A highly sensitive fluorescent immunoassay based on avidin labeled nanocrystals

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ABSTRACT

Nanocrystals of the fluorogenic precursor fluorescein diacetate (FDA) were applied as labels to enhance assay sensitivity in our previous studies. Each FDA nanocrystal can be converted into ~2.6 x 10^6 fluorescein molecules, which is useful for improving sensitivity and limits of detection of immunoassays. NeutrAvidin was simply adsorbed on the surface of the FDA nanocrystals which was coated with distearoylglycerophosphoethanolamine (DSPE) modified with amino(poly(ethylene glycol))(PEG(2000)-Amine) as an interface for coupling biomolecules. It can be applied to detect different kinds of analytes which are captured by corresponding biotinylated biomolecules in different bioanalytical applications. The applicability of the NeutrAvidin-labeled nanocrystals was demonstrated in an immunoassay using the labeled avidin-biotin technique. Biotinylated antibody and FDA-labeled avidin were applied to the assay sequentially. The performance was compared with the traditional sandwich type assay for mouse immunoglobulin G detection. Following the immunoreaction, the nanocrystals were released by hydrolysis and dissolution instigated by adding a large volume of organic solvent/sodium hydroxide mixture. The limit of detection was lowered by a factor of 2.5–21, and the sensitivity was 3.5–30-fold higher compared with the immunoassay using commercial labeling systems, i.e. FITC and peroxidase. This study shows that the fluorescent nanocrystals combined with the avidin-biotin technique can enhance the assay sensitivity and achieve a lower limit of detection without requiring long incubation times as in enzyme-based labels.

Keywords
FDA, Avidin, NeutrAvidin, Biotin, Labeled avidin-biotin technique, Immunoassay, Fluorescent nanocrystals

1 INTRODUCTION

The high affinity of avidin for biotin was first exploited in histochemical applications in the mid-1970s. Avidin (pI of 10.5, ~67 kDa, 5.6 x 5 x 4 nm) is a basic tetrameric glycoprotein, isolated from hen egg-white, tissues of birds and reptiles [1-3]. There are four binding sites in each avidin molecule that can bind non-co-operatively up to four molecules of biotin with a very high affinity (K_d=10^{-15}M) [2]. The four binding sites together with the high affinity of the avidin-biotin interaction serve as an aid in amplifying the sensitivity of immunoassays [4-5]. The avidin-biotin complex is almost inseparable [6] and even stable under strong chemically denaturant conditions over a wide pH range [6][3]. The avidin-biotin technique is widely used to localize antigens in cells and tissues and to detect biomolecules in immunoassays and DNA hybridization techniques.

The advantages of FDA nanocrystals as labeling system over traditional enzymatic amplification systems and other directly fluorescent labels were reported in our previous studies [7-8]. In order to pursuit a higher sensitivity and a lower limit of detection, the labeled avidin-biotin technique was applied together with the FDA nanocrystals (Figure 1a).

Apart from the binding properties of the antibodies themselves, the sensitivity of fluorescence assays is determined by the number of light quanta emitted per analyte molecule. A high ratio of fluorescent dyes to protein (i.e., the F/P ratio) and minimal self-quenching effects are the key parameters to achieve superior signal amplifications. However, conventional fluorescence systems have self-quenching problems: if more than 10 -15 fluorophores are attached to 1 antibody, their distance is close to or within their Foerster radius, resulting in significant loss of fluorescence emission intensity due to energy transfer. The F/P ratio of the FDA nanocrystals to proteins was studied extensively in our previous studies [7-8]. In this report, combining the ultrasensitive FDA nanocrystals and the labeled avidin-biotin technique to achieve an even better assay performance was our main goal.

In our previous studies, a traditional sandwich immunoassay was used and the FDA-labeled antibody was added directly to the analyte (Figure 1a). In the immunoassay using the labeled avidin-biotin technique, biotinylated antibody and NeutrAvidin-labeled FDA were used. The biotinylated antibody was first incubated with the antigen. After washing, NeutrAvidin-labeled FDA was added. After further incubation and washing steps, the FDA associated with the antigen was hydrolyzed and dissolved (Figure 1b). The fluorescence intensity was finally measured.

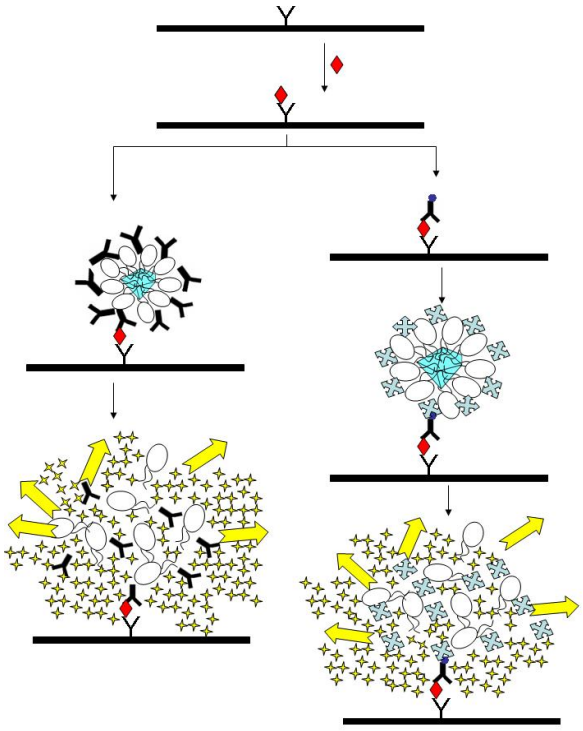

Figure 1. Principle of a labeled avidin-biotin fluorescent immunoassay using FDA-avidin conjugates. The analyte was first incubated with the capture antibody preadsorbed on the microtiter plate and then exposed to the biotinylated-antibody followed by FDA-avidin conjugates. High signal amplification was achieved after solubilization, release, and conversion of precursor FDA into fluorescein molecules by the addition of DMSO and NaOH. A low limit of detection of 0.0256 μg/L was achieved in the current study (b). A limit of detection of 0.0574 μg/L was achieved previously by using FDA-antibody conjugates as labels in traditional sandwich fluorescent immunoassay (a) [7].

2 EXPERIMENTAL SECTION

2.1 Pretreatment and Characterization of Nanocrystals

The size of the FDA nanocrystals was reduced to 107 nm in average by the milling the suspension of FDA in DSPE-PEG(2000)Amine for 48–72 h using a process developed at Elan Drug Delivery Inc. (King of Prussia, PA) process with DSPE-PEG-(2000)Amine solution. The hydrophobic DSPE-PEG-(2000)Amine molecules were adsorbed on the FDA nanocrystals surface that caused the FDA nanocrystals to disperse in water and prevent their aggregations, hence enhancing the stability. The surface charge (zeta-potential), morphology and size distribution of the milled FDA nanocrystals were reported in a previous study [7].

2.2 Physical Adsorption of Encapsulated Nanocrystals with NeutrAvidin

The pretreated particle suspension was diluted to 0.0626% (w/v) and incubated with 200 μg/mL of NeutrAvidin in 10 mM phosphate-buffered saline (PBS, pH 7.4) at room temperature for 1 h. The supernatant was then removed by centrifugation at 16000*g* for 10 min. The NeutrAvidin-coated particles were finally separated from the NeutrAvidin solution by three repeated centrifugation/washing cycles.

2.3 Solid-Phase Sandwich Fluorescence Immunoassay with a Labeled Avidin-Biotin Technique

The avidin-biotin bridged fluorescence immunoassay was used for detection of mouse Ig G (Figure 1). 1 μg/mL of goat anti-mouse IgG (100 μL/well) was coated on Nunc Maxisorp 96-well microplates (Nunc International, Rochester, NY) in 0.1 mol/L carbonate buffer (pH 9.6) at 4°C overnight. After rinsing three times withwashing buffer [10 mM PBS, 0.1% (w/v) BSA, 0.5% (w/v) Tween-20], the wells were blocked with 300 μL of 1.0% BSA solution for 0.5 h at 37°C. The plates were then washed four times and incubated with dilutions (100 μL/well) of mouse IgG as the analyte at 37°C for 1 h. After washing four times, the goat anti-mouse IgG-Biotin (750 ng/ml) was dispensed into the wells (100 μL/well) and incubated at 37°C for 1h. The plates were then washed five times, the NeutrAvidin-coated nanocrystal suspension (0.013%, w/v) was added into the wells (100μL/well) and the microplates were incubated again at 37° C for 1h. Soluble direct-label NeutrAvidin-FITC dilutions (1:1000, Molecular Probes) and avidin-peroxidase dilutions of (1: 3000, Sigma-Aldrich) were used for comparison. After incubation, excess antibody conjugates were washed off five times with the washing buffer. 100 μL of releasing reagent (DMSO and 1M NaOH in a 1:1 ratio) was added to each well. The fluorescence intensity was measured with FLUOstarOPTIMA multifunctional microplate reader (BMG Labtechnologies GmbH, Offenburg, Germany) with an excitation/emission wavelength of 485/520 nm. A fluorescence reader gain settings of 575 and 1500 were used for measurements of the FDA-labeled biomolecules and FITC labeled biomolecules. For the avidin-peroxidase label, TMB substrate solution (100 μL /well) was added to the plate. After 10 to 15 min for colour development, 50 μL of H_2SO_4 (2M) was added to each well to stop the enzymatic reaction. The absorbance was measured with a Dias Microplate Reader (Dynatech Laboratories, United States) at wavelength of 450 nm.

Negative controls were prepared by omitting the mouse IgG as the analyte, or by exchanging it with rabbit IgG and by

omitting the conjugate.

The detection limit is defined as the concentration of mouse IgG corresponding to the mean fluorescence or absorbance of the zero dose response plus three times the standard deviation (SD) of this measurement.

3 RESULTS AND DISSCUSSION

3.1 Biolabeled FDA Nanocrystal

DSPE-PEG-(2000)Amine contains one amino functional group. Biomolecules could be conjugated to FDA encapsulated with DEPE-PEG-(2000)Amine nanocrystals via different conjugation methods, for example, physical adsorption and covalent conjugation using different crosslinkers [9-10]. The adsorption of antibodies to FDA nanocrystals was confirmed in our previous study by microelectropheresis and adsorption spectroscopy [7]. Beside antibodies, other proteins e.g. NeutrAvidin were also used for conjugation to FDA nanocrystals by physical adsorption.

In our previous study, high sensitivity and low limit of detection could be achieved by physical adsorption. The main driving forces of the adsorption are electrostatic attraction, hydrophobic interaction and the van der Waals forces. However, the biomolecules on the encapsulated FDA nanocrystals surface may be detached after certain period of time. In order to increase the stability of the biomolecules-nanocrystals conjugation, covalent conjugation should be used [11-12]. Also, covalent conjugation chemistries have demonstrated that functional capability of conjugated protein can be preserved for up to two years [13].

3.2 Comparison of the FDA-Labeled Antibodies, FDA-Labeled Avidin, and Commercial Directly-Labeled Avidin

Figure 2 shows the calibration curves of assays using NeutrAvidin-FDA nanocrystals with labeled avidin-biotin technique, directly FITC-labeled NeutrAvidin conjugate and goat anti-mouse IgG-FDA nanocrystals as labels. A limit of detection of 0.0256 µg/L was achieved with the FDA-labeled NeutrAvidin while values of 1.18 µg/L and 0.0299 µg/L were achieved with the directly fluorescent-labeled NeutrAvidin from Molecular Probes and the enzyme-labeled avidin from Sigma-Aldrich respectively . A limit of detection of 0.0574 µg/L was reported in our previous study [7] with antibody-labeled FDA in a sandwich immunoassay (Figure 1a). The limit of detection achieved by NeutrAvidin-FDA nanocrystal labels is 2.5-fold lower than that of antibodies-FDA nanocrystal labels. The highest S/N ratio was obtained by using the NeutrAvidin-FDA nanocrystal labeling system. It was 3.5–30-fold higher than those obtained by using the other conjugates. The greatest increase in the signal-to-noise ratio was observed in the low concentration range. The immunoassay sensitivity and performance of FDA-protein conjugates are higher than the directly labeled protein conjugates. The high sensitivity of FDA-protein conjugates can be explained by the boosting effect of the higher ratio of dye molecules to binding molecules [7].

The assay sensitivity observed using FDA-labeled NeutrAvidin with labeled avidin-biotin technique was higher than using FDA-labeled antibodies in traditional sandwich immunoassay. It is because the adsorption of antibodies on the nanocrystals surface was a random process. The orientation of the antibodies attached on the surface could be affected by the surface charge of the nanocrystals, ionic strength of the medium, etc. [14-15]. All these may interfere with epitope recognition and ligand-antibody interaction [14-18]. Therefore, the binding of the antibody adsorbed on the nanocrystal surface and the captured analyte would be affected, hence affecting the assay performance and sensitivity [19-20].

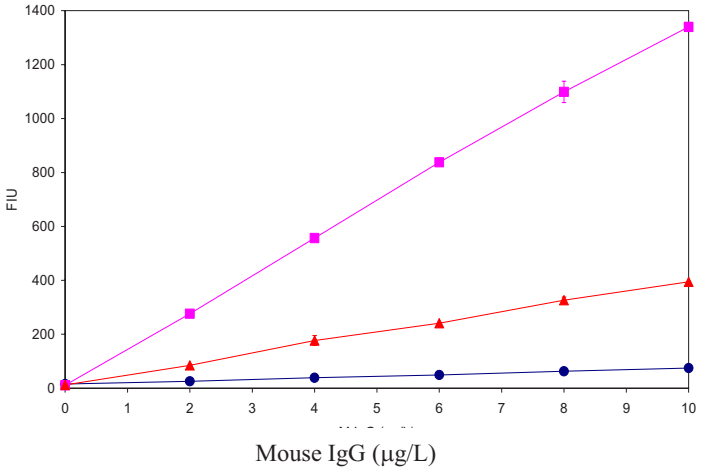

Figure 2. Sandwich fluorescence immunoassay of mouse IgG using NeutrAvidin-FDA nanocrystals (■), goat anti-mouse IgG-FDA nanocrystals (▲) and direct FITC-labeled NeutrAvidin from Molecular Probes (●) as labels. Good linearity is observed in all cases using NeutrAvidin-FDA nanocrystals (R^2=0.998), goat anti-mouse IgG-FDA nanocrystal labels (R^2=0.999) and direct FITC-labeled from Molecular Probes (R^2=0.999).

In the labeled avidin-biotin bridge system, the antibody-biotin conjugate was bound to the analyte first, followed by the affinity reaction with the functionalized nanocrystals. Due to the small size of the biotin molecules (FW: 244.31 g/mol), which would not affect the binding affinity of antibody to the analyte, the NeutrAvidin-labeled nanocrystals would be able to bind tightly to the analyte-antibody-biotin complex. Because of the exceptionally high affinity of the binding between avidin and biotin (K_d=10^{-15}M), which is much higher than the binding of antibody-antigen (typically in the range of 10^{-7} M to 10^{-11}M for IgG antibodies) the binding is extremely tight and difficult to reverse [24-25]. Moreover, one avidin molecule can bind non-co-operatively up to four molecules of biotin. Therefore, the use of NeutrAvidin labeled nanocrystals to detect the analyte in the labeled avidin-biotin system is more efficient than antibodies labeled nanocrystals in the traditional sandwich system.

4 CONCLUSION

A novel signal amplification technology based on FDA nanocrystals with the application of the labeled avidin-biotin technique was described. The system of NeutrAvidin-labeled FDA with the labeled avidin-biotin technique was observed to lower the limit of detection by a factor of 2.5–21 and to produce up to a 3.5–30-fold increase in assay sensitivity compared with commercial labels such as FITC-labeled NeutrAvidin and enzyme-labeled avidin. By conjugating different antibodies to the biotin molecules, different kinds of analytes can also be detected by using the avidin-labeled FDA with the labeled avidin-biotin technique. Moreover, the stability of the biomolecule-FDA conjugates could be further improved by covalent conjugation. FDA nanocrystals labels with labeled avidin-biotin immunoassay may also be applied to other areas such as DNA array.

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