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Time-resolved fluorometry: a sensitive method to quantify DNA-hybrids

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ABSTRACT

Europium and other lanthanides can be excitated with UV-radiation, whereafter the energy is released as fluorescence, delayed in time up to 1 ms after the excitation. Eu can be used as a sensitive label in biological assays. Here we report on the application of time-resolved fluorometry to detect nucleic acid hybrids. The probe DNA was tagged with a hapten, either a fluorene or a sulfone group. After hybridization the probe DNA was detected by a two-step immunological assay with the second antibody labelled with Eu. The method is quantitative with a detection limit of 0.3 pg of actual target regions of immobilized adenovirus genomic DNA. The label was also used in sandwich hybridization, which allowed analyzing nasopharyngeal mucus for the presence of adenovirus.

INTRODUCTION

Nucleic acid hybridization has become a method with widespread use not only in molecular biology, but also in applied fields like diagnostic medicine. This has created a need for stable, non-radioactive probes. So far the non-radioactive markers for hybridization probes have been: i) biotinylated nucleotide analogues, which are detected by avidin or streptavidin (1-3), ii) haptens for immunological detection (4,5) or iii) proteins crosslinked to the nucleic acid (6-8). In all of these procedures the final detection of the hybridized probe molecules is achieved by enzyme catalyzed colour development. Even if non-radioactively labelled probes can be used at high concentrations, which increases the hybridization rate, these detection procedures cannot compete with those using radioactively labelled nucleic acid probes (9-11) in terms of sensitivity or simplicity. Consequently alternative labelling and detection methods should be based on novel principles to replace the well established radioisotopes.

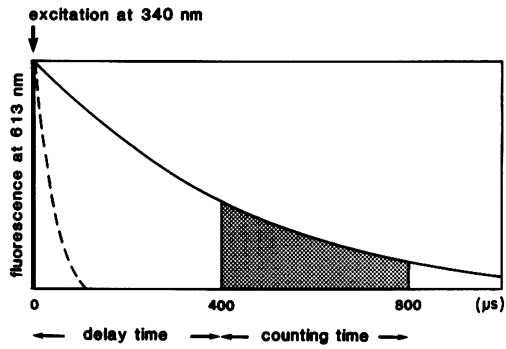

Figure 1.
Principle of the time-resolved fluorometric measurement. Fluorescent compounds with exceptionally long decay times (eg. chelates of the lanthanides) are excited with a light pulse and the emitted photons of the long-lived fluorescence (——) are counted. The measurement is done after a delay time, during which short-lived background fluorescence (---) decays (13). This cycle is repeated 1000 times per sample in 1 second.

Time-resolved fluorometry with europium (Eu) or some other lanthanide as label is theoretically more sensitive than radioisotopes. The exceptionally long fluorescent decay time of lanthanide chelates is the basis for time-resolved fluorometry. Eu-chelates are excited with UV-light pulses and the emitted fluorescence is measured by a photon counter after a delay time, during which short-lived fluorescence disappears (Figure 1). This background is a serious problem in fluorometric measurements of biological samples. (12,13)

Time-resolved fluorometry has been successfully applied to immunochemical assays. It has potentials to initiate a new generation of non-isotopic assays (14). In the time-resolved fluoroimmunoassays (TR-FIA) the Eu-label is bound to the antibodies with EDTA derivatives (15). After formation of the immunocomplex the specifically bound Eu is converted into a fluorescent chelate, which is measured in a time-resolved fluorometer.

Here we have applied time-resolved fluorometry to the detection of hybridized DNA. The method is based on introduction of antigenic groups into the probe DNA. After the hybridization reaction the probe DNA is detected immunochemically in two steps using a second antibody labelled with Eu. The method is quantitative and the sensitivity is ten-fold compared to the corresponding enzymatic detection method.

MATERIALS AND METHODS

Materials

The recombinant M13 bacteriophage (mKTH 1206) contains a 1.2 kb fragment and the pBR 322 plasmid (pKTH 1202) a 6.0 kb fragment of the adenovirus type 2 DNA (Ad2 DNA) (16). Rabbit antiserum against N-2-(guanosin-8-yl)acetylaminofluorene (anti-Guo-AAF) was obtained as described (17). IgG was isolated from the antiserum with Protein A-Sepharose CL-4B (18). A monoclonal anti-sulfone mouse antibody was obtained from Orgenics Ltd., Yavne, Israel. Eu-labelled anti-rabbit and anti-mouse IgG were purchased from LKB-Wallac, Turku, Finland, and anti-rabbit IgG conjugated to alkaline phosphatase from Orion Diagnostica, Espoo, Finland. Nasopharyngeal mucus aspirates from adult patients with acute respiratory infections were obtained from Dr. Kyösti Lehtomäki, Institute of Occupational Health, Helsinki, Finland.

Modification of probe DNA

5-7% of the bases in mKTH 1206 DNA were modified with 7-iodo-N-acetoxy-N-2-acetylaminofluorene (AAIF) essentially as described (4). At this level of modification no decrease in stability of hybrids formed by the AAIF-modified probe DNA can be observed in dot hybridization (4). Safe handling of AAIF was done as follows: newly synthesized AAIF was dissolved in 2-100 µg aliquots in 1.5 ml Eppendorf tubes. Aliquots were dried in a "Speed Vac" concentrator (Savant Instruments) and kept dessicated at -20°C under argon. 2 µg aliquots were used to modify 5 µg of DNA. Immediately prior to use 1 µl of ethanol was added to the tube containing the dessicated AAIF. Then 5 µg of the DNA to be modified dissolved in 20 µl of 2 mM sodium citrate, pH 7, was added. After incubation for 40 min at 37°C the unreacted AAIF was removed by extractions with cold diethyl ether, which was descarded into sulfochromic acid.

Sulfone groups were introduced into mKTH 1206, pKTH 1202 and uncloned Ad2 DNA using the "DNA Chemiprobe"-system according to the procedure of the manufacturer (Orgenics Ltd, Yavne,Israel).

Hybridization procedures

Ad2 DNA and plasmid pKTH 1202 were immobilized on nitrocellulose filters (BA 85, Schleicher and Schüll; diameter 4.4 mm) as described (11,16). Hybridizations to be detected by time-re-

solved fluorometry were done in individual polystyren microtiter wells in a reaction volume of 50 µl per sample with the filter discs carrying the immobilized target Ad2 DNA. In each hybridization a blank filter or a filter carrying 40 ng calf thymus DNA was included as control. Filters for enzymatic vizualization were hybridized in plastic bags using 0.1 ml reaction mixture per cm^2 of filter.

The filters were preincubated in 2 x SSC (SSC = 0.15 M NaCl, 15 mM sodium citrate), 0.1 % Ficoll 400, 0.1 % polyvinylpyrrolidone, 0.1 % glycine, at 65°C for at least two hours. Hybridization conditions were 2 x SSC, 0.02 % Ficoll, 0.02 % polyvinylpyrrolidone, 0.02 % glycine, 25 mM sodium phosphate, pH 7.5, 2 mM EDTA, 0.5 % sodium dodecyl sulphate (SDS), 65°C, (4). The AAIF-labelled M13 probe was used at a concentration of 1 µg/ml and the sulfone-modified DNA probes at 2 µg/ml and the hybridization time was usually 16 h. Sandwich hybridization was performed in the same conditions except that the filters carried plasmid pKTH 1202 DNA (400 ng) as capturing reagent, while denaturated target Ad2 DNA and the probe DNA were added to the reaction mixtures (16). After hybridization the filters were washed twice for 30 min in 0.1 x SSC, 0.2 % SDS at 50°C.

Immunochemical detection

The enzymatic detection of hybridized AAIF-modified DNA was performed essentially as described previously (4). The filters were saturated with 20 % horse serum (Behingwerke AG, Marburg, FRG) in 1 x SSC for 1 h at 20°C and incubated with anti-Guo-AAF serum diluted 1:200 in 20 % horse serum, 1 x SSC for 1 h at 20°C. After washing three times for 5 min in 1 % Triton X-100, 2 x SSC the filters were incubated for 1 h at 20°C with 1.7 µg/ml anti-rabbit IgG conjugated to alkaline phosphatase in 20 % horse serum, 1 x SSC and the filters were washed as above. The spots were visualized with 0.33 mg/ml nitro-blue tetrazolium (NBT) and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$.

All steps for detection by time-resolved fluorometry except the final wash were carried out in microtiter wells. The filters were saturated with 4 % bovine serum albumin in 0.15 M NaCl, 10 mM Tris-HCl, pH 7.8, for 1-1.5 h at 37°C and incubated for 1 h at 20°C with the first antibody at 1.4 µg/ml (anti-Guo-AAF IgG)

or 1:100 diluted (anti-sulfone antibodies) in 50 µl TR-FIA buffer (0.1 M Tris-HCl, pH 7.75, containing 0.15 M NaCl, 0.05% NaN_3, 0.5% bovine serum albumin, 0.05% bovine γ-globulin, 20 µM diethylenetriaminepentaacetic acid and 0.01% (v/v) Tween 40). After three 5 min washes at 20°C with 250 µl 0.05% Tween 20, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.8, the second incubation was performed with 0.5 µg/ml Eu-labelled IgG for 1 h at 20°C in 50 µl of the TR-FIA buffer. Finally the filters were washed three times for 20 min in 5 ml 0.1% Tween 20, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.8, per filter.

Measurement of europium fluorescence

To release the specifically bound Eu-label and develop the fluorescence the washed filters were incubated in 800 µl TR-FIA enhancement solution (0.1 M acetate-phtalate buffer, pH 3.2, containing 0.10% (v/v) Triton X-100, 15 µM 2-naphtoyltrifluoroacetone, 50 µM tri-n-octylphosphine oxide; LKB-Wallac) in polystyrene test tubes for 15 min with occasional mixing. The enhancement solution was decanted into another tube to dispose of the filter, and the fluorescence of the Eu-chelates was measured in an automatic time-resolved fluorometer ("Arcus" 1230, LKB-Wallac). The delay time was 400 µs and the counting time 400 µs per measurement cycle, which is repeated 1000 times giving a total measuring time of 1 s per sample. The excitation and measuring wavelengths were 340 nm and 613 nm, respectively.

RESULTS AND DISCUSSION

Here we describe a method to measure quantitatively the amount of hybridized nucleic acids based on the time-resolved fluorescence of europium. Eu can be bound to organic molecules by mediation of EDTA derivatives (15). These chelates are unstable at hybridization conditions (data not shown), why the probe DNA cannot be directly labelled with Eu-EDTA chelates. Therefore an indirect immunochemical detection method was used.

For the test the DNA probes were chemically modified with either immunogenic fluorene (4) or sulfone ("Chemiprobe"-system) groups. After hybridization these haptens were allowed to react with specific antibodies, which in turn were complexed to second antibodies labelled with Eu. Finally Eu was released from the complex with the fluorescence enhancement solution in which the

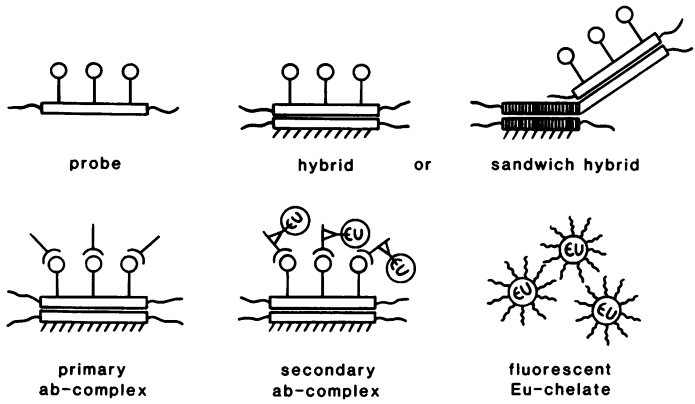

Figure 2.
Procedure for the quantification of DNA hybrids by time-resolved fluorometry. The method includes the following steps: i) tagging of the probe with a hapten, ii) hybridization (direct or sandwich), iii) binding of first antibody (ab), iv) binding of Eu-labelled second antibody, v) measurement of the time-resolved fluorescence of Eu-diketone chelates.

Eu becomes chelated to diketones and trapped into Triton micelles (Figure 2),(15).

Optimization of the detection procedure

In our standard test system the AAIF-modified single-stranded probe containing a 1.2 kb Ad2 specific insert (mKTH 1206) was hybridized to immobilized target Ad2 DNA and detected by the two-stage immunological reaction depicted in Figure 2.

In principle the measurement of Eu-fluorescence is extremely sensitive detecting 10^{-15} M solutions of Eu (12). In practice, however, the sensitivity is dependent on signal to noise ratios. Care was thus taken to avoid unspecific background fluorescence from the hybridization filters. Different hybridization conditions had little effect on the background, while changes in the procedure used for the immunoreactions caused significant variations. It was essential to saturate the washed hybridization filters with protein prior to the addition of the antibodies. Serum albumin was more satisfactory for this purpose than the whole serum used in the enzymatic detection system. The dilution level of the specific antibodies was critical and the use of purified IgG was advantageous. The background fluorescence signals

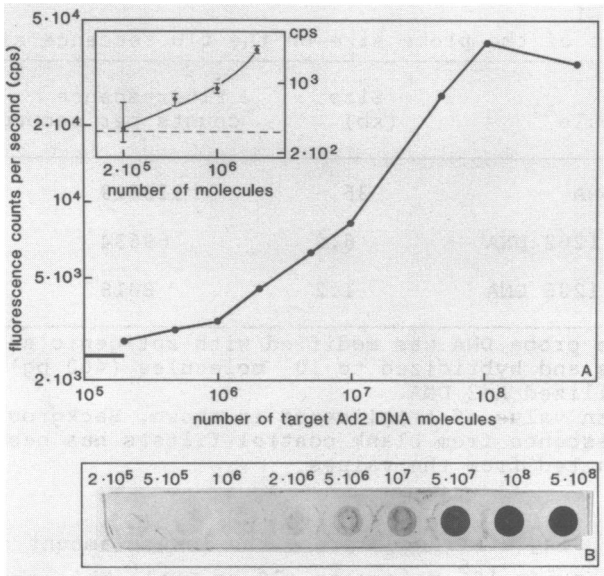

Figure 3.
(A) Detection by Eu-fluorescence of AAIF-modified probe DNA (mKTH 1206) hybridized to increasing amounts 2 x 10^5 - 5 x 10^8 molecules (8 pg - 20 ng) of target Ad2 DNA immobilized on filter discs. Each point represents the mean fluorescence of triplicates. The background fluorescence of control filters is indicated by the horisontal bar. In the inset the background fluorescence has been subtracted. The limit of detectability is indicated by the dashed line (two times the SD above the mean background, six determinations). The vertical bars indicate the variation range of the signals. (B) Enzymatic visualization of AAIF-modified mKTH 1206 probe hybridized to dots carrying 2 x 10^5 - 5 x 10^8 molecules (8 pg - 20 ng) immobilized Ad2 DNA.

from blank filters after the optimized detection procedure were 2000-4000 counts per second (cps) with a variation within an experiment of about 25%. This value should be compared to a background of 1100-1300 cps from enhancement solution alone. The latter "counter background" has been subtracted from all values presented.

Quantification of hybridized probe

From the standard curve presented in Figure 3A showing the Eu-fluorescence cps values as a function of the amount of target Ad2 DNA it can be seen that the DNA detection is quantitative. The plateau at high target amounts (ie. 5 x 10^8 molecules) was repeatedly seen. The inset of Figure 3A shows that using the

Table 1.
Effect of the probe size on the fluorescence signal

Probe molecule[a)]	Size (kb)	Fluorescence counts per second[b)]
Ad2 DNA	36	119819
pKTH 1202 DNA	6.0	69634
mKTH 1206 DNA	1.2	8018

a) The probe DNA was modified with antigenic sulfone groups and hybridized to 10^7 molecules (400 pg) of immobilized Ad2 DNA.
b) Mean value of triplicates is shown. Background fluorescence from blank control filters has been subtracted from the values.

small 1.2 kb single stranded probe the lowest amount of Ad2 DNA detectable was 5 x 10^5 molecules (20 pg DNA). This corresponds to 0.3 pg of actual target sequences.

In the dot hybridization experiment illustrated in Figure 3B, the same AAIF-modified probe was detected enzymatically after hybridization at conditions identical to those in the experiment shown in Figure 3A. In this case the detection limit was 5 x 10^6 Ad2 genomes (200 pg DNA, or 3 pg of actual target se quences). Thus use of the Eu-labelled second antibody increased the sensitivity of detecting AAIF-modified DNA ten-fold as compared to the enzymatic procedure.

The hybridization reaction was usually done over night for convenience. Since high probe concentrations are used, the hybridization time can be reduced without a serious loss of fluorescence signal. After a 2 h or a 4 h hybridization rection 51% and 74% of the signals of a 16 h reaction were obtained, respectively.

In the experiments described above we used AAIF-modified DNA detected by the polyclonal anti-Guo-AAF antibodies. We also tested sulfone-modified probe DNA, against which a monoclonal mouse antibody is available. Fluorescence signals were comparable to those obtained with the same AAIF-modified probe (cf. Table 1 and Figure 3A). In addition to the 1.2 kb single-stranded probe we used sulfone-modified full sized Ad2 DNA as well as

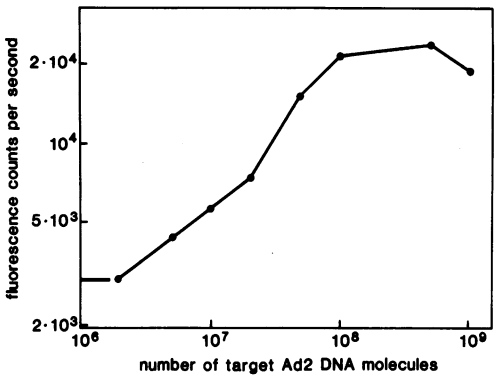

Figure 4.
Detection of sandwich hybridization by Eu-fluorescence using AAIF-modified mKTH 1206 DNA as a probe. Increasing amounts 2 x 10^6 - 10^9 molecules (80 pg - 40 ng) denaturated target Ad2 DNA were added to the 50 µl reaction mixtures. The mean fluorescence counts of triplicates are shown. The horisontal bar indicates the mean background on capture filters hybridized in the absence of target DNA.

a 6 kb fragment of the Ad2 genome in the plasmid pBR 322 as probes (16). As expected larger probes give stronger signals (Table 1). Consequently the detection sensitivity could be further increased by using a larger probe molecule. However, the signal per unit length of probe is within the same order of magnitude in all cases.

When comparing the sensitivities obtained with different labelling systems the size of the probe molecule used in each case has to be taken into account. The sensitivity achieved here, expressed as mass of actual detectable target DNA, is clearly higher than that of previously reported non-radioactive detection systems (2,4).

In the immunochemical applications of time-resolved fluorometry the detection sensitivity is limited by the affinity of the antibodies used. Monoclonal antibodies screened for high affinity could be a way to increase the sensitivity. A biotinylated probe DNA (2), detected by Eu-labelled streptavidin, is another way to increase the affinity between the probe and the reporter molecule. Preferably the time-resolved fluorometric detection of nucleic acid hybrids should be performed with probe molecules directly labelled with Eu. However, the methodology

Table 2.
Measurement of adenovirus type 2 DNA in nasopharyngeal mucus by sandwich hybridization and time-resolved fluorometry

Number of Ad2 genomes per test[a)]	Fluorescence counts per second[b)]
2×10^8	16762
7×10^7	11989
2×10^7	7666
0	3411

a) The given amount of Ad2 DNA was added to 15 µl adenovirus-free nasopharyngeal mucus aspirates. The samples were analyzed by sandwich hybridization using AAIF-modified mKTH 1206 DNA as probe.
b) Mean of four parallell tests is shown.

for direct and stable labelling of DNA with Eu is not yet available.

Measurement of target DNA in crude samples

DNA probes have recently come into use also in clinical routine laboratories. Here the use of non-radioactive material is desirable and the results should preferably be obtained as numerical values. Consequently time-resolved fluorometry is of particular interest for clinical applications. Here we have applied the methodology described above for the detection of adenovirus in nasopharyngeal mucus. For this aim the sandwich hybridization technique (16) was used (cf. Figure 2), even if it is not as sensitive as direct hybridization (Figure 4, Figure 3A). In sandwich hybridization the target DNA anneals to the immobilized capture DNA and thus mediates binding of the probe DNA to the filter. Background problems are avoided since the sample is kept in solution during the reaction, why sandwich hybridization allows testing of crude samples without prior DNA purification (19-21).

Ad2 DNA was added to a pool of adenovirus-free nasopharyngeal aspirates in amounts corresponding to those found in samples from infected patients (19). The mucus aspirates were treated with proteinase K and SDS prior to denaturation as described (19) and the samples were analyzed by sandwich hybridization. When comparing the fluorescence signals in Table 2 with the

standard curve in Figure 4 it is seen that 60-100% of the DNA added to the samples was detected using the 1.2 kb single-stranded AAIF-modified probe. No additional background originating from negative crude specimens was observed.

We conclude that the time-resolved fluorometry of Eu offers a non-radioactive measuring method with great potentials. Until the problem of direct labelling of DNA with a suitable lanthanide has been solved, we think that the indirect method described here may find a number of applications when non-radioactive monitoring of nucleic acid hybrids is desired.

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