

# An Organic Nanoparticle based High Signal Amplification Immunoassay with Improvement of Nonspecific Binding

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## Abstract

A high signal amplification immunoassay method based on Fluorescein Diacetate (FDA) organic nanoparticle was proposed here. FDA nanoparticles were prepared in a ball mill and centrifugation was applied to get the size-uniformed SDS stabilized particle (FDA@SDS). Further SEM images confirmed the particle size was well dispersed in solution and particle size analysis results showed that the particle size 90% was distributed in the range of 200 nm to 500 nm. Mass Spectrometry results showed that the antibody can directly attach to the FDA@SDS particles and standard sandwich immunoassay confirmed the bioactivity of the antibody on the FDA@SDS particle surface (FDA@SDS-IgG). And then Zeta potential test results showed that the blocking reagents BSA and Casein can successfully block the unsatisfied sites on the FDA@SDS-IgG surface, which resulted in the improvement of nonspecific binding and stabilization. Further experiments were carried out using the standard immunoassays which showed the analyte signal can be greatly amplified when Casein was used as block buffer in the immunoassays. The limitation of detection was lower than 10 pg/mL, which is 100 times lower than Cy3 or FITC directly labeled antibody. The improved procedure simplifies previously reported protocols and provides a simple high signal amplification immunoassay.

**Keywords:** Fluorescent immunoassays; Organic nanoparticles; Non-specific binding; Signal amplification; Detection limitation

## Introduction

Some viruses, such as HCV and HIV, can duplicate in very low concentration in the host and so the early infection period diagnosis is very important in such diseases [1-5]. Diagnosis depends on the sensitivity of the detection method and, generally, the lower the detection limit, the shorter the window period between infection and diagnosis [4-7]. And with the development of science in all the fields, loads of new methods and concepts have been designed and developed with high sensitivity and low limit of detection both in serological and molecular approaches [8-13]. Due to the low cost and convenience, labels related immunoassays are the commonly used methods in the clinic practice and thus various formats were developed such as enzyme-linked immunosorbent assays (ELISA), fluorescent immunoassays and radio immunoassays. Among all these immunoassay methods, fluorescent immunoassays are the promising technique both in the laboratory and in the practice with well detection performance.

The detection performance of a fluorescent immunoassay is determined by the number of fluorescent photons emitted per analyte molecule. In order to improve this detection ability, high excitation and high quantum yield fluorophores are pursued [14] or high signal-to-noise materials are applied [7,15-17] or high-load of label molecules systems are designed [11]. These methods are trying to overcome the conventional label system which suffers from the energy transfer and quenching problems when too many of fluorescent molecules are labelled on the targets, while improve the signal amplification either by increasing the fluorescent molecules per analyte (i.e. F/P ratio) or increasing the emitted photons per fluorophore. However, high signal amplification is by far not the only issue the analysts are focusing on in the immunoassays. The lower limitation of detection (LOD) which is affected by nonspecific binding and other aspects is as important as the sensitivity [1-4,12,13]. High signal amplification systems increase the analyte signal; however, they will amplify the nonspecific binding and background signals at the same time, especially in the solid-phase fluorescent immunoassays.

Recently, C.P Chan and Dieter Trau et al reported a DSPE-PEG (2000) amine based polyelectrolyte multilayers fluorescein diacetate (FDA) nanocrystals [18-21]; Yvonne Bruemmel et al. [18] studied the influence of different surface modifications on the performance of fluorescent based immunoassays. These methods increase the F/P ratio by releasing fluorescein molecules through the hydrolysis of the FDA nanoparticles after dissolution in DMSO, which undoubtedly can achieve very high sensitivity and very low limit of detection in principle. Beside their study, there are some other aspects should also be considered such as how to deal with the noise signals which might cause by the nonspecific binding or the cross-reactions from the analyte and other proteins in the immunology analytical systems. And this noise signal can also be enlarged by this signal amplification system and it might eliminate the advantages for the related immunoassays.

Here we present a direct antibody adsorbed and blocking reagent coated IgG encapsulated FDA nanoparticles system for fluorescent immunoassay, with the advantages of the low nonspecific binding, easy-preparation and high signal amplification, low limit of detection.

## Materials and Methods

### Materials

Fluorescein Diacetate (FDA), Sodium dodecyl sulfate (SDS); Goat anti-human IgG, rabbit anti-goat IgG, human IgG, Cy3 labelled rabbit anti-goat IgG, BSA and PBS were ordered from Sigma. The immunoassays were run on Inverted Fluorescent Microscopy (IX71,

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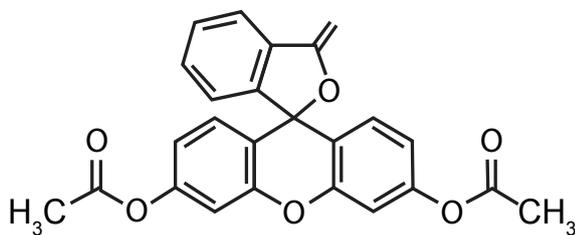
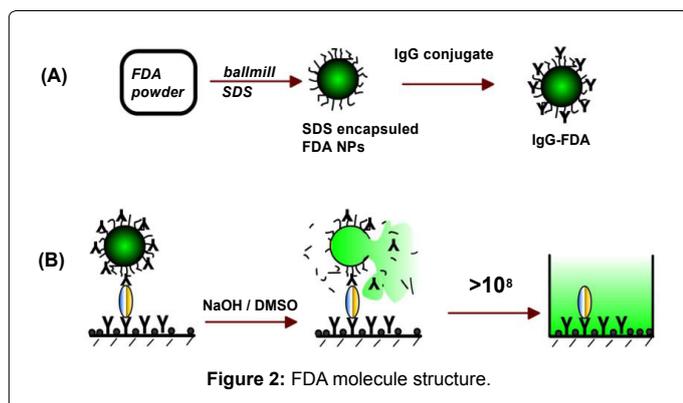


Figure 1: Illustration of FDA based immunoassay.



Olympus) with 96-well plastic-bottom COSTAR plate (catalogue number 3603).

### FDA nanoparticles preparation

0.2 g FDA powder and 0.15 g SDS was added to 10 mL distilled H<sub>2</sub>O, and then the mixture was loaded into the ballmill for 12 hours, at 200 rpm. After that, the solution in the mill was taken out and centrifuged to get the sediment, and then the sediment was dissolved with 5% SDS. The FDA particle solution was then prepared for SEM by applying it to a surface and coating with Au, and then the coated sample viewed in by SEM (JEOL, Japan).

### Preparation of antibody encapsulated FDA@SDS

1 mL of the SDS encapsulated FDA solution was added to 2 mL 50 µg/mL rabbit anti-goat IgG solution and the mixture was kept shaking for 24 hours before centrifuging at 5,000 rpm to get the FDA@SDS-IgG sediment. Then the sediment was dissolved in 5 mL PBS solution and divided into 5 tubes (1mL/tube) for further experiments. The process was shown in Figure 1A.

Casein coated FDA@SDS-IgG (FDA@SDS-IgG-Casein): 1 mL of FDA@SDS-IgG and 1mL of 0.5% Casein buffer were mixed and centrifuged to get the sediment and then the sediment was dissolved in PBS solution for further applications.

BSA coated FDA@SDS-IgG (FDA@SDS-IgG-BSA): 1 mL of FDA@SDS-IgG and 1mL of 1% BSA buffer were mixed and centrifuged to get the sediment, and then the sediment was dissolved in PBS solution for further applications.

### FDA based Immunoassay

FDA@SDS-IgG particles were applied to standard immunoassays (Figure 1B). Human IgG was immobilized onto the plate surface by overnight incubation and followed by 1% BSA block; goat anti-human

IgG in series concentrations was loaded into the wells as analyte; and then FDA@SDS-IgG was loaded at 30ml/well and followed by PBS (pH 6.8) buffer washing for 6 times; and then NaOH/DMSO (1:1, v/v) was loaded into the wells at 50ml/well; fluorescence was measured after 10 min.

### Zeta potential and particle size distribution analysis

The samples (FDA solution and FDA@SDS-IgG solution) were loaded into the cell for Zeta potential and particle size distribution test on MALVERN Zetasizer Instrument (Zetasizer Nano model). The temperature was set as 25°C and the dispersant was water. For the Zeta potential tests, only pH was adjusted by 1N HCl or Na<sub>2</sub>CO<sub>3</sub> solution. All the conditions were the same.

### Mass spectrometry

The samples (FDA, FDA@SDS-IgG and IgG solutions) were applied to the plate for the Matrix Assisted Laser Desorption Ionization Mass Spectrometry (SHIMADU, Kratos Axima CFR<sup>+</sup> MALDI-TOF). Three proteins from laserbiolabs were used for the calibration in these measurements, i.e. Insulin oxidised B chain, Horse myoglobin, Trypsinogen.

### Results and Discussion

#### FDA@SDS nanoparticles preparation

Fluorescein diacetate (FDA) is a fluorescein derivative and water insoluble, and it is easy to hydrolyze in the two ester bonds in alkaline condition, as we can see from the molecule structure (Figure 2). Based on these properties of FDA, an organic nanoparticle related immunoassays can be developed by using ball mill with the help of surfactant SDS solution which makes the FDA water soluble. The interaction between the SDS and FDA particle is mainly by the hydrophobic interaction. As the SDS is an amphiphilic molecular, the hydrophobic alkyl tail contact with the FDA surface and the hydrophilic head faces outside to the aqueous phase. As shown in Figure 1, FDA nanoparticle are easily hydrolysed at high pH and with the help of DMSO, the SDS encapsulation on the FDA particle is dissolved and thus FDA nanoparticle is released into the NaOH solution and then FDA is converted into fluorescein which is highly fluorescent.

In the ballmill, the smaller particle size increases the surface/volume ratio so that more surfactant SDS molecules can be adsorbed to the surface of FDA by hydrophobic interaction, and thus SDS encapsulated FDA nanoparticle was formed (FDA@SDS). As a result, the SDS encapsulation at the surface of FDA particle makes the FDA particles aqueous suspendable. The SEM and particle size analysis were applied for the resultant particles and the results indicated that the FDA@SDS are well dispersed in the solution (Figure 3A) and no aggregations were observed during the preparations. The size distribution is shown in Figure 3B. From these results we can see that the particle size 90% was distributed in the range of 200 nm to 500 nm.

Assuming the FDA particle is 250 nm in sphere, the number of IgG on a single FDA particle in theory is in the range of 2800 to 5600, depending on the orientations of the absorbed IgG molecules, the percentage of IgG coverage, et al. In our experiments, it is possible the number of the IgG in the range of 100~500 on a single 500nm sized FDA particle, as more protein such BSA and Casein can be farther absorbed. And also it is possible monolayer may be formed. Furthermore, due to the space hinder resistance of the sandwich immunoassays, large number of IgG on single particle is not necessary.

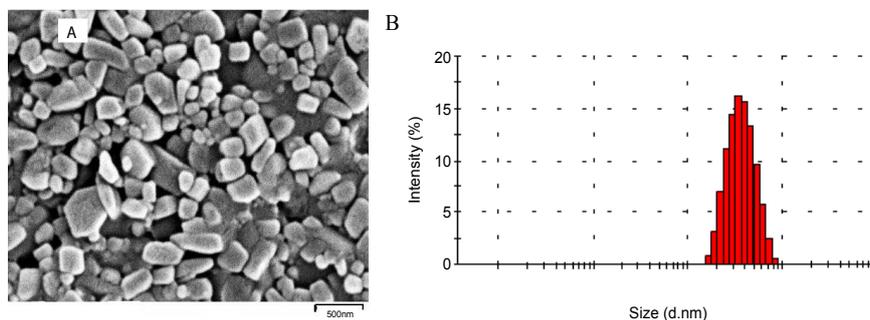


Figure 3: SEM image of FDA nanoparticles (A) and size distribution by intensity (B).

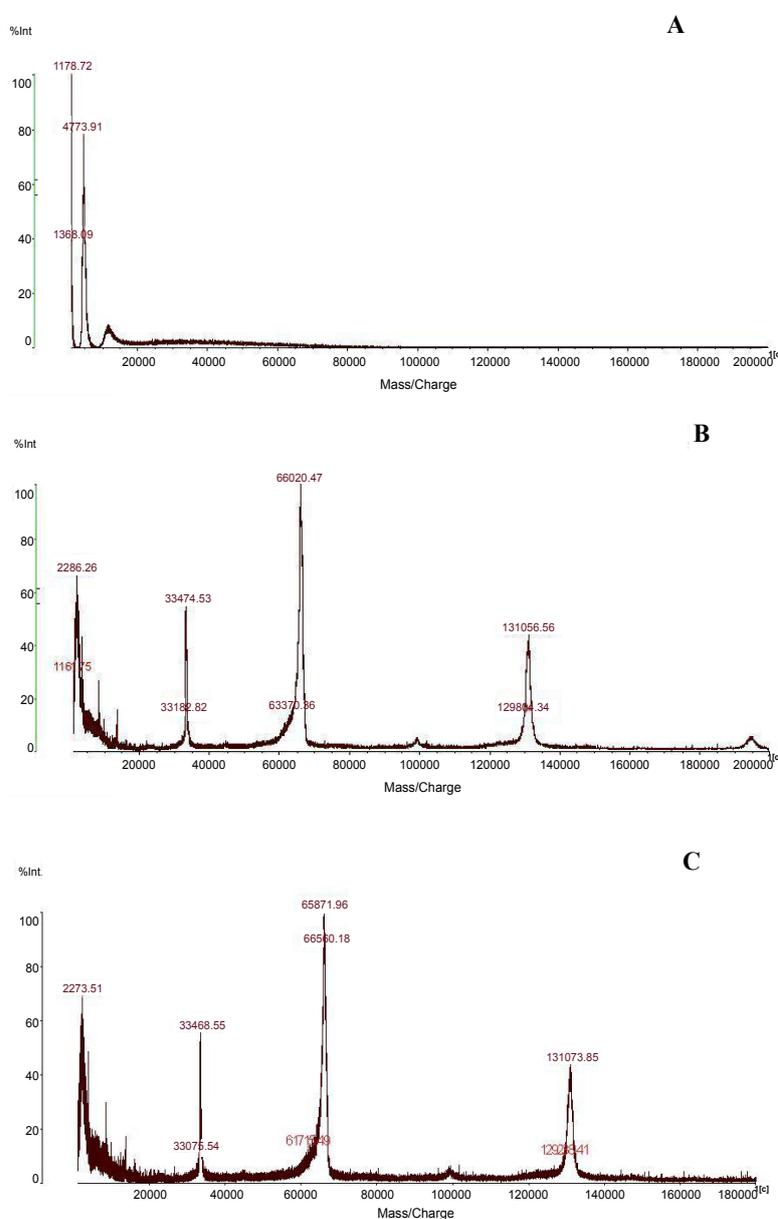
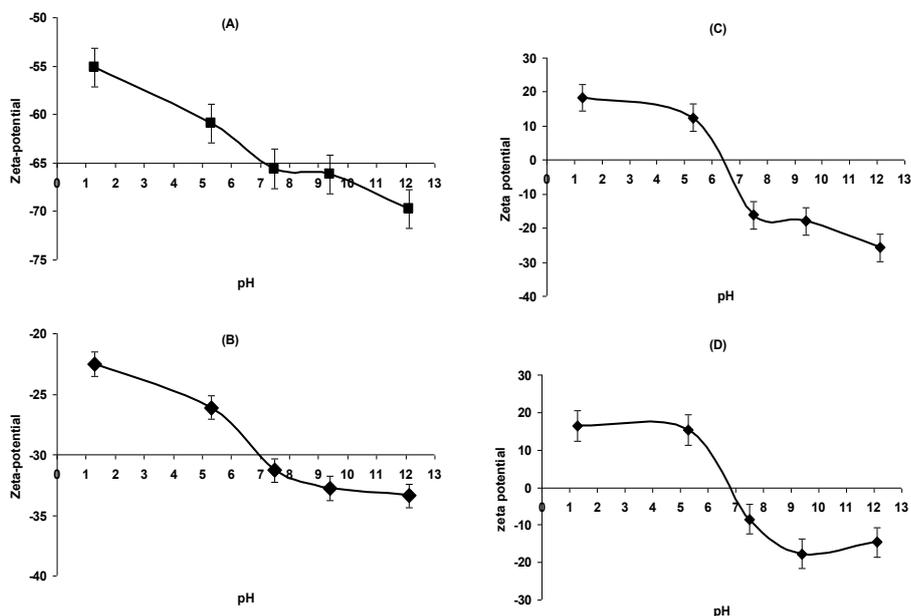


Figure 4: MALDI-MS spectrum of FDA@SDS nanoparticles (a), FDA@SDS-IgG (b) and 50 µg/ml IgG (c). Other conditions were the same. The IgG was Cy3 labelled rabbit anti-goat antibody.



**Figure 5:**  $\xi$ -potential of FDA@SDS (A), FDA@SDS-IgG (B), Casein/FDA@SDS-IgG (C) and BSA/FDA@SDS-IgG (D) at different pH. The data were averaged by 5 repeated measurements. All the other conditions were the same.

### FDA@SDS and antibody encapsulation and characteristic

In order to prove that antibody encapsulated on the FDA@SDS particles, MALDI-TOF mass spectrometry was applied to measure the protein on the nanoparticles' surface. The results were shown in Figure 4. In the mass spectrum of FDA@SDS (Figure 4A), there is no ionization peak in high molecule weight (>10 kDa); while in the spectrum of FDA@SDS-IgG (Figure 4B) there are several ionization peaks at 131056.56, 66020.47 and 33474.53, which is correspond to the ionization of  $[\text{IgG}+\text{H}]^{1+}$ ,  $[\text{IgG}+2\text{H}]^{2+}$  and  $[\text{IgG}+3\text{H}]^{3+}$  respectively. And these ionization peaks can be proved by the pure antibody mass spectrum (Figure 4C) which has the similar results. From the results we can see that the antibody in the solution can be encapsulated to the surface of FDA@SDS surface directly. And following experiments of SEM observations and fluorescent microscopy tests showed the FDA@SDS-IgG was stable in PBS solution in room temperature without aggregation or hydrolysis. Although antibody can be attached to the FDA@SDS surface, whether the surface becomes satisfied or not is another issue which affects the surface electrostatic property. And the unsatisfied surface charge might increase the non-specific binding during the following immunoassays, because the FDA@SDS-IgG particles may adsorpt to the plate surface which immobilized with proteins by electrostatic interaction. In order to satisfy the unoccupied sites on the FDA@SDS-IgG particles, minimize the non-specific binding and stabilize the FDA@SDS-IgG particles in the solution, commonly used blocking reagents BSA (66kDa) and Casein (23kDa) were used to coat the FDA@SDS-IgG particles which can get the resultant FDA@SDS-IgG-BSA and FDA@SDS-IgG-Casein particles, respectively. The excess of blocking reagent can also satisfy the surface charge after the antibody encapsulation.

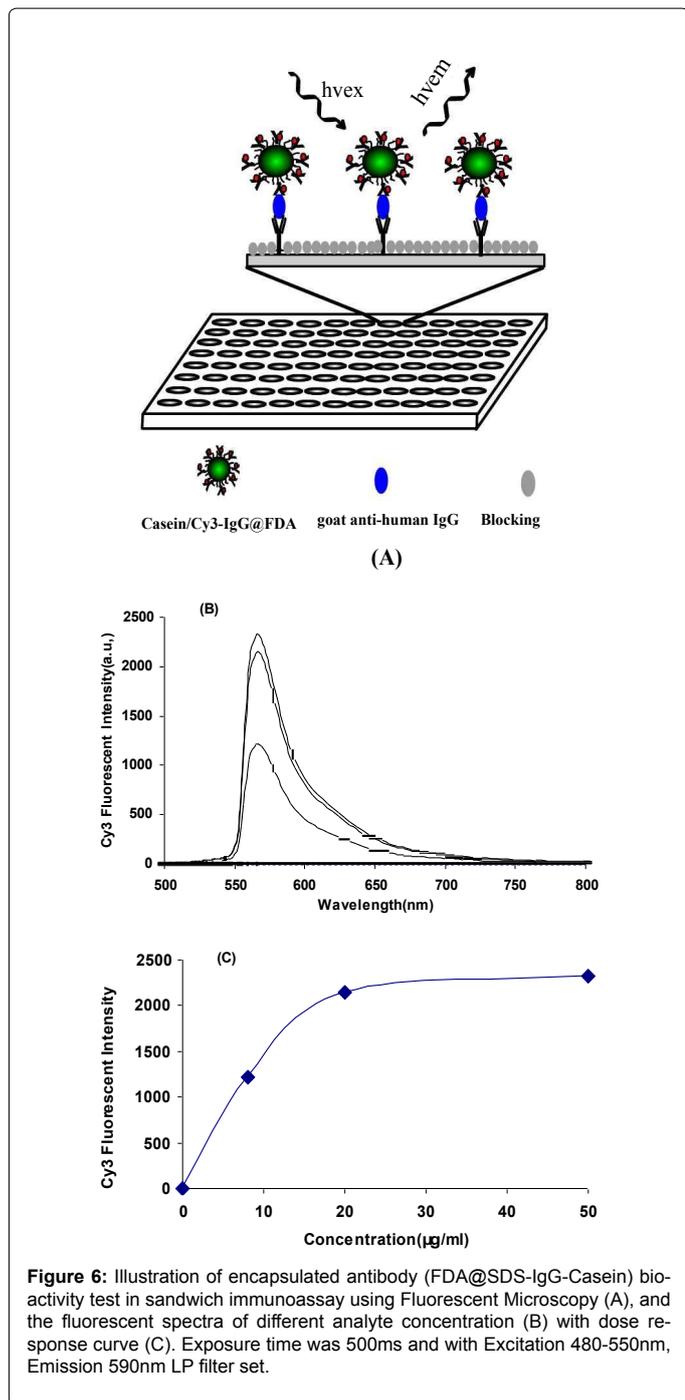
To monitor the surface charge, Zeta potential analyzer was applied to measure the charge property of the FDA@SDS, FDA@SDS-IgG, FDA@SDS-IgG-BSA and FDA@SDS-IgG-Casein particle. The results are shown in Figure 5. From the results we can see that both the

$\xi$ -potential of FDA@SDS-IgG nanoparticles (Figure 5A) and FDA@SDS nanoparticles (Figure 5B) are much negative either in high pH value or low pH value, though it changed a little at different pH value; however, for the Casein and BSA coated FDA@SDS-IgG nanoparticles (Figure 5C and D), the  $\xi$ -potential changed steadily from positive to negative when the pH value was changed from 1.3 to 12.1.

And by comparing the individual  $\xi$ -potential at different pH value, the antibody only satisfied part of the FDA@SDS charged surface while the excess of smaller molecules such as BSA or Casein can satisfy the rest of the charge. The results indicate that this interaction is mainly caused by electrostatic attraction. As in the interaction of SDS molecules and FDA nanoparticles, the dodecyl groups attach to the surface of FDA by hydrophobic interaction, leaving the sulfate groups out of the surface which makes the FDA@SDS nanoparticles in negative charge. These results indicate that the antibody in solution attaches on the surface of FDA@SDS nanoparticle with strong interaction.

### Antibody bioactivity test

Whether the SDS on the FDA nanoparticle surface can denature the encapsulated antibody or not is an important issue in the immunoassays, as the encapsulated antibody has to keep its bioactivities such as antigen-antibody recognition and specificity. In order to test the antibody bioactivities after the encapsulation on the FDA@SDS nanoparticle surface, dose response experiments were carried out when Cy3 labeled rabbit anti goat IgG was encapsulated on the FDA@SDS particles. According to the standard sandwich immunoassay (Figure 6A), human IgG was immobilized on the 96-well plate and goat anti-human IgG was used as analyte which the concentration was from 0, 10, 20 and 50  $\mu\text{g}/\text{mL}$ , when FDA@SDS-IgG-Cy3 was used as fluorescent reporter, we found that the Cy3 fluorescent intensity increased as the analyte concentration increased (Figure 6B) and the intensity was linear relation with the analyte concentration (Figure 6C) in the range from 0 to 20  $\mu\text{g}/\text{mL}$ , while it was satisfied when the analyte concentration increased from 20  $\mu\text{g}/\text{mL}$  to 50  $\mu\text{g}/\text{mL}$ . The results showed that the



**Figure 6:** Illustration of encapsulated antibody (FDA@SDS-IgG-Casein) bio-activity test in sandwich immunoassay using Fluorescent Microscopy (A), and the fluorescent spectra of different analyte concentration (B) with dose response curve (C). Exposure time was 500ms and with Excitation 480-550nm, Emission 590nm LP filter set.

antibody on the FDA@SDS surface can react with the analyte in a very good dose response which indicates that the encapsulated IgG can keep the bioactivities after the encapsulation. Presumably the SDS molecules and antibody interaction are contributed by the electrostatic interaction without further denature of the antibody. These attached IgGs may in the well-ordered status, i.e. sulfate group outside and lauryl group inside.

### FDA@SDS-IgG organic particles based immunoassay

Nonspecific binding exists in most of the solid-phase immunoassays which has relation with many aspects such as the solid surface property,

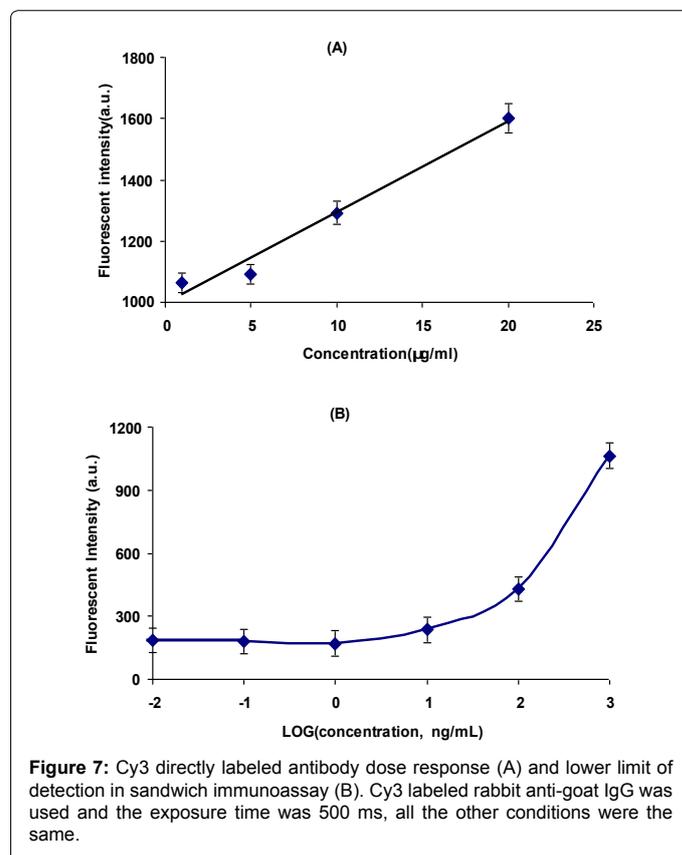
immunoassay conditions, property of samples and the analysis methods [22,23]. Here, for example, we compared two commonly used blocking buffers, i.e. BSA (66 kDa) and Casein (23kDa), in the FDA particle based immunoassays; and the results are shown in Table 1. From the table we can see that the FDA@SDS-IgG-Casein particles had better performance in nonspecific binding issue when Casein was used as blocking buffer. We assume this may due to the size of the Casein molecular is much small than BSA, which lead to higher surface protein.

In order to test the analytical performance of the Casein coated FDA@SDS-IgG particles, goat anti-human IgG (used as detection analyte) in series concentration from 50 µg/mL to 10<sup>-3</sup> ng/mL were applied to the standard immunoassays. And at the same time, the commonly used Cy3 (F/P=3.0~9.0, as refer to the Sigma reagent documents) and FITC (F/P=3.0~8.0, as refer to the Sigma reagent documents) directly labeled rabbit anti-goat IgG were also applied as comparisons respectively. From the results we can see that the work range for Cy3 directly labelled system was from 100 ng/ml to 20 µg/mL and could give out a lower limitation of detection (LOD) about 100 ng/mL (Figure 7) in the experiments. And the results for FITC

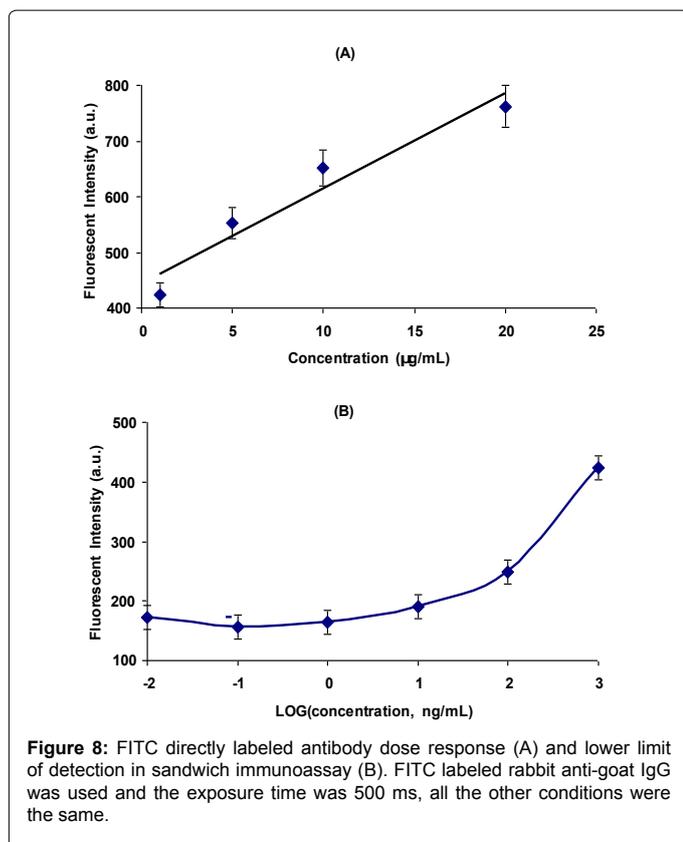
Label	BSA Blocking buffer	Casein Blocking buffer
System	NB Fluorescent Intensity (a.u.)	NB Fluorescent Intensity (a.u.)
FDA@SDS-IgG-Casein	835 ± 46.6	146.5 ± 45
FDA@SDS-IgG-BSA	1506 ± 414.8	418.3 ± 46.2

(1) The Filter set was 470 nm excitation with 515 Long pass emission; the exposure time was 100 ms with the intensifier profile Math DC 500 and Cath - 50.  
(2) NB: Nonspecific Binding (Unit: a.u.).

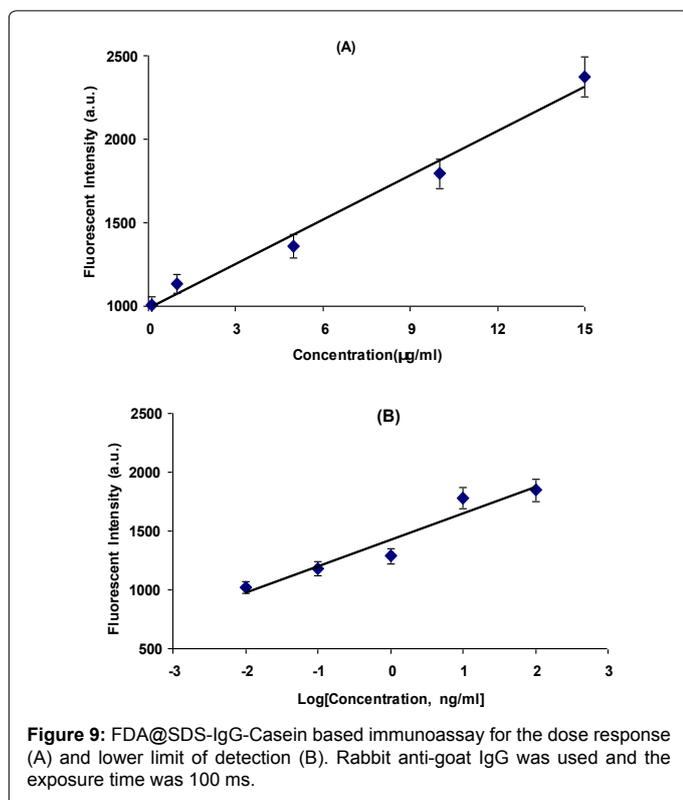
**Table 1:** Nonspecific binding in different labels and blocking buffers system <sup>(1)</sup>(n=4).



**Figure 7:** Cy3 directly labeled antibody dose response (A) and lower limit of detection in sandwich immunoassay (B). Cy3 labeled rabbit anti-goat IgG was used and the exposure time was 500 ms, all the other conditions were the same.



**Figure 8:** FITC directly labeled antibody dose response (A) and lower limit of detection in sandwich immunoassay (B). FITC labeled rabbit anti-goat IgG was used and the exposure time was 500 ms, all the other conditions were the same.



**Figure 9:** FDA@SDS-IgG-Casein based immunoassay for the dose response (A) and lower limit of detection (B). Rabbit anti-goat IgG was used and the exposure time was 100 ms.

directly labeled system gave out the similar results, as shown in Figure 8. For both of these two dyes directly labeled antibody, if the detection

analyte concentration is lower than 100ng/ml, the analyte signal is not distinguishable from nonspecific binding and background signal. For the FDA@SDS-IgG-Casein particles, the higher limitation of detection could only reach to 15 µg/mL under the same conditions (Figure 9A), and this is because the size of coated FDA particle used as labeled antibody is much larger than the dye directly labeled antibody. But the LOD was not reach at the concentration of  $10^{-2}$  ng/mL in our experiments (Figure 9B), and that is to say, the detectable concentration is about  $10^{-5.5}$  µg/mL which is 67 atto mol/mL [24]. Therefore, from the results we can see that the FDA@SDS-IgG-Casein particles have advantages in the very low analyte concentration samples and this is very important for the clinic diagnostics for the virus such HCV, HIV, which can greatly shorten the “detection window”. This method is promising to diagnose in the early weeks of virus infections.

## Conclusions

FDA nanoparticles can be successfully made by ball mill in SDS surfactant solution. The size distribution can be uniformed further by speed-gradient centrifugation. And the SDS encapsulated FDA nanoparticles can be further encapsulated with small blocking protein molecules such as casein to satisfy the surface charge by monitoring the zeta potential. And this process can also make the FDA particles more stable in the solution without aggregation and self-dehydrate. The blocking proteins can effectively eliminate the nonspecific binding which caused by the electrostatic absorption in the applied immunoassays. The FDA@SDS-IgG-Casein based immunoassay can achieve high signal amplification and low analyte detection. By further reducing nonspecific binding, the expected detectable lower concentration should be lower than 10 pg/mL.

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