Quantum dots-gold(III)-based indirect fluorescence immunoassay for high-throughput screening of APP†

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Here we report a indirect fluorescence immunoassay for high-throughput screening of APP based on the fluorescence quenching of quantum dots by gold(III), which were dissolved from gold nanoparticles-Rabbit anti-Pig IgG conjugate, and further utilize this system to detect APP in pig serum with high sensitivity and specificity.

Actinobacillus pleuropneumoniae (APP), the causative agent of a highly contagious disease-porcine pleuropneumonia, contributes substantially to economic losses in the swine industry worldwide.[1] The main pathogenicity factors of APP include capsular polysaccharides, lipopolysaccharides, outer membrane proteins and exotoxins (ApxI, ApxII, ApxIII and ApxIV). Among them, ApxIV is specific to the species of APP. Therefore, it is optimal to detect ApxIV in clinical diagnosis of APP.[2] Currently, several methods have been developed to test ApxIV, including polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA) and indirect hemaglutination assay (IHA).[3] However, these techniques have their limitations. For example, the PCR for detection of APP was expensive and require sophisticated instrumentation.[3]

With the development of nanobiotechnology, semiconductor nanocrystals (quantum dots, QDs) have attracted a great deal of attention.[4] Compared with the traditional organic dyes, QDs exhibit some unique properties such as size-controlled fluorescence, high fluorescence quantum yields, and stability against photobleaching.[5] As excellent complements to the organic dyes, QDs have been used in optical sensors, cell labelling, immunoassays, and so on.[6] QDs-based fluorescence immunoassay (FLIA) has already been developed as a powerful and sensitive technique for protein analysis.[7] In this sandwich-type protocol, QDs were functionalized with antibodies and used as fluorescent probes for antigens. In comparison with functionalized QDs, gold nanoparticles (NPs) have some advantages in immunoassays, such as simple preparation and easy conjugation with antibodies.[8] Moreover, the sensitivity in immunoassays can be improved by indirect detection of gold(III), released from gold NPs-protein conjugates (2.3×10^5 gold atoms are theoretically contained in a 20 nm spherical gold NP).[9] Limoges reported an electrochemical immunoassay for IgG with indirect detection of gold(III).[10] Lu *et al.* described a magnetic bead-based chemiluminescent immunoassay for the indirect determination of a IgG by the amplification feature of gold(III).[11] Most recently, our group developed a sensitive method for the indirect determination of APP based on gold(III)-enhanced luminol-chemiluminescence reaction.[12]

Inspired by these, we attempted to design an indirect FLIA for screening of the ApxIV antibody. In this strategy, gold NPs instead of functionalized QDs were used as labels for the immunorecognition event. After an oxidative treatment in acidic solution, a large amount of gold(III) that was released from gold NPs-IgG conjugate, acted as a bridge connecting the fluorescent signals of the QDs and the ApxIV antibody, and were determined by a fluorescence quenching method. The proposed method avoided the complex process of QDs conjugated with IgG and improved the sensitivity of detection of ApxIV antibody. Moreover, when integrated with microarray techniques, it is easy to perform high-throughput screening of the ApxIV antibody. Therefore, quantum dots-gold(III)-based indirect FLIA is a highly sensitive, simple procedure and is also time saving.

Fig. 1 illustrates the principle of high-throughput screening of the ApxIV antibody of APP with a 96-well microplate. The procedure for this immunoassay is as follows: (1) the recombinant ApxIV protein was immobilized on the well of a microplate through an electrostatic interaction between the protein and the well. (2) After washing and blocking steps with

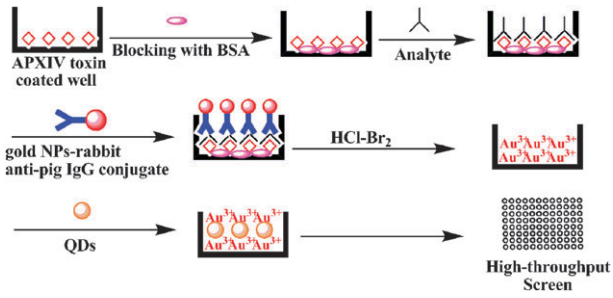

Fig. 1 Schematic representation of the QDs-gold(III)-based indirect FLIA for the high-throughput screening of APP.

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bovine serum album (BSA), the ApxIV antibody of APP with different concentrations was added for the immunorecognition event. (3) After washing three times, the gold NPs-Rabbit anti-Pig IgG conjugate was introduced into the well for the second immunorecognition event. (4) After HCl–Br_2 dissolution and volatilization, QDs were added to the well, and the fluorescent signals were recorded by a microplate reader.

In this study, water-soluble CdTe QDs capped with glutathione (GSH) were synthesized according to the procedure described in the literature with some modifications.[13] The size of the nanocrystals were estimated to be 2.9 nm from the first absorption peak (518 nm), and the center wavelength of the emission spectrum was 544 nm (see Fig. S1).† In the presence of gold NPs-Rabbit anti-Pig IgG conjugate (see Fig. S2 and Fig. S3†), the luminescence intensity of the GSH-capped CdTe QDs decreased by 8.7%, when compared to the emission of the QDs in the free solution. Under the same conditions, the luminescence of these QDs is quenched by 85.8% in a solution containing gold(III), dissolved from the gold NPs-Rabbit anti-Pig IgG conjugate (see Fig. S4).† Moreover, the gold(III) quenched the fluorescence intensity of the GSH capped CdTe QDs in a concentration dependence (Fig. 2) that is best described by a Stern–Volmer type equation (Fig. 3):

$$I_0/I = 1 + K_{sv}[\text{gold(III)}]$$

where, I_0 and I are the luminescent intensities of the QDs in a gold(III)-free solution and at a given gold(III) concentration, and K_{sv} is the Stern–Volmer quenching constant. A good linear relationship ($R = 0.9951$) was observed up to gold(III) concentration ranging from 1.5×10^{-8} to 2.0×10^{-6} mol L^{-1} and the detection limit was 5.0×10^{-9} mol L^{-1} ($S/N > 3$). More importantly, the center of fluorescent band (544 nm) was not changed with the increase of gold(III) concentration. So, integrated with microarray techniques, QDs also exhibited a high sensitivity (4.0×10^{-8} mol L^{-1}) for the detection of gold(III) (Fig. 4).

Several mechanisms have been proposed to explain how metal ions quench fluorescence of QDs, including inner filter effects, non-radiative recombination pathways, electron transfer processes and ion binding interactions.[14] To understand the fluorescence quenching mechanism in our system, fluorescence quenching experiments were conducted in the presence of free GSH. Reduced fluorescence quenching in the presence of gold(III) was observed in QD solutions containing free GSH (see Fig. S5).† It was indicated that the capping agent (GSH) of QDs was related to the fluorescence quenching. It has been reported that the gold atoms have an extremely high affinity toward thiols.[15] Moreover, our experiments showed that an interaction existed between gold(III) and

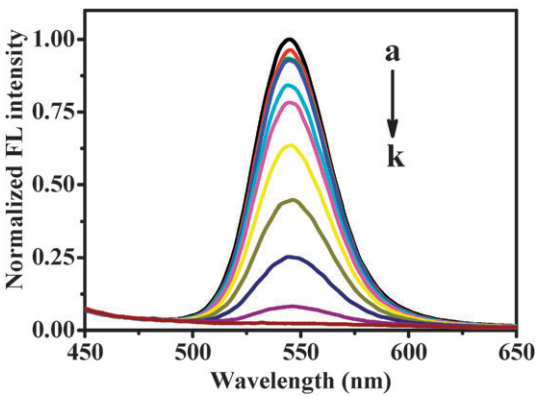

Fig. 2 FL response of GSH capped CdTe QDs to addition of gold(III) in 0.5 mol L^{-1} HCl-Tris buffer pH 7.4: (a) blank; (b) 1.5×10^{-8} mol L^{-1}; (c) 3.1×10^{-8} mol L^{-1}; (d) 1.2×10^{-7} mol L^{-1}; (e) 2.4×10^{-7} mol L^{-1}; (f) 4.9×10^{-7} mol L^{-1}; (g) 9.8×10^{-7} mol L^{-1}; (h) 2.0×10^{-6} mol L^{-1}; (i) 3.9×10^{-6} mol L^{-1}; (j) 7.8×10^{-6} mol L^{-1}; (k) 1.6×10^{-5} mol L^{-1}. Excitation wavelength = 390 nm.

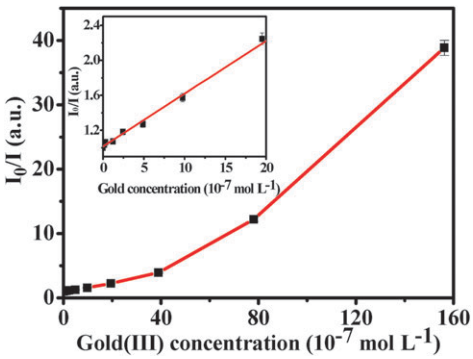

Fig. 3 Effect of gold(III) concentration on the I_0/I of GSH capped CdTe QDs. Inset: the relative fluorescence intensity of the GSH capped CdTe QDs *versus* the gold(III) concentration.

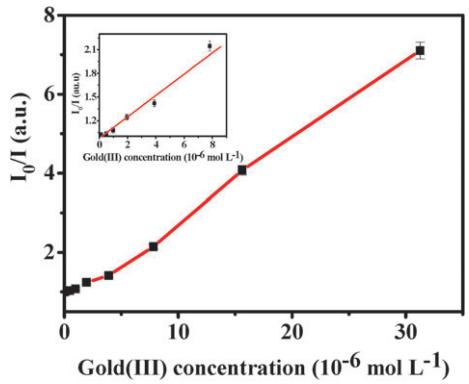

Fig. 4 Effect of gold(III) concentration on the I_0/I of GSH capped CdTe QDs. Inset: the relative FL intensity of the GSH capped CdTe QDs *versus* the gold(III) concentration. Using a multifunctional ELISA reader with excitation wavelength at 390 nm and emission wavelength at 528 nm.

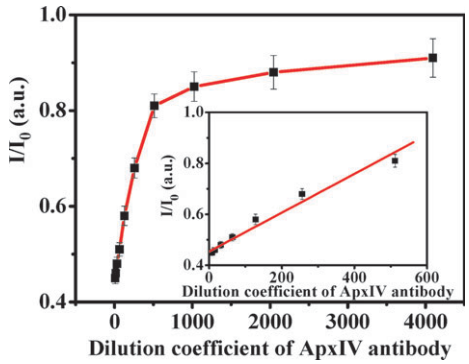

Fig. 5 The dilution coefficient of the ApxIV antibody *versus* the relative FL intensity (I/I_0). Inset: the relative FL intensity of the GSH capped CdTe QDs *versus* the dilution coefficient of the APxIV antibody.

Table 1 Comparative results of the indirect FLIA and ApxIV-ELISA of 30 clinical serum samples

		ApxIV-ELISA			Performance		
		Positive	Negative	Total	Efficiency (%)	Sensitivity (%)	Specificity (%)
Indirect FLIA	Positive	14	1	15	90.0	87.5	92.9
	Negative	2	13	15			
	Total	16	14	30			

GSH (see Fig. S6).† According to these findings, we concluded that the competitive binding of gold(III) with the GSH on the surface of the QDs was the primary mechanism for fluorescence quenching. The fluorescence intensity of the QDs was highly sensitive to their surface protection. Even the removal of a limited amount of surface-bound GSH would lead to a dramatic reduction in fluorescence.

Based on this fluorescence quenching, an indirect immunoassay for high-throughput screening of antibody against ApxIV of APP was developed. Under the optimal conditions (see Fig. S7, S8 and S9†), the linear range for the dilution coefficient of the ApxIV antibody (standard positive serum) was in the dilution range of 1 : 8–1 : 512. The limits of detection (LOD) for the assay was 1100 times dilution of standard positive serum (Fig. 5). When compared with the LOD of ApxIV-ELISA (320 times dilution) and IHA (128 times dilution), the proposed method had a higher sensitivity for the determination of ApxIV antibody of APP.[12]

To evaluate the diagnostic performance of the indirect FLIA, the clinical serum samples (n = 30), collected from naturally infected pig without an appropriate reference test to classify animals into truly infected and non-infected, were analyzed by indirect FLIA and ApxIV-ELISA. As shown in Table 1, the indirect FLIA had a efficiency of 90.0%, sensitivity of 87.5% and specificity of 92.9% compared with ApxIV-ELISA (see ESI†). The results show that indirect FLIA with high sensitivity and specificity can be used to detect ApxIV antibody of APP in clinical diagnostic test.

In conclusion, we detected the ApxIV antibody using a QDs-gold(III) system. This is the first report for measuring the ApxIV antibody *via* the quenching effect of gold(III) to QDs, and it showed great potential for numerous applications in immunoassays. We believe that the proposed method is extremely suitable for detecting, preventing, or controlling animal-borne disease outbreaks. Further studies are to be undertaken by employing a conventional gold-enhancement methods, or other kinds of metal nanoparticles instead of gold NPs, aiming to further increase the sensitivity and selectivity of the indirect FLIA.

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