Highly Sensitive Biotin-Labelled Hybridization Probe

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A simple chemical method for introducing biotin into nucleic acids has been developed for the synthesis of non-isotopic hybridization probes. The method is based on the reaction of biotin hydrazide with amino residues of nucleic acids by using glutaraldehyde as a bifunctional coupling reagent.

Biotin-labelled deoxyribonucleic acid (DNA) was detected by the use of alkaline phosphatase-labelled avidin, and alkaline phosphatase activity was measured by colorimetric and chemiluminescence methods. The chemiluminescence method using the nicotinamide adenine dinucleotide phosphate (NADP)/alcohol/alcohol dehydrogenase/microperoxidase/isoluminol system gave the highest sensitivity. A few picograms of λ-phage DNA coated on a microtiter plate well could be detected by this method.

Keywords biotin hydrazide; nucleic acid; hybridization; non-isotopic DNA probe; chemiluminescence assay; alkaline phosphatase; NADP

Since the nucleic acid hybridization technique in gene analysis was developed by Southern in 1975,[1] the use of the technique has expanded into various areas. It is now being widely used in genetic research, diagnosis of genetic and infectious diseases, forensic science, plant breeding, oncology and other fields.[2] Probes are commonly labelled with a radioisotope, ^{32}P, ^{125}I or ^{3}H, but problems of stability, safety and cost arise. Recently, nonisotopic probes using biotin derivatives (Biotin-11-dUTP[3] and Photobiotin[4]) and enzyme (Labezyme[5]) as labels have been developed as alternatives to radioactive probes. Although various methods for labelling biotin derivatives have been reported, most of them are tedious and complex. In this report, a simple and rapid procedure for obtaining the biotin derivative of deoxyribonucleic acid (DNA) using biotin hydrazide and glutaraldehyde is presented. The labelling of DNA with biotin by this procedure does not inhibit annealing between the biotin-labelled DNA probe and its complementary DNA, and the hybridized probe can be detected by the use of enzyme–avidin conjugate. In order to increase the sensitivity of the assay, a chemiluminecsence method for measuring alkaline phosphatase activity was used. Using this assay system, we could detect as little as 3×10^{-19} mol of λ-phage DNA coated per well of a microtiter plate.

Experimental

Materials Biotin hydrazide, horseradish peroxidase (HRP)–avidin conjugate and alkaline phosphatase (ALP)–avidin conjugate were purchased from Bio-Yeda, Ltd., Rehout, Israel, and biotin-X-hydrazide was obtained from Calbiochem. La Jolla, U.S.A., Calf thymus DNA, Ficoll (Type 400), microperoxidase and dextran sulfate (sodium salt, molecular weight 5000) were purchased from Sigma Chemicals Co., St Louis, U.S.A., and glutaraldehyde, polyvinylpyrrolidone (PVP) and bovine serum albumin (BSA) were from Wako Pure Chemicals, Osaka, Japan. λ-Phage DNA (bacteriophage cI 857 S7) was obtained from Takara Co., Kyoto, Japan. 2,2′-Azino-di (3-ethylbenzthiazoline sulfate) (ABTS), *p*-nitrophenyl phosphate and nicotinamide adenine dinucleotide phosphate (NADP) were from Boehringer Mannheim-Yamanouchi Co., Tokyo, Japan, Isoluminol was from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan, and 1-methoxyphenazinium methylsulfate from Nakarai Chemicals, Ltd., Kyoto, Japan. Microtiter plates were obtained from Nunc Co., Roskilde, Denmark. Other chemicals were of reagent grade.

Instruments A microtiter plate reader (Titertek Uniskan; Flow Laboratories, Irving, Finland) and an Aloka luminescence reader (Aloka Co., Tokyo, Japan) were used.

Reagent Solutions Phosphate-buffered saline (PBS) consisted of 50 mM KH_2PO_4, 50 mM K_2HPO_4, 257 mM NaCl and 0.1% BSA (pH 7.0); coating buffer consisted of 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl and 0.1 M $MgCl_2$ (pH 7.2); 10× SSC consisted of 1.5 M NaCl and 0.15 M sodium citrate; 2× SSC was 5-fold diluted 10× SSC. Prehybridization solution was prepared by mixing 5.2 ml of 10× SSC, 2.6 ml of 0.32% PVP and 0.32% Ficoll solution, 2.6 ml of 0.32% BSA solution, 0.13 ml of 5 mg/ml heat-denatured calf thymus DNA solution and 2.47 ml of redistilled water. Hybridization solution was prepared by mixing 5.2 ml of 10× SSC, 2.6 ml of 0.32% PVP and 0.32% Ficoll solution, 2.6 ml of 0.32% BSA solution, 2.6 ml of 50% dextran sulfate, 0.13 ml of 5 mg/ml heat-denatured calf thymus DNA and 25 μl of 500 μg/ml biotin-labelled DNA probe. HRP–avidin conjugate and ALP–avidin conjugate solutions were prepared by 1000 and 20000-fold dilution with PBS, respectively.

Preparation of Biotin-Labelled DNA Probe DNA (1 mg) was dissolved in H_2O (1 ml) and denatured by heating at 95 °C for 5 min. After cooling for 5 min in an ice bath, 12.5 μl of biotin hydrazide (biotin-X-hydrazide) solution (10 mg/ml in H_2O) and 15 μl of 5% glutaraldehyde solution were added to 25 μl of the heat-denatured DNA solution (1 mg/ml). The mixture was incubated at 37 °C for 10 min, and then 5.83 μl of 3 M sodium acetate solution and 146 μl of cold ethanol were added. The resultant solution was cooled at −20 °C overnight. After centrifugation for 10 min at room temperature, the precipitated biotin-labelled DNA was washed with cold 80 v/v% ethanol, dried *in vacuo* for 2 h, redissolved in 50 μl of 0.1 mM ethylenediaminetetraacetic acid (EDTA) solution, and stored at 4 °C until use.

Hybridization In order to quantitate a specific DNA a microtiter plate was used as a solid support to immobilize the DNA. Immobilization of DNA in wells of the microtiter plate was carried out by the procedure reported by Nagata *et al.*[6] The heat-denatured DNA (10 pg—100 ng) dissolved in coating buffer (200 μl) was transferred into each well of a microtiter plate and incubated overnight at room temperature.

After removal of the solution by aspiration, the plate was used for hybridization without ultraviolet (UV) irradiation for securing the immobilization of DNA. To each well of the microtiter plate in which DNA was immobilized, 200 μl of prehybridization solution was added. The plate was covered with a shield, put in a plastic bag and incubated for 1 h at 65 °C by floating in a water bath. The prehybridization solution was removed by aspiration, then 200 μl of hybridization solution was added and the plate was incubated overnight at 65 °C. After aspiration of the solution, each well was incubated with 2× SSC at 65 °C for 30 min and then washed 3 times with 200 μl each of PBS.

Detection of Biotin Probe Hybridized to DNA To each well of the washed microtiter plate, 100 μl of HRP-avidin conjugate solution (× 1000) or ALP–avidin conjugate solution (× 20000) diluted with PBS was added and the plate was incubated for 4 h at room temperature. After incubation, each well was washed 2—4 times with 200 μl each of PBS, and then the enzyme–avidin conjugate bound to the hydridized DNA was measured by the following colorimetric or chemiluminescence method.

Colorimetric Method: The HRP activity was measured by the ABTS–H_2O_2 method.[7] To each washed well, 150 μl of 2.5 mM ABTS–5 mM H_2O_2

in 0.3 M citrate buffer (pH 4.0) was added. After incubation for 30 min at room temperature, the reaction was stopped by adding 50 μl of 0.1% NaN_3 solution and then the absorbance of each well was measured at 420 nm by using a microtiter plate reader.

The ALP activity was measured by the conventional method using *p*-nitrophenyl phosphate as the substrate. To each washed well, 200 μl of 1 mM *p*-nitrophenyl phosphate in 0.9 M diethanolamine buffer containing 0.01% $MgCl_2$ was added. After incubation for 1—2 h at room temperature, the reaction was stopped by adding 100 μl of 2 N NaOH and then the absorbance of each well was measured at 405 nm.

Chemiluminescence Method: The alkaline phosphatase activity was measured by the chemiluminescence assay of nicotinamide adenosine dinucleotide (NADH) generated from NADP.[8] To each washed well, 100 μl of 0.1 mM NADP solution in 50 mM diethanolamine buffer (pH 9.5) was added and the plate was incubated for 30 min at room temperature. After incubation, 200 μl of enzyme reagent (0.08 mg/ml alcohol dehydrogenase, 0.375% ethanol, and 1.25×10^{-5} M 1-methylphenazinium methyl sulfate in 25 mM phosphate buffer (pH 7.0) were mixed and the plate was incubated for a further 20 min at room temperature. After stopping the reaction by addition of 50 μl of 0.2 M sulfuric acid, 10 μl of the reaction mixture was added to a glass tube containing 200 μl of a chemiluminescent reagent (1:1, v/v mixture of 2.4×10^{-4} M isoluminol solution and 1×10^{-6} M micro-peroxidase in carbonate buffer (pH 9.5)).

The chemiluminescence intensity was measured for 6 s by an Aloka luminescence reader from 15 to 21 s after agitation of the chemiluminescent reagent.

Results

In order to determine the optimum conditions for preparing an active probe, various parameters (glutaraldehyde concentration, biotin hydrazide–DNA ratio, reaction time) were examined.

Effect of Glutaraldehyde Concentration Biotin-labelled DNA probes were prepared with various concentrations of glutaraldehyde solution ranging from 0.1% to 10%. The prepared probes were examined by hybridization to immobilized DNA in wells. As shown in Fig. 1, hybridization activity (absorbance at 420 nm) reached the maximum at 5% glutaraldehyde.

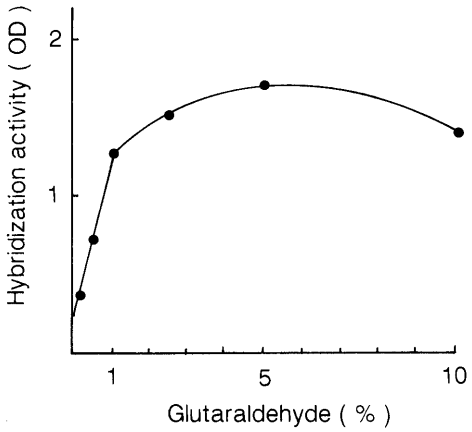

Fig. 1. Hybridization Activity of Biotinylated DNA Probe Prepared at Various Concentrations of Glutaraldehyde

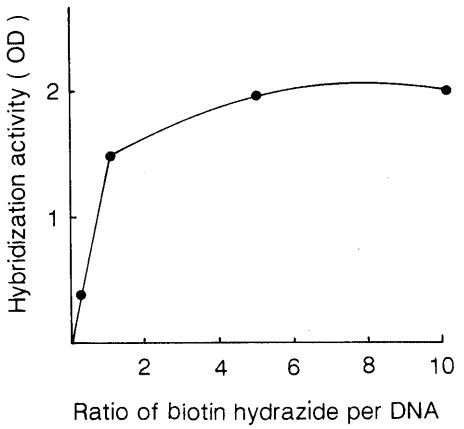

Fig. 2. Hybridization Activity of Biotinylated DNA Probe Prepared at Various Weight Ratios of Biotin Hydrazide to DNA

Effect of the Weight Ratio of Biotin Hydrazide to DNA Biotin-labelled DNA probes were prepared by varying the weight ratio of biotin hydrazide to DNA in the range from 0.1 to 10. As shown in Fig. 2, hybridization activity reached a plateau at the ratio of 5.

Effect of Reaction Time The effect of incubation time for conjugation of biotin hydrazide to DNA is shown in Fig. 3. The probe prepared by 10 min incubation showed the maximum hybridization.

From the above results, 5% glutaraldehyde, a biotin hydrazide: DNA ratio of 5:1 and an incubation time of 10 min were selected as the optimal conditions for preparing biotin-labelled DNA probes.

Determination of λ-Phage DNA λ-Phage DNA immobilized in the wells of a microtiter plate was determined by using the biotin-labelled DNA probe prepared by the method described above. The biotin-labelled DNA hybridized to the λ-phage DNA was assayed by the colorimetric method using HRP–avidin or ALP–avidin conjugate, and by the chemiluminescence method using ALP–

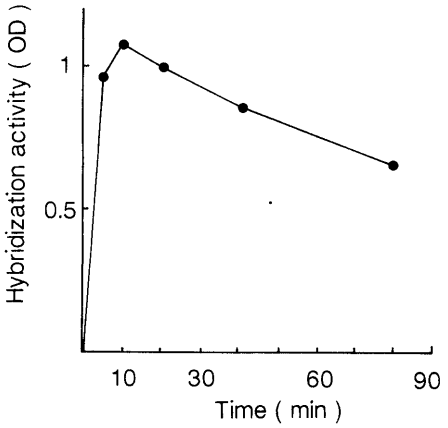

Fig. 3. Hybridization Activity of Biotinylated DNA Probe Prepared for Various Reaction Times at 36 °C

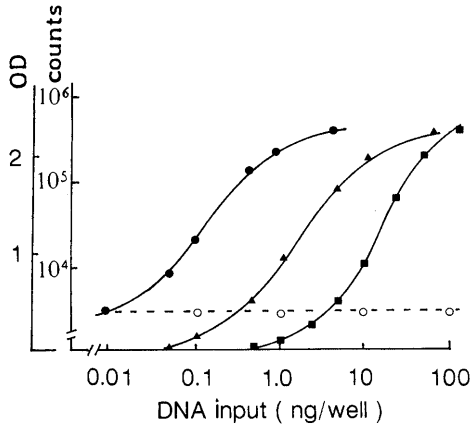

Fig. 4. Standard Curves of λ-Phage DNA

—●—, chemiluminescence (ALP–avidin); —▲—, colorimetry (ALP–avidin); —■—, colorimetry (HRP–avidin). The specificity of biotin-labelled λ-phage DNA probe was examined with various amounts of DNA of PBR 322 and HL-60 cells by the chemiluminescence method (ALP–avidin) (---○---).

TABLE I. Detection Limits of Hybridized DNA Probes

Labelling reagent	ALP–avidin		HRP–avidin
	Chemi-luminescence assay (mol)	Colorimetric assay[a] (mol)	Colorimetric assay[b] (mol)
Biotin hydrazide	10 pg (3×10^{-19})	50 pg (15×10^{-19})	500 pg (15×10^{-18})
Biotin-X-hydrazide	1 pg (3×10^{-20})	50 pg (15×10^{-19})	250 pg (7.5×10^{-18})

a) *p*-Nitrophenyl phosphate method. *b*) 2,2′-Azino-di(3-ethylbenzthiazoline sulfate) method.

avidin conjugate.

As shown in Fig. 4, typical standard curves of λ-phage DNA were obtained in the range from 250 pg to 100 ng/well by the colorimetric method and from 10 pg to 5 ng /well by the chemiluminescence method. Detection limits of λ-phage DNA were determined with various systems. The results are shown in Table I. Hybridization with the system using biotin-X-hydrazide-labelled DNA probe and ALP-avidin conjugate showed that 1 pg is detectable by the chemiluminescence method.

Specificity of Biotin-Labelled DNA Probe The specificity of biotin-labelled λ-phage DNA probe was examined. As shown in Fig. 4, the biotin-labelled λ-phage DNA probe did not hybridize with PBR322 and HEL-60 cell DNA immobilized to microtiter plate wells. The biotin-labelled DNA probe prepared by the method mentioned above hybridized specifically with the target DNA.

Discussion

Various non-isotopic DNA probes for hybridization assays have been reported.[9] Among them, the biotin-labelled DNA probe is the most suitable one because of its stability, sensitivity and detectability. In general, biotin-labelled probes have been prepared by the enzymatic incorporation of biotin derivatives of dUTP and UTP into DNA, or by photochemical reaction with a photo-activatable derivative of biotin, *N*-(4-azido-2-nitrophenyl)-*N*′-(*N*-*d*-biotinyl-3-aminopropyl)-*N*′-methyl-1,3-propanediamine (photobiotin). The reagent is commercially available as an assay kit. However, the enzymatic labelling procedure requires different protocols and enzymes for different types of nucleic acids.

Photobiotin must be kept in very subdued light before the coupling reaction, and the kits are expensive for routine assay. Recently, Reisfeld *et al.*[10] reported a new chemical method for introduction of biotin into DNA, based on the interaction of biotin hydrazide with unpaired cytosine residues. Though the sensitivity of the biotinylated probe prepared by this method is as high as that of any of the previously designed probes, the labelling procedure is not simple because of the long labelling reaction time, the need for strict adjustment of pH, and the dialysis step for purification. Therefore, a simple, rapid and cheap method for preparing a DNA probe is still required for routine diagnostic assays of nucleic acids by hybridization.

In a preliminary experiment, we attempted to prepare DNA probes by using biotin-*N*-hydroxysuccinimide ester and caproylamido biotin-*N*-hydroxysuccinimide ester. Highly active DNA probes could not be obtained with these esters. It seems that the coupling reaction between amino groups of nucleic acid and active esters of biotin derivatives is very poor.

In the present study, we used biotin hydrazide derivatives as the hapten and glutaraldehyde as the coupling reagent, respectively.

Extremely sensitive DNA probes could be prepared under mild reaction conditions. The biotin-labelled DNA prepared by the presented procedure did not inhibit the annealing between probe and complemetary DNA, and the hybridized biotin-labelled DNA probe could be detected by using avidin–enzyme conjugate.

Although the detection limits of hybridization assays using radioisotopes as the label go down to attomole levels of DNA, a lengthy autoradiographic step is needed. Horseradish peroxidase- and alkaline phosphatase-labelled probes can be detected by colorimetric methods, but the sensitivities are lower than those obtained by the radiometric method.

Recently, an enhanced chemiluminescence assay[11] has been adopted for hybridization assay using peroxidase as the label, and the detection limit attained the attomol level, comparable to that of the radiometric method.

The detection limits of the presented hybridization assay also attained the attomole level. In the case of the colorimetric method, the detection limit is about 15 amol, which is similar to those of published reports. On the other hand, the detection limit obtained with the chemiluminescence assay is 0.3 amol, which is superior to those of the methods reported previously.

The new biotin-labelling method using biotin-hydrazide derivatives and glutaraldehyde is very simple and inexpensive. The probe prepared by this method is highly active and stable. Moreover, a highly sensitive method has been established by using a plastic microtiter plate as the support for immobilization of DNA and a chemiluminescence method for detection of hybridized biotin-labelled DNA probe. Moreover, the proposed method has made it possible to determine a specific DNA and to assay a large number of samples at the same time by using a microtiter plate.

We are now attempting to develop this method for the diagnosis of infections diseases or genetic disorders.

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