Efficient Detection of Membrane-Bound Nucleic Acids Using New Fluorescein Derivative

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A number of fluorescein derivatives were synthesized and their fluorescent properties were studied. Phosphates of the fluorescein derivatives were tested for the alkaline phosphatase-linked fluorescence assay of membrane-bound DNA and were found to give high resolution for detection of DNA, in which DNA was detectable to the amount of 5 fg in the spot test and 0.1 pg in Southern blot hybridization .

Development of a convenient and efficient method for DNA diagnosis is of prime importance. The radioactive method[1] has been hitherto widely used for this purpose. In order to avoid using radioactive substances, the enzymatic method using chemiluminescent substrates such as adamantyldioxetane phosphate (CSPD)[2] has been developed for detection of membrane-bound nucleic acids. This method is as sensitive as the RI method for the detection of nucleic acid. However, fast decay of chemiluminescence limits the way of recording and generally X-ray films are used for this purpose, but its narrow dynamic range requires cumbersome operations such as multi-step exposure of the DNA bands to the X-ray film. We have been working to develop more efficient nonradioactive methods using fluorogenic substrates. Although fluorescence assays of the DNA sequence have been studied,[3] there are few reports on the fluorescence immunoassay, *e.g.*, europium[4] or terbium chelates[5] were used to detect DNA hybrids in solutions, but were not applied to membrane-bound nucleic acids. All these methods were based on the use of water-soluble fluorescent compounds. Recently, we reported the first successful study on a fluorescence immunoassay for detection of membrane-bound nucleic acids using phosphates derived from a number of hydroxynaphthalene- and hydroxyanthracenecarboxamides as fluorogenic substrates for alkaline phosphatase (ALP).[6] In our search for fluorogenic substrates which would give, after hydrolysis with ALP, strong fluorescence different in color from the above fluorochromes, we now wish to report a highly sensitive detection of DNA on the nylon membrane with the use of phosphates of fluorescein derivatives as substrates for ALP.

Fluorescein is known as an extremely high fluorescent compound.[7] Its high water-solubility,[8] however, has so far precluded application to the detection of membrane-bound nucleic acids in which high substantivity of the fluorochrome on the membrane is essentially required. We, therefore, planned to prepare a number of fluorescein derivatives which would be minimally soluble in water as well as highly fluorescent. Among the 5-aminofluorescein derivatives we prepared, N-(4-biphenylcarbony)-5-aminofluorescein **1**, prepared from 5-aminofluorescein, was found to show excellent properties; its fluorescence intensity was 100 times higher than that of the parent 5-aminofluorescein. In order to reduce the water-soluble property and to enhance substantivity on the membrane, one of the phenolic OH was converted to an ether linkage. Thus, 3'-O-aralkyl-5-aminofluorescein derivatives **2** were prepared by treatment of **1** with aralkyl bromide in the presence of K_2CO_3 in dimethylformamide at room temperature, giving 3'-O-(2,4-dimethylphenyl)methyl-, 3'-O-(2,4,6-trimethyl- phenyl)methyl-, 3'-O-(3,4-dimethylphenyl)methyl-, 3'-O-(1-naphthyl)methyl-, and 3'-O-(3-indolyl)ethyl-5-aminofluorescein derivatives[9] **2a, 2b, 2c, 2d,** and **2e** in high yields as depicted in Eq. 1. It is noteworthy that no formation of bisalkylated compounds was observed under these conditions.[10] 3'-O-Aralkyl-5-aminofluorescein derivatives **2a**, **2c**, **2d** and **2e** showed

Table 1. The DNA detection using monophosphates **3**

compound	relative fluorescence intensity of **2**[a]	DNA detection limit, fg	substantivity	nonspecific adsorption
3a	1.1	5	high	moderate
3b	0.31	5	high	moderate
3c	1.2	5	very high	low
3d	1.2	5	very high	low
3e	1.2	80	low	high

[a] The fluorescence intensities of the corresponding hydrolyzed forms **2a-2e** measured in 5×10^{-6} M NaH_2PO_4/NaOH (pH 11.0) 0.5% DMF are shown as relative values to that of FITC. The excitation and emission maxima were 492 and 512 nm, respectively.

1 → 2: 1) 1.1 eq RBr, 1.0 eq K_2CO_3, DMF, r. t., 4h; 2) 6N HCl, r. t., 10min (yield=89-99%)
2 → 3: 1) 1 eq $POCl_3$, pyridine, 0°C, 1h; 2) H_2O (yield=70-80%) (1)

a: R=—CH_2-(2,4-dimethylphenyl) **b:** R=—CH_2-(2,4,6-trimethylphenyl) **c:** R=—CH_2-(3,4-dimethylphenyl) **d:** R=—CH_2-(1-naphthyl) **e:** R=—$(CH_2)_2$-(3-indolyl)

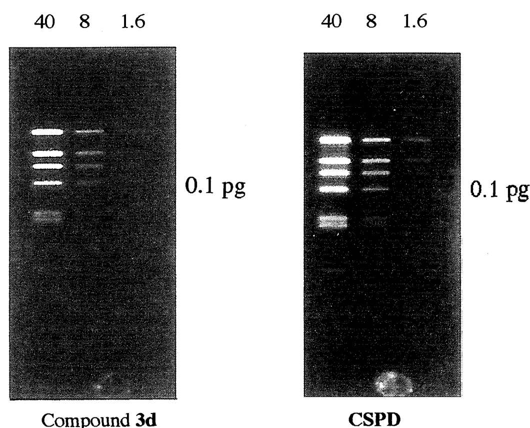

Figure 1. Southern blot hybridization using λDNA digested HindIII (40, 8, and 1.6 pg). From the top bands (base pairs), 23,130; 9,416; 6,557; 4,361; 2,322; 2,027; 564.

fluorescence a little higher than FITC in 3×10^{-7} mol dm^{-3} phosphate buffer (pH 11.0) containing 0.5% DMF,[1] whereas **2b** had weak fluorescence. These derivatives had excitation and emmision maxima in similar regions. The fluorescence intensities of **2** are shown in Table 1. Derivatives **2a-2e** were then efficiently converted to phosphates **3a-3e**, substrates for ALP, on treatment with phosphorusoxy chloride and pyridine.

We studied the DNA detection using the phosphates **3a-3e**, as substrates for ALP bound to the probe λDNA through the digoxigenin antibody. First, they were subjected to the spot test.[11] The detection limits for λDNA on the membrane using various fluorescein derivatives are shown in Table 1.

λDNA was found to be detectable to the amount of 5 fg by using phosphates **3a**, **3b**, **3c** and **3d**. The spots were slightly blurred by nonspecific adsorption in the case of the phosphate **3a**, whereas phosphates **3c** and **3d** gave distinguishably clear spots without diffusion and nonspecific adsorption. Fluorescence intensity is not always related to the sensitivity for the DNA detection, *e.g.*, the phosphate derivative **3b** having weak fluorescence, showed high sensitivity (5 fg DNA). On the contrary, the lowest sensitivity (80 fg) was obtained in the detection using the phosphate **3e**, **2e** showing strong fluorescence. This was due to both the low substantivity of its OH form and the high nonspecific adsorption which caused significant background signals. The high sensitivity in the detection of membrane-bound DNA is ascribed to not only the intrinsically strong fluorescence that fluorescein phosphate derivatives showed after hydrolysis with ALP, but also the high substantivity of the hydrolyzed compounds and the low nonspecific adsorption of the phosphates. In addition, it should be noted that phosphate derivatives revealed substantially non-fluorescence[12] and, thus, a highly efficient off-and-on system for fluorescence could be attained before and after hydrolysis of the phosphates with ALP .

The result of Southern blot hybridization[13] using the phosphate derived from 3'-O-(1-naphthyl)methyl derivative **3d** is shown in Figure 1. The result using CSPD is also shown for comparison. The fluorescence signals were recorded on a fluorescence detector, whereas the CSPD chemiluminescent signals were recorded on exposure on an X-ray film. DNA could be detected to the amount of 0.1 pg (3.6×10^{-20} mol) in both methods.[14] The operation transferring the target DNA to membrane and hybridizing the probe to the target DNA may bring about a reduction of the sensitivity of Southern blot hybridization in comparison with the results in the spot test.

In summary, the present study proved phosphates of N-(4-biphenylcarbonyl)-3'-O-aralkyl-5-aminofluoresceins to be excellent flurogenic substrates for alkaline phosphatase bound to the DNA probe, providing an efficient method for the detection of membrane-bound DNA. The high sensitivity for the DNA detection using fluorescein derivatives owes much to the non-fluorescent property of the phosphate form as well as the water-insoluble, highly fluorescent property of the fluorescein derivatives after hydrolysis with alkaline phosphatase. Furthermore, the alkaline phosphatase-linked fluorescence assay provides a convenient way of DNA detection because the high and long-lasting fluorescence enables the measurement by the ccd camera.

References and Notes

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