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One-step *in situ* solid-substrate-based whole blood immunoassay based on FRET between upconversion and gold nanoparticles

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ABSTRACT

Despite their general clinical applications, current fluorescence-based immunoassays are confronted with serious challenges, e.g. the advance serum/ plasma separation and the tedious washing process in current heterogeneous approaches, and aggregation of particles, low sensitivity and the narrow linear range in homogeneous approaches. In this paper, these urgent problems were solved in a novel one-step *in situ* immunoassay of whole blood samples by combining the traditional fluorescence resonance energy transfer (FRET) technology (between upconversion nanoparticles (UCNPs) and gold nanoparticles (GNPs)) and the solid-substrate based immunoassay technology. The low detection limits of goat IgG (gIgG) as 0.042 μg/mL in buffers, 0.51 μg/mL in 20-fold diluted whole blood samples and a wide linear range from 0.75 μg/mL to 60 μg/mL in blood samples were achieved. To the best of our knowledge, it is the first one-step *in situ* solid-substrate-based immunoassay of whole blood samples with large linear detection range. This development provides a promising platform for a rapid and sensitive immunoassay of various bio-molecules directly in whole blood without tedious separation, washing steps and aggregation problems.

1. Introduction

Fluorescence-based immunoassay methods, mainly divided into heterogeneous and homogeneous assays, have been generally applied in current clinics. However, these techniques are constantly suffering from the following problems, *e.g.* aggregation, low sensitivity and narrow linear range in homogeneous assays, as well as the tedious process in heterogeneous assays, which have been not solved (Algar and Krull, 2009; Kim et al., 2009; Kreisig et al., 2011; Ma et al., 2010; Wang et al., 2015; Zhang, 2015). The heterogeneous immunoassay on a solid-support (paper disc, cellulose, microtiter plates, glass or plastic beads, *etc.*), such as enzyme-linked immunosorbent assay (ELISA), is one of the earliest and most mature methods (Apilux et al., 2013; Clark and Adams, 1977; Engvall and Perlmann, 1971). Although, this method is highly sensitive and matches well with the relevant shelf instruments, the serum/ plasma separation of whole blood and the tedious washing processes have to be conducted to avoid strongly fluorescent background and the nonspecific absorption, which not only are labor-intensive and time-consuming, but also may result in the change of the structure or conformation of the biomolecules and fade of the specificity and sensitivity of the immunoassay (Algar and Krull, 2009; Apilux et al., 2013; Kim et al., 2009; Ma et al., 2010; Noor and Krull, 2013). Confronted by these problems, homogeneous immunoassay methods have been proposed and developed. Most of them were based on FRET principle which is extremely sensitive to the nanoscale changes of distance between energy donors (D) and acceptors (A) and the spectral overlap of the D emission and A absorption (Sapsford et al., 2006; Yun et al., 2005). However, with the solving of the complicated washing/separation problems, new problems emerge such as low sensitivity, narrow linear range and random aggregation of particles (easy to cause some false immunoassay results) (Börner et al., 2011; Chen et al., 2013; Kreisig et al., 2011; Rantanen et al., 2008; Sapsford et al., 2006; Wang et al., 2005, 2015). Therefore, it is imperative to develop novel immunoassay methods which take the advantages of heterogeneous and homogeneous assays, but avoid the serum/ plasma separation, complicated multi-steps process, aggregation of particles. Also important is that it should be compatible with current detection instruments.

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The potential of FRET in solid-substrate-based assays, is dimmed by, for example, the concern that the properties of the energy donor/acceptor are susceptible to environment, which might be the reason behind the fact that only few relevant reports have appeared up to now (Algar and Krull, 2009; Kim et al., 2009) where dyes or GNPs were used as acceptors and quantum dots (QDs) as donors whose properties were easily affected by the interaction of particles and the interaction between particles and the solid surface. Furthermore, the separation problem of serum and the multi-steps washing still remained.

Rare earth ions (RE)-doped UCNPs show excellent fluorescence stability benefiting from the shielding effect of the $5s^25p^6$ electronic shell (Tu et al., 2015), which makes them the best candidates of energy donors for solid-substrate-based detection. Moreover, the UCNPs own many other advantages, such as NIR-excitation, narrow emission band, large anti-Stokes shifts (> 300 nm) and no bio-fluorescence. Recently, these nanomaterials have been extensively applied in bio-detection and bio-imaging of complex biological systems, such as plasma, cells, tissues, *etc* (Cen et al., 2015; Chen et al., 2013; Long et al., 2015; Peng et al., 2011, 2015; Wang et al., 2005; Xu et al., 2014). For UCNPs-FRET based detections, GNPs were popularly used as acceptors for a large extinction coefficient and a long FRET half quenching distance (up to 28 nm) (Chen et al., 2013; Long et al., 2015; Peng et al., 2011; Samanta et al., 2014; Wang et al., 2005). However, up to now, UCNPs-FRET based detection was, without exception, executed in solution-based homogeneous immunoassays, which still faced the above-mentioned problems.

Our strategy in this work was to circumvent the above drawbacks of the current immunoassays by innovatively applying UCNPs-based FRET technology (UCNPs as donors, GNPs as acceptors) on a solid-substrate, the novel one-step *in situ* approach on glass substrate realized a direct immunoassay of whole blood samples (gIgG as a proof-of-concept) without any separation and washing steps in the detection. This approach offers a rapid, sensitive, economic and simple operation detection of a variety of bio-molecules directly from whole blood.

2. Materials and methods

2.1. Fabrication of the surface of solid-substrates

To fabricate the bio-functional substrate, the substrates (5 mm×5 mm) were cleaned and functionalized following the procedure of our previous report (Zhang et al., 2010). Briefly, the substrates were firstly cleaned to get rid of the contaminant and activated the hydroxyl. Then, the substrates were immersed in a 10% (v/v) solution of aminopropyltriethoxysilane (APTES) in ethanol for 12 h to form an amino surface. After being rinsed thoroughly and dried in 120 °C oven full of nitrogen, the amino-substrates were modified with 5% glutaraldehyde (GA) solution in methanol containing 0.1 wt% Sodium cyanoborohydride for 12 h to obtain the aldehyde-functionalized substrates. After aldehyde-functionalization, the substrates were immersed in 1 mg/mL polyethyleneimine (PEI)-UCNPs solution containing 0.1 wt% Sodium cyanoborohydride to covalently immobilize UCNPs on the substrates by forming an amine linkage between the aldehyde groups and the amino groups. To obtain a proper surface coverage, the reaction time was set to be 8 h, 12 h, 24 h and 36 h, respectively. To evaluate the immobilized stability of the UCNPs on the substrates, the upconversion fluorescence spectra were measured when the UCNPs-substrates were continuously oscillated in 10 mM PBS buffer for 0 h, 6 h, 12 h, 24 h, 48 h and 96 h, respectively. The UCNPs-substrates were aldehyded again using GA. They were then coated overnight with 0.2 mg/mL rabbit-anti-goat (rIgG) at 4 °C and dipped into 2% bovine serum albumin (BSA) solution for 2 h. Finally, the substrates were immersed in an excess amount of gIgG-GNPs solution with different times from 0 min to 75 min, the upconversion fluorescence spectra were detected. Up to now, the FRET-based bio-functional substrates have been completely constructed. To verify the stability of the bio-functional substrates, the upconversion fluorescence spectra of the substrates were detected after being kept in 4 °C for 4 h, 12 h, 24 h, 36 h, 48 h and 72 h, respectively.

2.2. Immunoassay in buffers

In the typical FRET-based immunoassay, the constructed bio-functional substrates were vertically inserted into the cuvette containing 10 mM PBS buffers (pH=7.4), and the upconversion fluorescence spectra were recorded. Series concentrations of gIgG (0.09, 0.75, 3.75, 7.5, 15, 30, 45, 60, 75, 90 μg/mL) were prepared, respectively. After reaction, the fluorescence measurements were directly conducted without any washing steps. The schematic diagram of the instrumental setup is shown in Fig. S2. The gIgG concentration can be quantitatively determined according to the recovery of upconversion fluorescence. To obtain the optimum detection time, the fluorescence spectrum was measured every 10 min until the fluorescence was almost unchanged with time. To ensure the precision of the detection, the standard deviations were calculated from four measurements conducted with four independent bio-functional substrates for four samples.

2.3. Specificity of the immunoassay

To determine the detecting specificity, other biomarkers, such as rabbit-anti-human IgG, rabbit IgG, human IgG and BSA, were detected under the same experimental conditions. The concentrations of gIgG, non-specific IgGs and BSA were set as 60 μg/mL, 1 mg/mL and 50 mg/mL, respectively. Meanwhile, for studying the mechanism of the novel immunoassay, the absorption spectra of BSA-gIgG-GNPs (BgGNPs) solutions, BgGNPs bound on rIgG-UCNPs-substrate, BgGNPs bound with rIgG-UCNPs in solution were measured, respectively.

2.4. Immunoassay in whole blood

To evaluate the ability of UCNPs-GNPs-FRET-based approach for whole blood sample assay, the whole blood were firstly thawed at room temperature and mixed well. The immunoassay was performed in 20-fold diluted whole blood. The diluted blood was spiked with a series of concentrations of gIgG (0, 0.75, 1.5, 3.75, 7.5, 15, 30, 45, 60 μg/mL), respectively, and a detection procedure identical to that in buffers was followed.

3. Results and discussion

3.1. Principle of one-step in situ immunoassay on glass substrate

UCNPs-GNPs as FRET D-A pair on glass substrates was designed to realize the *in situ* immunoassay and to take advantage of the enrichment effect of the substrate to circumvent the problems of low fluorescent probes concentration and aggregation of nanoparticles in FRET-based homogeneous immunoassay methods. On top of that, the red shift of GNPs absorption spectrum due to the dense assembly on the substrate should encourage better spectral overlap with the emission of UCNPs. The implementation plan of the immunoassay is schematically illustrated in Fig. 1. The UCNPs covalently attached on the substrate are modified by rIgG, and GNPs by gIgG. The specific binding between gIgG and rIgG pushes the donor (UCNPs) and the acceptor (GNPs) in close proximity, which shall result in the fluorescence quenching of UCNPs following mainly FRET mechanism. When the bio-functional substrate is immersed into the analyte (gIgG) solutions, a competitive immunoassay takes place between gIgG-GNPs and free gIgG. Thus, the FRET process is inhibited to some extent, and the fluorescence intensity of UCNPs will be recovered in a gIgG concentration-dependent manner, which is the foundation of the quantitative detection of gIgG.