Biofunctional Organic Nanocrystals for Quantitative Detection of Pathogen Deoxyribonucleic Acid

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Abstract

Advances in nanotechnology have had significant impacts on the field of biodiagnostics. In this study, we describe the novel application of dissolvable, organic, and biofunctional nanocrystals for the quantitative detection of a PCR product. Fluorescein diacetate (FDA), a fluorogenic precursor of fluorescein, was milled in a solution of a polymeric surfactant to create a stable, nanosized colloid with an interface for coupling streptavidin molecules. The application of these particulate labels for the quantitative detection of biotinylated human papillomavirus (HPV) DNA, amplified in a standard PCR procedure, was demonstrated. After the affinity reaction, the FDA molecules were dissolved and concomitantly converted into fluorescein. This approach resulted in a high selectivity, short incubation times and a sensitivity up to 147 times greater than obtained from state-of-the-art, directly fluorescent-labeled streptavidins. This innovative method offers rapid detection of small amounts of nucleic acids because less target material and thus fewer PCR cycles are required.

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1. Introduction

Human papillomavirus (HPV) is a relatively small, non-enveloped virus, 55 nm in diameter, with a circular genome [1]. HPV is one of the most common sexually transmitted infections (STIs) and is considered to be a major cause of cervical cancer [2,3]. Cervical cancer has the second highest mortality rate (after breast cancer) among female patients worldwide [4]. Annually, about 240,000 women worldwide die from cervical cancer, accounting for 10% of all cancer-related deaths in women [5].

Over 100 HPV genotypes have been identified, some 40 of which are known to infect the ano-genital tract, and are thus closely related to cervical cancer [1,4,6-8]. Based on their potential to induce cervical cancer and preneoplastic lesions of cervical intraepithelial neoplasia, HPV genotypes can be classified into high-risk (HR) and low-risk (LR)-HPV groups. HR-HPV genotypes are found in the majority of cervical cancer patients [1,4,6]. Infection with high-risk genotypes HPV 16, HPV 18 and HPV 45 accounts for almost 80% of all squamous cervical carcinomas worldwide [5,9,10].

Cytology and histology are the conventional diagnostic tools for cervical cancer. The detection of HPV-DNA offers an alternative or complement to the regular cytological screening of cervical cancer. Various HPV-DNA detection methods have been developed based on DNA amplification using either Polymerase Chain Reaction (PCR) techniques and/or liquid hybridization [1,11]. PCR techniques to amplify HPV

gene fragments are now used in routine HPV diagnostics [12]. Most studies on HPV-DNA employ consensus primers for the amplification of the L1 open reading frame region in order to cover a broad spectrum of HPV genotypes [13]. The amplified HPV-DNA is identified by sequencing or hybridization with a specific sequence of a DNA or RNA probe. The hybridized DNA-DNA or DNA-RNA complex is then detected by means of colorimetric or chemiluminescence techniques [2,14].

Advances in nanotechnology have had significant impacts on the field of biodiagnostics [15-17]. In a previous study, we applied a novel signal amplification technology based on a new class of biofunctional fluorogenic nanocrystals to improve the sensitivity and the limits of detection in immunoassays [18-21]. A simple two-step approach was used to encapsulate nanocrystalline fluorescein diacetate (FDA) with an average size of 107 nm with ultrathin polyelectrolytic distearoylphosphatidylethanolamine (DSPE) lipids coupled with amino(poly(ethylene glycol)) (PEG2000-Amine) [19]. This polyelectrolyte coating was subsequently used as an interface for the attachment of biorecognition molecules through adsorption (Scheme 1). A high fluorescein equivalent (FE) value (2.6×10^6) and a high molar ratio of released fluorescent molecules per binding event (2.8×10^4) were achieved in this nanocrystal biolabel system. The FDA[DSPE-PEG(2000)Amine]-modified

biolabels could be stored as a highly stable colloidal suspension, which resulted in minimal nonspecific interactions. The limit of detection was lowered by a factor of 5 to 28 and the sensitivity was 400 to 2700-fold higher compared with a state-of-the-art immunoassay using directly fluorescent-labeled antibodies. In addition, the applicability of NeutrAvidin-labeled nanocrystals was demonstrated in an immunoassay [20]. The limit of detection was lowered by a factor of 2.5 to 21 and the sensitivity was 3.5 to 30-fold higher than immunoassays using commercial labeling systems [i.e. fluorescein 5-isothiocyanate (FITC) and horseradish peroxidase (HRP)]. The biofunctional nanocrystals were also applied to detect different kinds of clinically interesting plasma proteins with high sensitivity, low limits of detection and short incubation times [21]. These results suggested that these functionalized FDA nanoparticles would be useful in the detection of nucleic acids as the final step in PCR-based tests.

In this study, a set of consensus primers, 5' biotinylated PGMY09/11 [22], was used to amplify HPV-DNA from various concentrations of HPV 16, HPV 18 and HPV 45 plasmids. Biotinylated HPV-DNA was amplified and hybridized specifically with DNA probes that had been immobilized on a NucleoLinkTM microplate (Scheme 2). The biotin molecule of the specific hybridized HPV-DNA was finally coupled with streptavidin that had been conjugated with FDA nanocrystals. The performance of the

nanocrystal biolabel system was then compared with various directly labeled systems (i.e., FITC and peroxidase).

2. Experimental

2.1. Materials

Fluorescein diacetate (FDA), fluorescein 5-isothiocyanate (FITC), dimethyl sulfoxide (DMSO) and all other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) and were of analytical grade. 1,2-Distearoyl sn -glycero-3-phosphoethanolamine- N -[amino(poly(ethylene glycol))-2000] (ammonium salt) [DSPE-PEG(2000)Amine] with a molecular weight of 2,790.52 was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Streptavidin was supplied by Pierce Chemical Company (Rockford, IL). NucleoLink™ Breakapart 96-well microplate was purchased from Nunc International (Rochester, NY). Bovine serum albumin (BSA, fraction V) was from Roth (Karlsruhe, Germany).

2.2. Instrumentation

An *fmax* Fluorescence Microplate Reader and a SPECTRAmax 340PC Microplate Reader were purchased from Molecular Devices Corporation (Sunnyvale, CA) for measurements of fluorescence intensity and absorbance respectively.

2.3. Physical adsorption of streptavidin onto encapsulated FDA nanocrystals

A suspension of 5% (w/w) FDA in 1.25% (w/w) DSPE-PEG(2000)Amine was milled for 48 to 72 h using a process developed by Elan Drug Delivery Inc. (King of Prussia, PA). The temperature was maintained at 20°C to prevent the material from hydrolyzing. The surface charge, morphology and size distribution of the milled FDA nanocrystals were all described in a previous publication (Figure S-1) [19].

The pretreated particle suspension was diluted to 0.0626% (w/v) and incubated with 200 µg/mL streptavidin in 10 mM phosphate-buffered saline (PBS, pH 7.4) at room temperature for 1 h. The supernatant was removed after centrifugation at 16,000g for 10 min. After three repeated centrifugation/washing cycles, the streptavidin-coated particles were finally separated from the streptavidin solution.

2.4. Immobilization of DNA probes in microplate

NucleoLink™ Breakapart 96-well microplates were used for DNA probe immobilization and hybridization. Each well of the 96-well plate was immobilized with a specific 5' amine-labeled DNA probe²³ (HPV 16, HPV 18 or HPV 45) or a 5' biotinylated DNA probe for detection or as a positive control for the color development, respectively. The probe was immobilized based on the protocol

provided by Nunc for the NucleoLink™ product [24]. 100 µL of coating buffer, containing 100 nM 5' amine-labeled DNA probe, 10 nM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in 10 mM 1-methyl-imimidazole (pH 7.0), were added to each well. The plates were then incubated in a humidity chamber at 50°C over night and finally washed five times with 200 µL of washing buffer, containing 0.5 × SSC and 0.1% Tween 20 (pH 7.4), for 5 min at room temperature. The plates were stored in ethylene bags at 4°C until use.

2.5. HPV-DNA amplification

Clones containing unique L1 fragments of different HPV genotypes (HPV 16, HPV 18 and HPV 45) were used at standard concentrations (10^5 , 10^4 , 10^3 and 10^2 copies/µL) as templates for the PCR. A standard PCR was run for the amplification of different HPV genotypes using the set of consensus primers. The DNA amplification and labeling was conducted in 40 µL of a reaction system containing 1× PCR buffer, 3.5 mM MgCl₂, 300 µmol (each) dATP, dCTP, dGTP and dUTP, 1 nM of forward and reverse primer set, 2 U Taq polymerase, 1 U UNG and various concentrations of plasmid. Amplifications were performed in an Eppendorf Mastercycler by activation of UNG at 37°C for 15 min and 95°C for 10 min, and 45 cycles of 95°C for 30 s, 52°C for 45 s, 65°C for 30 s. This was followed by a final extension at 65°C to 5 min. The

amplification reaction mixtures were examined by gel electrophoresis in 1.5% agarose gel with ethidium bromide staining and stored at 4°C.

2.6. Amplified HPV-DNA hybridization

Amplified HPV-DNA, labeled with biotin during the amplification reaction, was separately hybridized with the immobilized probes of HPV 16, HPV 18 or HPV 45 in 96-well microplates. A volume of 10 µL of amplified HPV-DNA was added to 10 µL of 1N NaOH solution and incubated at room temperature for 10 min. Then 80 µL of neutralization buffer, containing 6 × SSC, 0.125% Tween 20, 0.5 M NaH₂PO₄, 10% Denhardt solution (pH 6.5) and 10 µg/L salmon sperm DNA (pH 6.5), was added to the tube. 100 µL of the mixture was transferred to the DNA probe-immobilized wells and incubated at 55°C for 1 h. Then, the plates were washed five times in 200 µL of washing buffer at room temperature for 5 min each.

100 µL of diluted streptavidin-FDA or streptavidin-FITC (1:50) in a coupling buffer, containing 2 × SSC with 0.1% SDS (pH 7.4), was added to each well and incubated at 37°C for 30 min. Subsequently, the microplates were washed five times in 200 µL of washing buffer at room temperature for 5 min each. An aliquot of 100 µL/well of releasing reagent (DMSO and 1 M NaOH in a 1:1 ratio) was added to the FDA system. Emission from the plate wells was excited at 485 nm and recorded at

538 nm in an *fmax* Fluorescence Microplate Reader. In the 3,3',5,5'-tetramethylbenzidine (TMB) system, 100 μ L of 0.1 U/mL streptavidin-HRP conjugate in coupling buffer was added to each well and incubated at 37 °C for 30 min. The microplates were washed five times in 200 μ L of washing buffer at room temperature for 5 min each. A substrate solution, 0.1 mg/mL of TMB and 0.003 % H₂O₂ in 0.1 M sodium citrate solution (pH 5.0), was freshly prepared. Each well was filled with 100 μ L of this solution and incubated at room temperature for 10 min. The color development was stopped by adding 100 μ L of 0.5 M H₂SO₄. The microplate was examined by a SPECTRAmax 340PC Microplate Reader at 450 nm.

3. Results and discussion

3.1. Application of Nanocrystalline FDA Biolabels in DNA Hybridization Assays

The assays were conducted in 96-well microtitre plates utilizing internal primers labeled with biotin. The amplicons were hybridized with the immobilized HPV 16, 18 or 45 probes and detected using FDA/FITC/HRP-labeled streptavidin. Figure 1 shows the calibration curves of the assays performed with the streptavidin-FDA nanocrystal labels. The fluorescence signal is directly proportional to the amplicon concentration in the range of 10^3 to 10^5 copies/ μ L (Figure 1). To minimize nonspecific binding, low concentrations of the fluorescent-labeled streptavidin conjugate are typically preferred.

No increase in the signal intensity was registered in our assays with increasing concentrations of unmatched amplicons hybridized with the immobilized HPV probes up to 10^5 copies/ μ L (Figure 1).

3.2. Comparison of Nanocrystalline FDA-Labeled Streptavidin with Commercially Available Directly Labeled Streptavidins

The performance of the streptavidin-FDA nanocrystal label was compared with directly FITC-labeled and HRP-labeled streptavidin conjugates. Although the HRP system allowed ultra-sensitive detection of HPV-DNA (down to 100 copies/ μ l), the method did not provide quantitative information on the viral load. Neither did the FITC-labeled streptavidin conjugates. In contrast, the nanocrystalline FDA system did produce quantitative results without nonspecific interactions with the other probes (Figure 1). Moreover, depending on the amplicon concentration and the HPV genotype, the FDA system yielded much higher S/N ratios (Table 1; absolute data shown in Table S-1) with a concomitant sensitivity enhancement by a factor of 2.8 to 147.0 compared with the directly FITC-labeled streptavidin (Table 2).

The high sensitivity of the particle-based conjugates probably results from the boosting effect of the dissolving dye molecules, but the minimized quenching could also have contributed to the improved signal.

As well as detecting HPV, it is also important to discriminate between HR and LR-HPV genotypes. The assay described above reliably detects 11 HR-HPV genotypes and yields the highest S/N ratios (Table 3; absolute data shown in Table S-3). We immobilized the 11 HR-HPV genotypes in a single well and hybridized them with different amplicons by using FDA/FITC/HRP-labeled streptavidin for detection. The labeled amplicons and the FDA/FITC/HRP labeled streptavidin were added sequentially to coated microtitre plates to permit maximal binding. Compared with other reagents used to detect PCR products (e.g., ethidium bromide and radioisotopes), FDA is non-hazardous and fast because there is no color processing. After addition of the releasing reagent, the instant fluorescence intensity is over 90% of the maximum and reaches the maximum within 5 minutes. On the other hand, common metal ions in human body have no interference with FDA. Furthermore, the fluorescence detection is performed in microtitre plates rather than in tubes, which permits rapid dispensing of reagents with multichannel pipetting equipment and the possibility of automation using robotic arms.

4. Conclusion

We have demonstrated a novel signal amplification technology for a HPV-DNA hybridization assay based on FDA nanocrystals encapsulated within

PEG-Amine-modified phospholipids (DSPE-PEG-(2000)Amine). The surface modification of the nanocrystalline biolabel system prevents nonspecific binding and particle aggregation. It provides an interface for conjugation with streptavidin. The FDA nanocrystals are nearly water-insoluble precursors of fluorescein, which is dissolved and hydrolyzed by treatment with an organic solvent/hydroxide mixture. This technology allows easy quantification of PCR products and a markedly increased sensitivity and S/N ratio over established systems. It should be noted that there is a clear potential for further improvement, to develop early-stage and instant detection systems for various diseases.

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Legends

Scheme 1. Preparation of Biofunctional Fluorescent Labels^a

Scheme 2. Amplified HPV-DNA Hybridization using Nanocrystalline FDA as Label^b

Figure 1. Quantitative evaluation of the nanocrystalline FDA conjugates as shown in scheme 2 by using different amplified HPV-DNA labeled with biotin which were separately hybridized with the immobilized probes of HPV 16 (■), 18 (▲) or 45 (◆) in the microtitre plate. Error bars correspond to standard deviations ($\pm$ SD, n = 3).

Table 1. Signal-to-Noise Ratios (S/N) of FDA-Labeled, FITC-Labeled and HRP-Labeled Streptavidin

Table 2. Sensitivity-to-Sensitivity Ratios (S/S) of FDA-Labeled and FITC-Labeled Streptavidin

Table 3. Signal-to-Noise Ratios (S/N) of FDA-Labeled and HRP-Labeled Streptavidin for Detection of Multiple Genotypes in a Single Well