
Engineering Center for Biomedicine
Key Laboratory for Bionantechnology and
Molecular Engineering of Hunan Province
Hunan University, Changsha 410082 (China)
Fax: (+86) 731-882-1566
E-mail: kmwang@hnu.cn

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merase, this approach can offer selective, sensitive, and simultaneous information about the dephosphorylation process.

To demonstrate the feasibility of this method, three samples were prepared: sample A contained MB1 and Oligo C, which is complementary to almost the full length of the loop in MB1; sample B contained MB1 and Oligo A, which binds to the 3′ end of the loop on MB1; sample C contained MB1 only. As illustrated by curve A, which corresponds to sample A (Figure 1), when Oligo C annealed to MB1 the beacon was unlocked and a fluorescent signal was generated. Consequently, after addition of the Klenow fragment exo$^-$ and T4 PNK to sample A at $t_1$ and $t_2$, respectively, there was no fluorescence change. This result implies that the MB1–DNA duplex was not affected by Klenow fragment exo$^-$ and T4 PNK. In sample C (Figure 1, curve C) fluorescence did not change after the addition of Klenow fragment exo$^-$ and T4 PNK. This result implies that the enzymes did not affect MB1's conformation. The only factor that affected the conformation of MB1 and altered its fluorescence was the presence of the phospohorylated target DNA, Oligo A, which hybridized with MB1 and acted as substrate for T4 PNK and Klenow fragment exo$^-$.

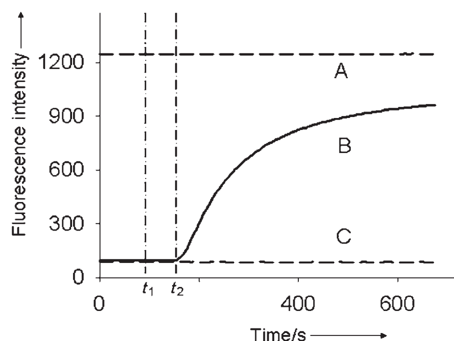


**Figure 1.** Monitoring nucleic-acid dephosphorylation in real time. Sample contained A) MB1 and Oligo C, B) MB1 and Oligo A, C) MB1 only. At time $t_1$, 2.0 U of Klenow fragment exo$^-$ was added to the samples; at time $t_2$, 1.0 U of T4 PNK was added.

In sample B (Figure 1, curve B) the fluorescence did not change after the addition of Klenow fragment exo$^-$ at $t_1$. However, after the addition of T4 PNK at $t_2$ the fluorescence rapidly increased; this implies that MB1 hybridized with the target oligonucleotide and acted as substrate for T4 PNK. According to the mechanism demonstrated in Scheme 1, the "dephosphorylation and polymerization" enzyme-coupled reaction took place, and the newly produced oligonucleotide could "stretch" MB1. Based on these data, the dephosphorylation of nucleic acids can be monitored in real-time with this approach.

To analyze the 3′-phosphatase activity of T4 PNK, we assumed that the initial velocity of the polymerase reaction was sufficiently high so as to have no effect on the overall measured rate. A series of reactions that contained various amounts of T4 PNK were investigated (Figure 2A). The results indicate that the fluorescence-enhancement rate increased in response to increasing concentrations of T4 PNK. Consistent with our hypothesis, when the enzyme concentration ranged from 0.1 to 15.0 U mL$^{-1}$, the initial velocity of fluorescence increased proportionally to the concentration of T4 PNK, and the detection time was only 5 min. The detection limit for T4 PNK was determined to be 0.1 U mL$^{-1}$ based on the 5 min reaction. This assay was convenient, rapid, and sensitive, and had a wide detection range, which makes it a useful approach for analyzing the 3′-phosphatase activity of T4 PNK.

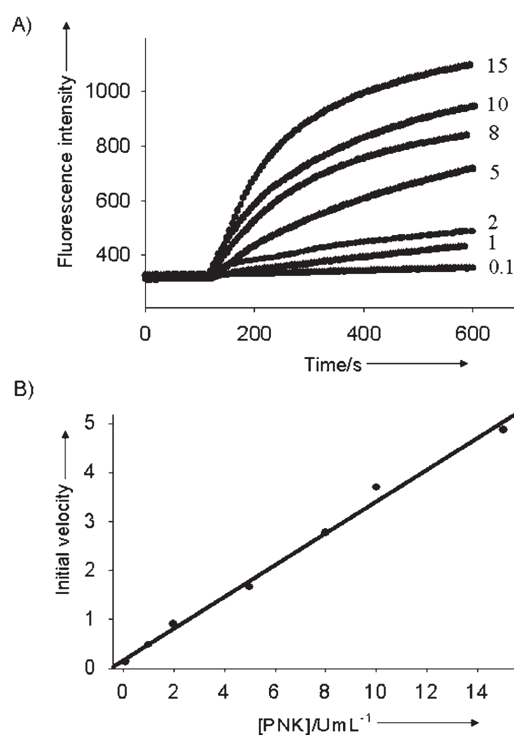


**Figure 2.** A) Time courses for real-time monitoring of the dephosphorylation process catalyzed by various concentrations of T4 PNK (U mL$^{-1}$). B) Relationship between initial reaction velocity and concentration of T4 PNK. Assays were carried out at 37 °C in a final volume of 100 µL and contained MB1 (400 nM), Oligo A (400 nM), and Klenow fragment exo$^-$ (2.0 U).

To further verify our hypothesis, a polymerase reaction mixture that contained MB1 and Oligo B (equivalent to unphosphorylated Oligo A) was incubated under the same conditions as described in Figure 2A. As shown in Figure 3, it is clear that the polymerase reaction was far more rapid than the phosphatase activity; these results support our hypothesis.

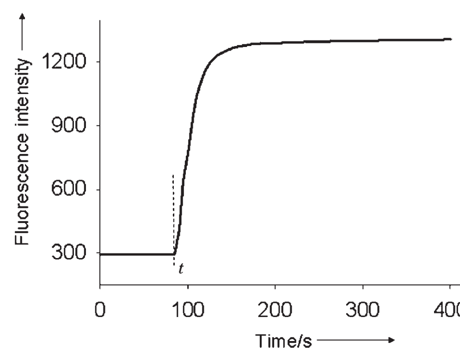


**Figure 3.** Monitoring of the polymerase reaction. Assays were carried out at 37 °C in a final volume of 100 µL, and contained MB1 (400 nM) and Oligo B (400 nM). At time $t$, 2.0 U of Klenow fragment exo$^-$ was added to the sample.

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This assay is suitable for screening the effects of inhibitor compounds on the phosphatase activity of T4 PNK. Heparin, a drug commonly used for unstable angina and silent ischemia, is reported to be an inhibitor of kinase[19] and leukocyte acid phosphatase.[20] To test whether the phosphatase activity of T4 PNK could be inhibited by heparin, we carried out a phosphatase assay in the presence of varying concentrations of heparin. The phosphatase activity of T4 PNK was monitored by an initial fluorescence-enhancement rate, which was calculated from a curve obtained by real-time monitoring of the polymerization reaction. The activity of Klenow fragment exo⁻ was not found to be affected with heparin concentrations below 800 nM. Therefore, the effect of heparin on the phosphatase activity of T4 PNK was directly measured. As shown in Figure 4,

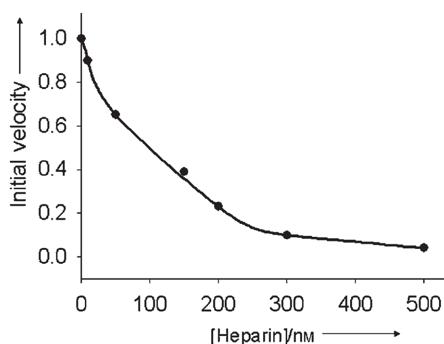


**Figure 4.** Inhibition of T4 PNK activity by heparin. Assays were carried out at 37 °C in a final volume of 100 μL, and contained MB1 (200 nM) and Oligo A (250 nM). The amounts of Klenow fragment exo⁻ and T4 PNK were 2.0 U and 1.0 U, respectively.

the phosphatase activity of T4 PNK was inhibited by heparin. This might be due to the binding of heparin to the C-terminal phosphatase domain of T4 PNK,[6] although the mechanism of inhibition has not been well studied. The $IC_{50}$ value was estimated to be about 110 nM, and the phosphatase activity of T4 PNK was inhibited by more than 90 % when the concentration of heparin was higher than 500 nM. Therefore, we have shown that the assay is suitable for the identification and characterization of enzyme inhibitors.

In summary, we have developed a novel approach for real-time monitoring of nucleic-acid dephosphorylation catalyzed by T4 PNK by using MBs. T4 PNK and MB DNA probe were used to detect nucleic-acid dephosphorylation. Compared to current assay methods, this approach is simple, rapid, and highly sensitive, and takes advantage of MBs and DNA polymerase. It also has excellent specificity for the DNA substrate and is capable of real-time monitoring in homogenous solutions. It provides a new tool for studying the interactions between proteins and nucleic acids, such as in DNA replication, recombination, and repair, as well as in inhibitor screening.

## Experimental Section

**Materials**: MB1 probe (5′-TAMRA–CCTCTCCGTGTCTTGTACTTCCCGTCAGAGAGG–DABCYL-3′), Oligo C (5′-GACGGGAAGTACAAGACAC-3′), Oligo A (5′-GACGGGAAG-P-3′; where P is phosphate), Oligo B (5′-GACGGGAAG-3′), and T4 PNK were purchased from Dalian Takara Bio Inc. (Dalian, China). Klenow fragment exo⁻ was purchased from MBI Fermentas (Vilnius, Lithuania). Heparin was purchased from Amresco (Solon, OH, USA). All other reagents were of analytical grade. Deionized water was obtained through a Nanopure Infinity™ ultrapure water system (Barnstead/thermolyne Corp., Dubuque, IA, USA).

**Fluorescence measurements**: All fluorescence measurements of samples were carried out by using an F2500 (Hitachi, Japan) with excitation at 521 nm and emission at 578 nm for the TAMRA label at the 5′ end of MB1. Assays were carried out in a final volume of 100 μL under optimized conditions: Tris-HCl (50 mM, pH 8.0), $MgCl_2$ (10 mM), DTT (1 mM), and dNTPs (50 μM). Each sample was incubated at 37 °C in a water bath (Amersham) for about 8 min so that a steady state was reached before the addition of Klenow fragment exo⁻. After this the samples were incubated until the fluorescence reached equilibrium, then T4 PNK was added to the solution and mixed for 4 s, and the fluorescence intensity of the samples were recorded simultaneously.

**Monitoring of the dephosphorylation process**: Three samples were prepared: sample A contained MB1 (200 nM) and Oligo C (250 nM); sample B contained MB1 (200 nM) and Oligo A (250 nM); sample C contained MB1 (200 nM) only. The fluorescence difference between the samples demonstrated the capability of MB1 to hybridize with the different oligonucleotides.

In experiments for the real-time monitoring of nucleic acid dephosphorylation, the aforementioned three samples were incubated at 37 °C with the sequential addition of Klenow fragment exo⁻ (2.0 U) and T4 PNK (1.0 U).

**3′-phosphatase activity assay of T4 PNK**: To assay the T4 PNK 3′-phosphatase activity, samples were prepared with MB1 (400 nM) and Oligo A (400 nM). Before the addition of T4 PNK at various concentrations (0.1–15.0 U mL$^{-1}$), Klenow fragment exo⁻ (2.0 U) was added to each sample. The dephosphorylation velocities of each sample were evaluated by analyzing the time courses.

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**Keywords:** activity assay · dephosphorylation · DNA detection · molecular beacons · real-time assays

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