Simultaneous Determination of Human Enterovirus 71 and Coxsackievirus B3 by Dual-Color Quantum Dots and Homogeneous Immunoassay

ARTICLE *in* ANALYTICAL CHEMISTRY · MARCH 2012

Impact Factor: 5.64 · DOI: 10.1021/ac203172x · Source: PubMed

8 AUTHORS, INCLUDING:

Xiaowei Zhang
Chinese Academy of Sciences
77 PUBLICATIONS **1,307** CITATIONS
SEE PROFILE

Guohua Zhou
Lingnan Normal University
27 PUBLICATIONS **330** CITATIONS
SEE PROFILE

Zhenhua Zheng
Wuhan Institute of virology, Chinese Academy …
31 PUBLICATIONS **235** CITATIONS
SEE PROFILE

Hanzhong Wang
Chinese Academy of Sciences
83 PUBLICATIONS **1,570** CITATIONS
SEE PROFILE

analytical chemistry

Article

pubs.acs.org/ac

Simultaneous Determination of Human Enterovirus 71 and Coxsackievirus B3 by Dual-Color Quantum Dots and Homogeneous Immunoassay

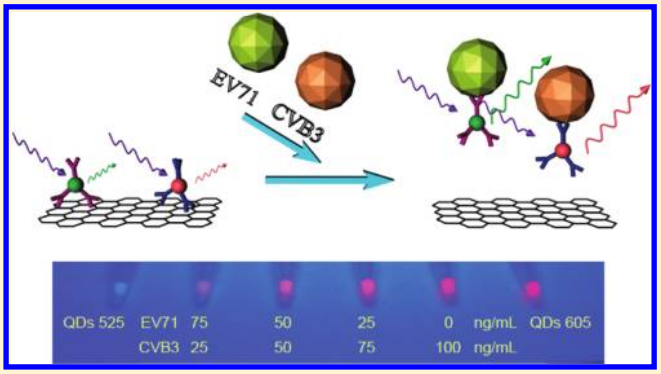
Lu Chen,[†] Xiaowei Zhang,[‡] Guohua Zhou,[†] Xia Xiang,[†] Xinghu Ji,[†] Zhenhua Zheng,[‡] Zhike He,*[,†] and Hanzhong Wang*[,‡]

[†]Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), College of Chemistry and Molecular Sciences, Wuhan University, Wuhan, 430072, PR China

[‡]State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, 430071, PR China

Supporting Information

ABSTRACT: Human Enterovirus 71 (EV71) and Coxsackievirus B3 (CVB3) have high risks for morbidity and mortality. A virus quantitation immunoassay has been proposed by employing two colored quantum dots (QDs), antibodies of the virus, and graphene oxide (GO). The QDs are streptavidin-conjugated quantum dots (SA-QDs), and the antibodies are biotinylated antibodies. Biotinylated EV71 antibody (Ab1) was associated with 525 nm green colored SA-QDs via biotin-streptavidin interaction forming QDs-Ab1, whereas biotinylated CVB3 antibody (Ab2) was associated with 605 nm red colored SA-QDs via biotin−streptavidin interaction forming QDs-Ab2. GO was an excellent quencher to the fluorescence of both QDs-Ab1 and QDs-Ab2. The targets of EV71 and CVB3 can break up the complex of QDs-Ab and GO, recovering the fluorescence of QDs-Ab1 and QDs-Ab2, respectively. Using these two different colored QDs-Ab fluorescence recovery intensities upon the addition of targets EV71 and CVB3, the two enteroviruses can be simultaneously quantitatively determined with a single excitation light. The detection limits of EV71 and CVB3 are 0.42 and 0.39 ng mL^{-1} based on 3 times signal-to-noise ratio, respectively. More importantly, this strategy can be further used as a universal method for any protein or virus determination by changing the conjugated antibodies in disease early diagnosis, which can provide a fast and promising clinical approach for virus differentiation and determination. In a word, a simple, fast, sensitive, and highly selective assay for EV71 and CVB3 has been developed. It could be applied in clinical sample analysis with a satisfactory result. It was notable that the sensor could not only achieve rapid and precise quantitative determination of protein/virus by fluorescent intensity but also could be applied in semiquantitative protein/virus determination by digital visualization.



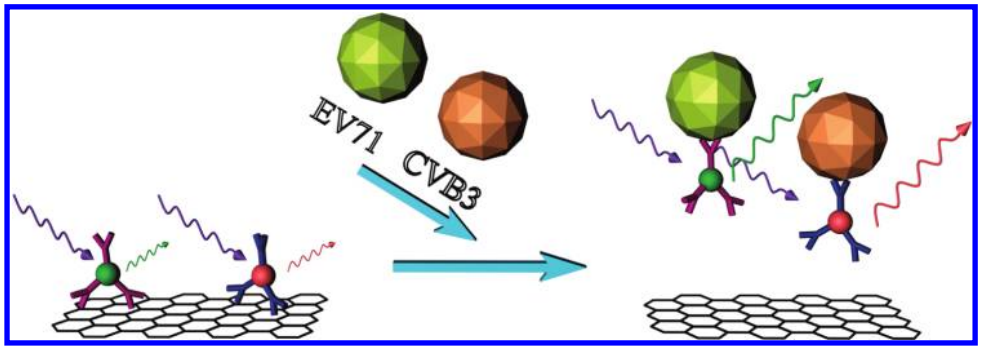
Human enteroviruses are small, single-stranded, positive-sense RNA viruses which belong to the *enterovirus* genus of the *picornaviridae* family.[1] On the basis of molecular and biological properties, human enteroviruses have been classified into four species (A−D), including some of the most common viruses causing diseases in humans like Poliovirus (PV), Enterovirus 71(EV71), Coxsakievirus B3 (CVB3), and Echoviruses (ECHO).[2] Collectively, human enteroviruses infection has a wide spectrum of clinical manifestations ranging from mild febrile illness to potentially fatal multisystem disease. Both EV71 and CVB3 are the most common and more extensively studied types of enteroviruses. However, EV71 and CVB3 may cause different diseases due to distinctive tissue tropisms, the former is the major etiological agents of hand, foot, and mouth disease (HFMD)[3] and the latter is involved in myocarditis and pancreatitis.[4] Children are considered to be more vulnerable to the infection of EV71 and CVB3 due to an immature immune system which can not protect individuals completely against subsequent lethal infection. Up until now, there are no obvious findings showing that EV71 and CVB3 could coinfect the same individual, but the symptoms caused by these two viruses infected are exactly the same. With high risk for morbidity and mortality in neonates and for accurate diagnosis in the clinical application, there is an urgent need for a rapid and specific virus screening method to differentiate these viruses for disease early diagnosis.

The availability of protein arrays has led to protein analysis a revolutionary process in the past decade for their ability to simultaneously detect multiple analytes in a sample by an affinity event at the surface of a solid interface.[5,6] Multiple

Received: November 29, 2011
Accepted: March 5, 2012
Published: March 5, 2012

   dx.doi.org/10.1021/ac203172x | *Anal. Chem.* 2012, 84, 3200−3207

Scheme 1. Schematic Presentation of the Multicolored QDs-Ab and GO Based EV71 and CVB3 Determination Biosensor



proteins are immobilized on a surface and share the same superficial chemical environment regardless of their physicochemical properties and their different binding vocations in a protein microarray, which has greatly limited its effectiveness for such application of this technology.[7] Faced with this challenge, homogeneous suspension arrays have been discovered with a number of advantages over traditional solid protein arrays, regarding, for instance, solution kinetics, ease of assay modification, higher sample throughput, and better quality control by batch synthesis.[8] However, for both the protein arrays and suspension arrays described above, tedious performance is required for completing the whole screening and determination process.[9] To develop a high-throughput and easy operation homogeneous approach for virus determination, semiconductor quantum dots (QDs) have the potential to circumvent some of the functional limitations encountered by protein arrays and suspension arrays approaches due to their unique optical features.[10] Recent advances in QDs based biosensors open up a new possibility for protein or virus multiplex detection.

QDs are currently under intensive attention due to their predominant chemical and physical properties such as broad excitation spectra,[10] photobleaching resistance,[11] narrow, symmetric, and tunable emission spectra and simultaneous excitation of multiple fluorescence colors,[12] a larger Stokes shift over conventional organic fluorophores,[13] combined with the recent research progress on QDs surface chemistry for biocompatibility and bioconjugation has made QDs labeling for the detection of various biological analytes, such as DNA,[14] protein,[15] cells,[16] and small organic molecules, possible.[17] For instance, Goldman and co-workers have reported a preparation method of bioinorganic conjugates made with highly luminescent semiconductor CdSe/ZnS core shell QDs and antibodies for use in fluoroimmunoassays.[18] Li's group has developed an immunoasay for detecting *E. coli* O157:H7 by QDs labeled antibody with a detection limit at least 100 times lower than that of the FITC-based method.[19] Banin et al. have addressed the fluorescence resonance energy transfer processes in CdSe/ZnS QDs-DNA conjugates.[20] In this present work, we take two different colored QDs for fluorescence signal output in simultaneously detecting EV71 and CVB3. As QDs have a large Stokes shift and can be excited at a single excitation wavelength, two contrasting colored fluorescence signals will be obtained at the same time without spectra overlap, which cannot be fulfilled by traditional organic dyes.

To produce a fluorescence "turn on" detection strategy, graphene oxide (GO) is selected as a superquencher to QDs fluorescence first. Recently, the rise of GO with its amazing nanoscale properties has stimulated great interest in various fields,[21] including molecular sensors,[22] drug delivery,[23] photovoltaic cells,[24] nanocomposites,[25] nanoelectronic devices,[26] and transparent conductors.[27] Particularly, in terms of its large cross section,[28] two-dimensional distribution of disorders, and random orientation of active sites, two-dimensional GO appears to behave differently from these zero-dimensional gold nanoparticles (Au NPs).[29] To date, GO has been employed as an efficient fluorescence quencher based on the photoinduced electron transfer mechanism or energy transfer mechanism. Xie et al. designed a novel and promising "turn-on" fluorescent Cu^{2+} biosensor based on a graphene-DNAzyme catalytic beacon due to the essential surface and quenching properties of two-dimensional graphene.[30] Chandra and co-workers reported a facile chemical route to synthesize the polypyrrole-reduced graphene oxide composite showing a highly selective Hg^{2+} removal capacity.[31] Now, we further take advantage of GO as an effective quencher to QDs fluorescence for creating a dual-color fluorescence "turn on" strategy to simultaneously detect EV71 and CVB3. In our previously reported work,[32] we have developed a new fluorescent immune ensemble probe comprised of a conjugated lower toxic water-soluble CdTe:Zn^{2+} QDs and Ru(bpy)$_2$(mcbpy-O-Su-ester)$(PF_6)_2^-$ antibody complex (Ru-Ab) for the dual-color determination of EV71 in homogeneous solution. Though Ru-Ab was an effective quencher to QDs fluorescence for EV71 detection, this strategy was not able to achieve simultaneous detection of multiplex viruses due to the quenching effect of Ru-Ab to QDs fluorescence without selectivity.

In this present work, we designed a novel detection strategy to realize multiplex viruses simultaneous determination by labeling EV71 antibody (Ab1) and CVB3 antibody (Ab2) with different colored QDs. Our detection strategy is shown in Scheme 1. Ab1 and Ab2 are biotin labeled by biotinylated reagent at first. Streptavidin conjugated green colored QDs (525 nm) and streptavidin conjugated red colored QDs (605 nm) are coupled with Ab1 and Ab2 via biotin−streptavidin interaction, respectively. The fluorescence of these QDs-Ab bioconjugates (QDs-Ab1 and QDs-Ab2) are quenched by GO to produce a virus capture probe. Once the virus capture probe is exposed to the targets of EV71 and CVB3, green color and red color fluorescence will be turned on by releasing the QDs-Ab due to the antibody antigen combination. If only one target exists, the corresponding colored QDs will be released and emit strong fluorescence, indicating the existence of the corresponding target virus (i.e., when EV71 exists alone, only green color fluorescence emission will be turned on). By taking advantage

dx.doi.org/10.1021/ac203172x | *Anal. Chem.* 2012, 84, 3200−3207

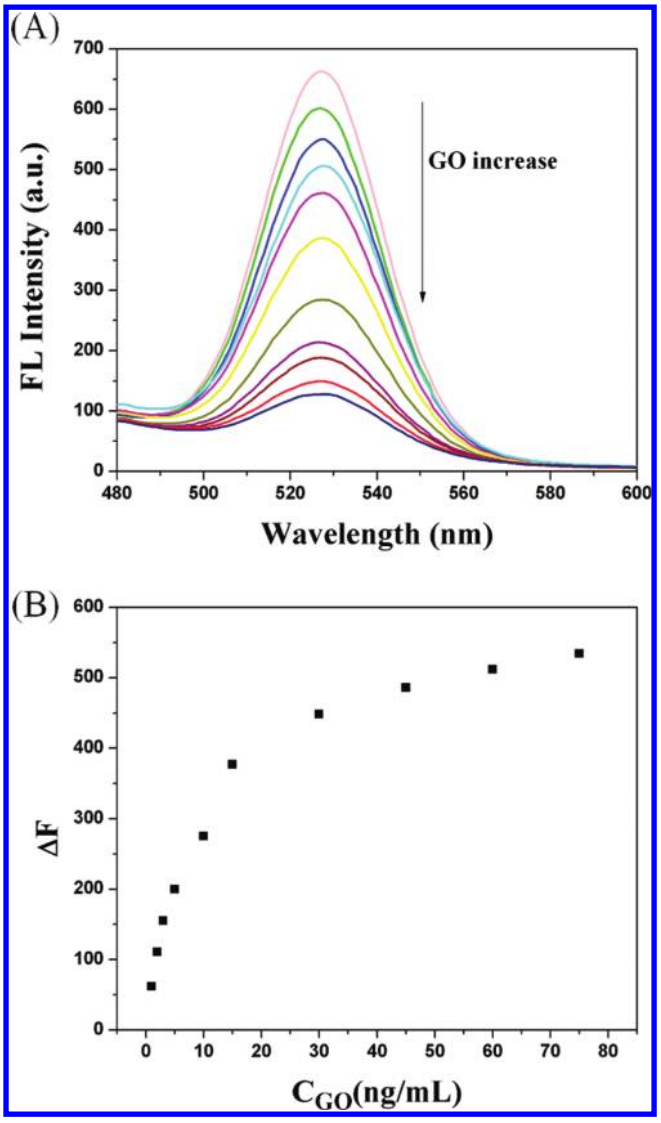


Figure 1. (a) Changes in the fluorescence spectra of 525 nm QDs-Ab1 (1 nM) in 15 mM PBS solution (pH 8.0; λex = 350 nm) with an increasing concentration of GO, with concentrations of 0, 1, 2, 3, 5, 10, 15, 30, 45, 60, 75 ng mL^{-1} (from top to bottom). (b) The curve of quenched fluorescence intensity of 525 nm QDs-Ab1 upon increasing of the GO concentration.

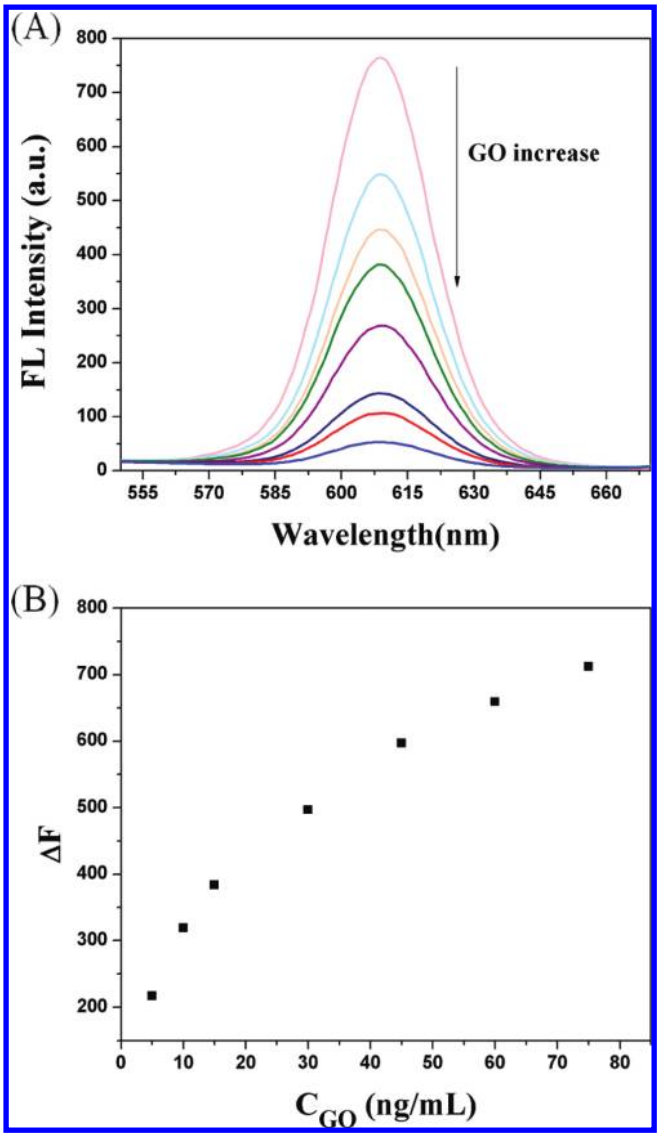


Figure 2. (a) Changes in the fluorescence spectra of 605 nm QDs-Ab2 (1 nM) in 15 mM PBS solution (pH 8.0; λex = 350 nm) with an increasing concentration of GO, with concentrations of 0, 5, 15, 30, 45, 60, 75 ng mL^{-1} (from top to bottom). (b) The curve of quenched fluorescence intensity of 605 nm QDs-Ab2 upon increasing of the GO concentration.

of this novel detection strategy, EV71 and CVB3 can be determined simultaneously. More importantly, this detection model cannot only determine the virus by fluorescence recovery intensity but also be realized by photo visualization.

EXPERIMENTAL SECTION

Material and Reagents. Sodium phosphate monobasic dihydrate ($NaH_2PO_4{\cdot}2H_2O$), sodium phosphate dibasic dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$), sodium chloride (NaCl), and biotin *N*-hydroxysuccinimide ester were commercially available from Sigma (St. Louis, MO). Goat antimouse IgG, goat antirabbit IgG, mouse IgG, and rabbit IgG were obtained from Pierce Biotechnology. Streptavidin modified QDs (525 nm) and QDs (605 nm) were bought from Wu Han Jia Yuan Quantum Dots Co., Ltd. (Wuhan, China). All chemicals used were of analytical grade or of the highest purity available. All solutions were prepared using Milli-Q water (Millipore) as the solvent.

Apparatus and Procedures. UV−vis absorption spectra data were recorded by a UV-2550 spectrophotometer (Shimadzu, Tokyo, Japan). Fluorescence spectra data were collected with a RF-5301PC fluorescence spectrophotometer (Shimadzu, Tokyo, Japan). The sensor was put in a ZF-20D black-box type UV analyzer (Shanghai Electro-Optical Instrument Factory), and the fluorescent photos were taken by a digital camera. Solutions containing appropriate concentrations of QDs-Ab1, QDs-Ab2, GO, EV71, and CVB3 (The preparation of QDs-Ab1, QDs-Ab2, EV71, and CVB3 was detailed in the Supporting Information) were made up to 0.6 mL in 15 mM PBS buffer solution (pH 8.0, 15 mM NaCl). The fluorescence emission spectrum of the solution was then measured 15 min later. All optical measurements were performed at room temperature under ambient conditions, and the excitation wavelength (λex) was 350 nm.

dx.doi.org/10.1021/ac203172x | *Anal. Chem.* 2012, 84, 3200−3207

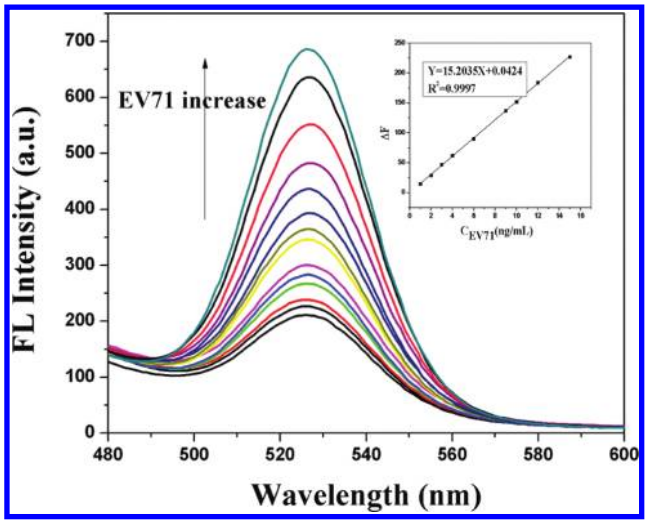


Figure 3. Changes in the fluorescence spectra of 525 nm QDs-Ab1 upon increasing the concentration of EV71 from 0, 1, 2, 3, 4, 6, 9, 10, 12, and 15 ng mL^{-1} (from bottom to top). (Inset: linear curve of single determination for EV71).

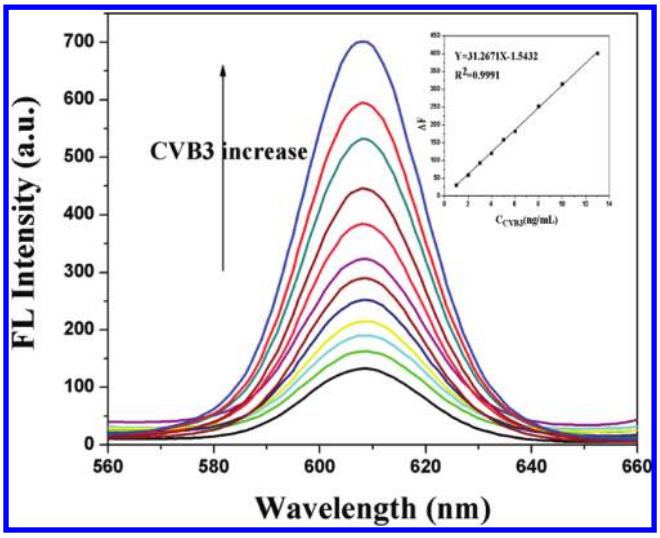


Figure 4. Changes in the fluorescence spectra of 605 nm QDs-Ab2 upon increasing the concentration of CVB3 from 0, 1, 2, 3, 4, 5, 6, 8, 10, and 13 ng mL^{-1} (from bottom to top). (Inset: linear curve of single determination for CVB3).

RESULTS AND DISCUSSION

GO As an Excellent Quencher to Dual-Color QDs-Ab Fluorescence and Forming a Virus Capture Probe with Them. In the recent studies, GO has attracted considerable attention in bioassays because of its unique electronic, mechanical, and thermal properties.[33] As reported by the pioneering work of Swathi et al., it was proposed through theoretical calculations that graphene could act as a super-quencher of organic dyes, as a result of nonradiative transfer of electronic excitation energy from dye excited states to the π system of graphene.[34] Inspired by this unique property, we take advantage of GO as a good quencher to QDs fluorescence. Graphene oxide nanosheets were synthesized according to the reported method,[35] and the obtained dispersion was dialyzed through semipermeable membranes to remove impurities. The products were characterized by the XRD pattern (Figure S3 in the Supporting Information), which exhibits the characteristic

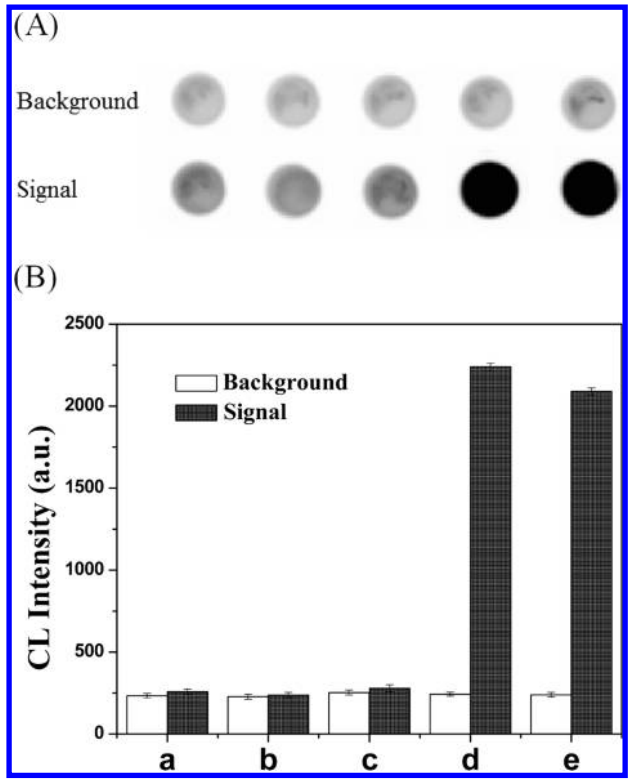


Figure 5. Chemiluminescence images (A) and chemiluminescence intensities (B) of ELISA results by these two antibodies and antigens cross reaction. The ELISA reactants are of (a) Ab1 and Ab2, (b) Ab1 and CVB3, (c) Ab2 and EV71, (d) Ab1 and EV71, and (e) Ab2 and CVB3, respectively.

diffraction peak of GO at $2\theta = 11.42$, and the mean thickness of GO is 0.7 nm (Figure S4 in the Supporting Information). Green colored QDs-Ab1 and red colored QDs-Ab2 got close to GO, and superquench occurred by the GO excellent quenching capacity. As shown in Figures 1 and 2, the effect of the concentration of GO was studied over the range between 5 and 75 ng mL^{-1}. When the GO concentration was less than 1 ng mL^{-1}, there was rarely a quenching effect to 1 nM QDs-Ab which could be due to incomplete absorption between GO and QDs-Ab. ΔF was augmenting with the GO concentration increase between 5 and 75 ng mL^{-1} and reached a plateau after GO concentration was beyond 75 ng mL^{-1}, which indicated that QDs-Ab was adsorped on the surface of GO completely. An excess amount of GO was unfavorable for desorption of QDs-Ab from it through virus capture probe formation, and ΔF was no longer increased with the increase of GO when the concentration was higher than 75 ng mL^{-1}. Therefore, 60 ng mL^{-1} GO was used for this research. The high quenching efficiency was mainly attributed to the $\pi-\pi$ stacking interaction between GO and QDs-Ab, as well as hydrogen bonding interaction presented between −OH or −COOH groups of GO and −OH or $-NH_2$ groups of the QDs-Ab. The strong interactions between the GO and QDs and the high stability and water-solubility made GO a good quencher for this virus detection probe design.

Determination of EV71 and CVB3 by the Fluorescence Turn on Detection Model, Respectively. Under the optimum conditions, EV71 and CVB3 can be determined by this fluorescence turn on detection model, respectively. As indicated by Figure 3, fluorescence intensity increased linearly with

dx.doi.org/10.1021/ac203172x | Anal. Chem. 2012, 84, 3200−3207

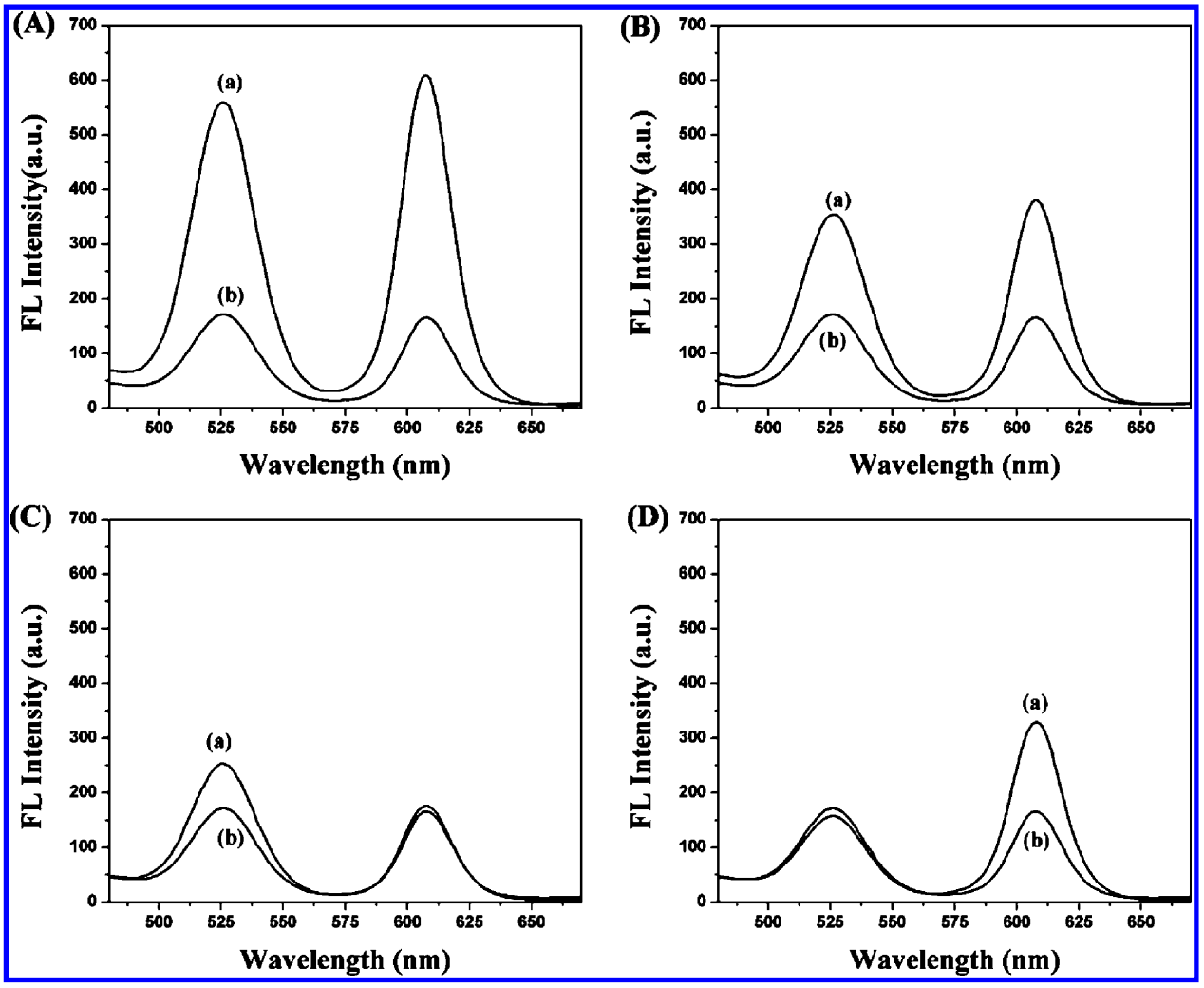


Figure 6. (A) Fluorescence spectra of (a) QDs-Ab, (b) QDs-Ab, and GO. (B) Fluorescence spectra of (a) QDs-Ab, GO, EV71, and CVB3, (b) QDs-Ab and GO. (C) Fluorescence spectra of (a) QDs-Ab, GO, and EV71, (b) QDs-Ab and GO. (D) Fluorescence spectra of (a) QDs-Ab, GO, and CVB3, (b) QDs-Ab and GO.

the EV71 concentration increase in the range between 1 and 15 ng mL^{-1} with a detection limit of 0.38 ng mL^{-1} based on 3 times the signal-to-noise ratio. For CVB3 determination, as shown in Figure 4, a 1−14 ng mL^{-1} linear relationship was obtained with a detection limit of 0.26 ng mL^{-1} based on 3 times the signal-to-noise ratio. As QDs can be simultaneously excited with multiple fluorescence colors and have a larger Stokes shift than organic fluorophores, we can realize EV71 and CVB3 simultaneous detection by the above-described methods by exciting different colored QDs at the same time.

Cross-Reaction Analysis. Before simultaneous detection of EV71 and CVB3, it is necessary to demonstrate whether there is cross reaction between Ab1 and Ab2, as the cross-reaction is a crucial analytical parameter regarding the assay specificity and thus the reliability of the multiplex immunoassay. A solution of each antibody at 30 ng mL^{-1} was analyzed by the enzyme linked immunosorbent assay (ELISA) by comparing horseradish peroxidase (HRP) labeled secondary antibody to catalyze the luminol and hydrogen-peroxide chemiluminescence intensity in the absence and in the presence of the corresponding antigen. As indicated by Figure 5, it was found that there was no obvious cross reaction between Ab1 and Ab2, neither between Ab1 and CVB3, nor between Ab2 and EV71. By contrast, the specificities between Ab1 and EV71 and Ab2 and CVB3 were very remarkably strong. Furthermore, the specificity of this method was also investigated straightforward by fluorescence adopting QDs-Ab1, QDs-Ab2, and GO complex tested against Ab1, EV71, and Ab2 and QDs-Ab1, QD-Ab2, and GO complex tested against Ab1, CVB3, and Ab2 (see Figure S5 in the Supporting Information). The result has shown that there is no cross reaction between the two virus antibodies. Thereafter, this QDs-Ab and GO based technology can be employed for the simultaneous determination of EV71and CVB3 in a single sample solution.

Selectivity of the Dual-Colored QDs-Ab and GO Virus Capture Probe. It has to be stated that the detection system can differentiate the two enteroviruses by QDs with different color fluorescence emission. Figure 6 has shown the determination results. The dual-colored QDs emitted strong fluorescence emission alone (Figures S1 and S2 in the Supporting Information have shown the QDs fluorescence and UV−vis spectroscopy) but exhibited weak fluorescence with GO quenching, which was composed to be the virus capture probe to identify EV71 and CVB3. When EV71 alone existed in the sample solution, increasing fluorescence emission of green colored QDs was observed. In contrast, when CVB3 alone existed in the analysis sample, only red colored QDs

dx.doi.org/10.1021/ac203172x | *Anal. Chem.* 2012, 84, 3200−3207

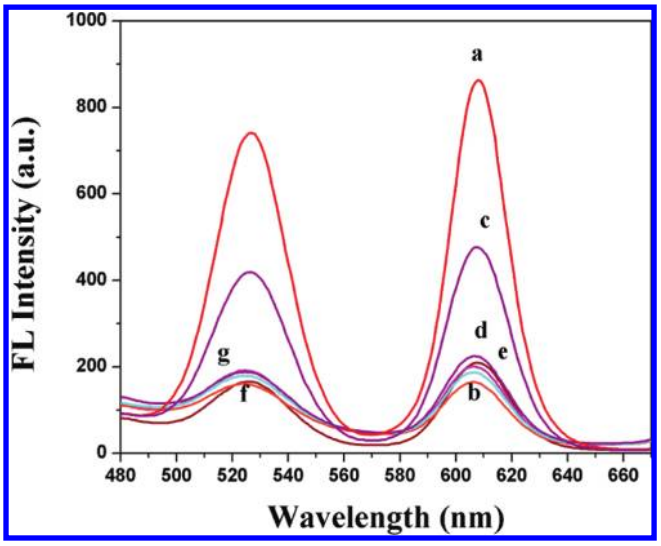


Figure 7. Fluorescence spectra of (a) QDs-Ab, (b) QDs-Ab with GO, (c) QDs-Ab, GO with EV71, and CVB3, (d) QDs-Ab, GO with PRV, (e) QDs-Ab, GO with mumps virus, (f) QDs-Ab, GO with CVA9, (g) QDs-Ab, GO with ECHO9.

fluorescence emission intensity increased. By utilizing these two different colored QDs, we can differentiate the two viruses easily and effectively. When both EV71 and CVB3 existed in the sample solution, the two distinguishing colored QDs fluorescence emission increased at the same time and the solution exhibited its mixed color yellow. The present multicolored QDs based virus identification method can be applied to other more kinds of virus differentiation by employing more colored QDs and virus antibodies based on this simple, fast, and cost-effective detection method.

To testify the selectivity of this detection system, Coxsakievirus A9 (CVA9), Enteric Cytopathic Human Orphan virus (ECHO virus), mumps virus, and pseudorabies virus (PRV) were chosen as controls to investigate selectivity of the method. As the results in Figure 7 indicated, CVA9 and ECHO virus, which belong to enterovirus family, could not recover the fluorescence intensity. Mumps virus and PRV could not interact with QDs-Ab1 or QDs-Ab2, either. However, there is somewhat a little fluorescence recovery in the control experiment, which may be due to the antibody's nonspecific interaction to other antigens. Other interferences such as calf thymus DNA, ATP, and enzyme proteins are also under investigation (see Figure S6 in the Supporting Information). The result has shown that these biomolecules, DNA, and proteins have little interference to the virus determination. Overall, this detection system has shown a good selectivity over EV71 and CVB3 and can be applied for quantitative determination of them.

Novel Multicolored QDs-Ab and GO Based Biosensor for Dual-Color Simultaneous Determination of EV71 and CVB3. Under the optimized conditions, the multicolored QDs-Ab and GO based virus capture probe can be used for quantitive detection of EV71 and CVB3 simultaneously. As shown in Figure 8, once this virus capture probe was exposed to the targets of EV71 and CVB3, antigen antibody combination occurred and QDs-Ab1 as well as QDs-Ab2 were released from the GO surface, resulting in fluorescence recovery. With the increased concentration of EV71 and CVB3, the green and red colored QDs fluorescence intensities

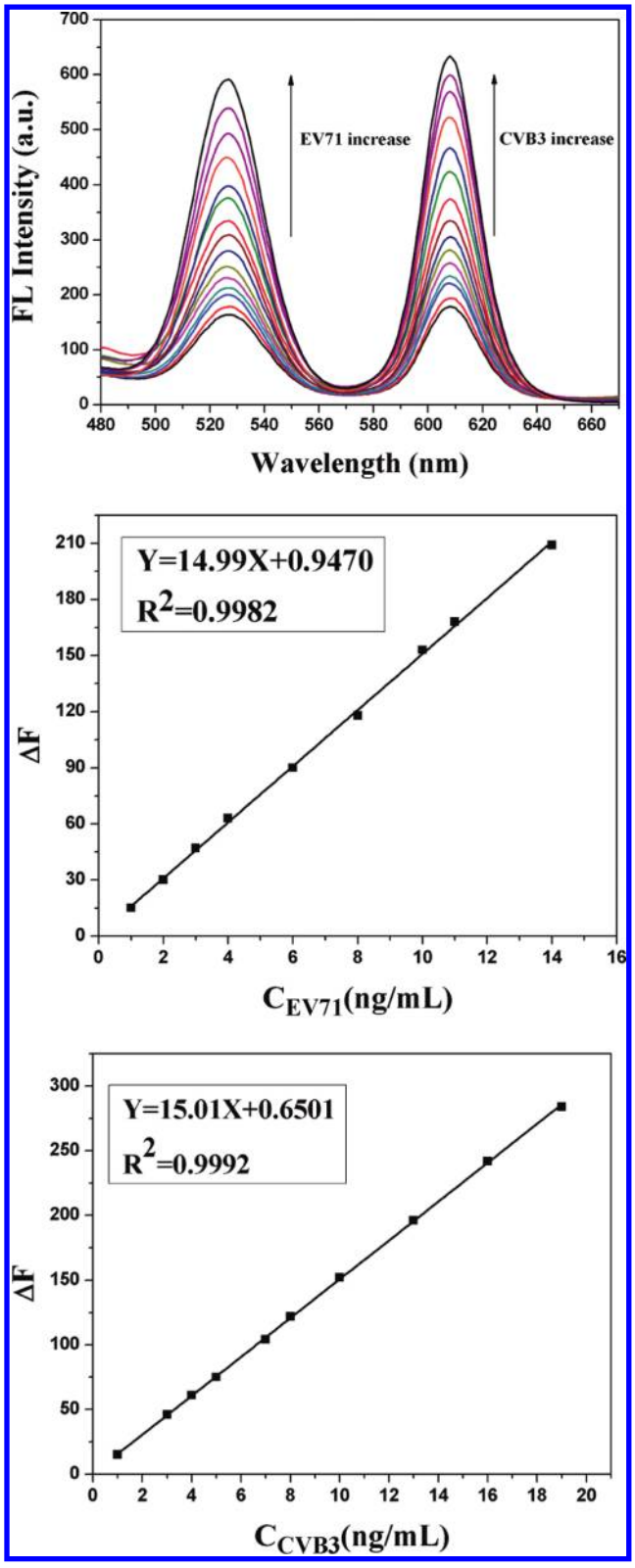


Figure 8. Fluorescence spectra of (A) QDs-Ab, GO and increasing concentration of EV71 and CVB3; (B) linear curve for EV71 detection; (C) linear curve for CVB3 detection.

were augmented at the same time, respectively. The virus concentration was quantified by monitoring the change in the fluorescence intensity. The detection was accomplished with a single excitation light. Multiplexed detection was demonstrated

dx.doi.org/10.1021/ac203172x | *Anal. Chem.* 2012, 84, 3200−3207

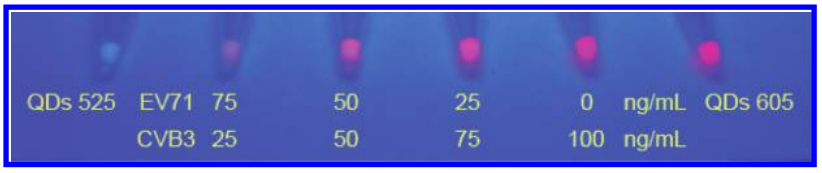


Figure 9. Photo visualizations of semiquantitative simultaneous determination of EV71 and CVB3.

Table 1. Analytical Results for the Determination of EV71 and CVB3 in Human Throat Swab Clinical Samples

		EV71	CVB3	mean recovery for EV71 (%)	mean recovery for CVB3 (%)	RSD for EV71 (n = 3)	RSD for CVB3 (n = 3)
	0	6.32 ± 0.02	2.44 ± 0.05			2.4	2.8
sample 1	2.0	8.35 ± 0.04	4.46 ± 0.06	100.3	100.4	0.6	1.2
	4.0	10.29 ± 0.03	6.42 ± 0.04	99.7	99.6	1.2	2.0
	8.0	14.30 ± 0.02	10.38 ± 0.06	99.8	99.4	2.6	1.8
	0	9.60 ± 0.03	3.65 ± 0.06			1.0	1.5
sample 2	2.0	11.62 ± 0.03	5.61 ± 0.03	100.1	99.3	1.4	1.6
	4.0	13.61 ± 0.06	7.68 ± 0.04	100.1	100.4	3.2	2.7
	8.0	17.56 ± 0.04	11.58 ± 0.07	99.8	99.4	1.9	2.3
	0	8.83 ± 0.02	1.68 ± 0.04			2.0	1.6
sample 3	2.0	10.85 ± 0.05	3.60 ± 0.06	100.2	97.8	0.8	1.2
	4.0	12.80 ± 0.06	5.64 ± 0.03	99.8	99.3	1.1	2.6
	8.0	16.80 ± 0.03	9.62 ± 0.05	99.8	99.3	2.1	1.8
	0	4.83 ± 0.06	1.12 ± 0.03			1.7	1.8
sample 4	2.0	6.85 ± 0.02	3.15 ± 0.05	100.3	101.0	2.0	2.2
	4.0	8.84 ± 0.07	5.18 ± 0.04	100.1	101.1	0.7	1.5
	8.0	12.80 ± 0.02	9.08 ± 0.05	99.8	99.6	2.7	1.9
	0	7.53 ± 0.03	1.20 ± 0.05			1.3	0.8
sample 5	2.0	9.52 ± 0.06	3.16 ± 0.04	99.9	98.7	2.5	1.6
	4.0	11.60 ± 0.03	5.26 ± 0.05	100.6	101.1	1.4	2.8
	8.0	15.48 ± 0.04	9.14 ± 0.07	99.7	99.3	2.1	1.9
	0	5.90 ± 0.03	2.84 ± 0.06				
sample 6	2.0	7.80 ± 0.04	4.80 ± 0.06	98.7	99.2	2.6	1.8
	4.0	9.98 ± 0.02	6.88 ± 0.03	100.8	100.6	1.7	2.2
	8.0	13.85 ± 0.02	10.80 ± 0.06	99.6	99.6	1.3	1.9

by simultaneously assaying EV71 and CVB3 (as model analytes) using two different emissions of QDs. EV71 can be determined at 0.42 ng mL^{-1} with a linear range of 1−14 ng mL^{-1}, whereas CVB3 can be quantified at 0.39 ng mL^{-1} with a linear range of 1−19 ng mL^{-1}, respectively. This simultaneous detection method not only can realize virus differentiation but also virus determination at the same time with good sensitivity, selectivity. In addition, it has shortened the detection time compared with RT-PCR,[36] protein arrays,[37] etc. Finally, as a simple homogeneous immuoassay for virus quantitation methodology, it can also be realized by photo visualization (see Figure 9).

Application to Clinical Specimen Analysis. We also demonstrated that this homogeneous immune detection strategy could be applied to detect human throat swabs as clinical samples obtained from EV71 and CVB3 positive cases (see in the Supporting Information for the clinical sample preparation protocol). The analytical results for the clinical samples spiked with 2−8 ng mL^{-1} are given in Table 1. The concentrations of EV71 and CVB3 virions in the spiked clinical samples determined by the developed method were in good agreement with those added of EV71 and CVB3 viral particles, along with the quantitative recovery ranging from 98.7% to 101.8%, demonstrating the potential clinical applicability of the QDs-Ab and GO immunofluorescent sensor for the simultaneous quantification of EV71 and CVB3. Furthermore, the generality of this detection strategy to other proteins was also discussed (see Figure S7 in the Supporting Information).

CONCLUSIONS

A simple, sensitive, highly selective, and rapid QDs-based virus multiplex detection sensing ensemble is developed for simultaneous detection of EV71 and CVB3 with dual-color fluorescence output signals in homogeneous solution. As a proof of concept, we demonstrate that the proposed assay is effective for detecting EV71 and CVB3 simultaneously with high sensitivity and specificity. Apparently, the sensor has good potential to expand its application to the early diagnosis determination of any virus by changing antibodies. According to the proposed sensing strategy, it can be applied in real sample analysis with a satisfactory result. This optical sensor can be achieved only with an excitation light from a single wavelength, which makes the assay much easier to achieve multiplex detection than the conventional fluorescence methods using traditional organic dyes. More importantly, the detection strategy cannot only achieve rapid and precise quantitative determination of protein/virus by evaluating changes in fluorescent intensity but also could be applied in semiquantitative protein/virus determination by digital visualization. On the whole, it provides a novel option for virus species or subtype differentiation and screening simply and effectively.

dx.doi.org/10.1021/ac203172x | *Anal. Chem.* 2012, 84, 3200−3207

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: zhkhe@whu.edu.cn (Z.H.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financially supported by the National Science Foundation of China (Grant 21075093), the National Key Scientific Program Nanoscience and Nanotechnology (Grant 2011CB933600), the Science Fund for Creative Research Groups of NSFC (Grant 20921062), "863 Project" (Grant 2008AA10Z412) from the Ministry of Science and Technology, People's Republic of China, and National Natural Science Foundation of China (Grant 81071351). We appreciate the EV71 and CVB3 antibodies provided by Prof. P. Y. Mao from the Department of Virology, Institute of Infectious Disease of 302 Hospital in Beijing.

■ REFERENCES

(1) Lum, L. C. S.; Wong, K. T.; Lam, S. K.; Chua, K. B.; Goh, A. Y. T. *Lancet* **2000**, *355* (9198), 146−147.

(2) Foo, D. G. W.; Ang, R. X.; Alonso, S.; Chow, V. T. K.; Quak, S. H.; Poh, C. L. *Clin. Microbiol. Infect.* **2008**, *14* (3), 286−288.

(3) Kiyu, A.; Cardosa, J.; Mohd-Noh, K.; Matbah, R.; Ooi, C. H. *Int. J. Infect. Dis.* **2008**, *12*, 12−13.

(4) Xu, J.; Qian, Y.; Wang, S. X.; Serrano, J. M. G.; Li, W.; Huang, Z. H.; Lu, S. *Vaccine* **2010**, *28* (20), 3516−3521.

(5) Garibotti, A. V.; Perez-Rentero, S.; Eritja, R. *Int. J. Mol. Sci.* **2011**, *12* (9), 5641−5651.

(6) Cerf, A.; Alava, T.; Barton, R. A.; Craighead, H. G. *Nano Lett.* **2011**, *11* (10), 4232−4238.

(7) Liu, W. Y.; Zhong, H.; Wang, R. S.; Seeman, N. C. *Angew Chem., Int. Ed.* **2011**, *50* (1), 264−267.

(8) Su, H. Y.; Zhang, Y. L.; Zhang, C. M.; Zhou, X. F.; Li, J. P. *Bioresour. Technol.* **2011**, *102* (21), 9884−9890.

(9) Baumgartner, S.; Vasicek, M.; Bulyha, A.; Heitzinger, C. *Nanotechnology* **2011**, *22* (42), 1−9.

(10) Wu, P.; Li, Y.; Yan, X. P. *Anal. Chem.* **2009**, *81* (15), 6252−6257.

(11) Gosso, S.; Gavello, D.; Giachello, C. N. G.; Franchino, C.; Carbone, E.; Carabelli, V. *Biomaterials* **2011**, *32* (34), 9040−9050.

(12) Ratanatawanate, C.; Chyao, A.; Balkus, K. J. *J. Am. Chem. Soc.* **2011**, *133* (10), 3492−3497.

(13) Wang, X.; Lou, X. H.; Wang, Y.; Guo, Q. C.; Fang, Z.; Zhong, X. H.; Mao, H. J.; Jin, Q. H.; Wu, L.; Zhao, H.; Zhao, J. L. *Biosens. Bioelectron.* **2010**, *25* (8), 1934−1940.

(14) Narayanan, S. S.; Sinha, S. S.; Verma, P. K.; Pal, S. K. *Chem. Phys. Lett.* **2008**, *463* (1−3), 160−165.

(15) Shan, Y. M.; Wang, L. P.; Shi, Y. H.; Zhang, H.; Li, H. M.; Liu, H. Z.; Yang, B.; Li, T. Y.; Fang, X. X.; Li, W. *Talanta* **2008**, *75* (4), 1008−1014.

(16) Pan, J.; Feng, S. S. *Biomaterials* **2009**, *30* (6), 1176−1183.

(17) Fei, X. N.; Gu, Y. C.; Wang, J.; Jia, G. Z.; Liu, Z. J. *J. Lumin.* **2011**, *131* (2), 291−296.

(18) Goldman, E. R.; Anderson, G. P.; Tran, P. T.; Mattoussi, H.; Charles, P. T.; Mauro, J. M. *Anal. Chem.* **2002**, *74* (4), 841−847.

(19) Su, X. L.; Li, Y. B. *Anal. Chem.* **2004**, *76* (16), 4806−4810.

(20) Gill, R.; Willner, I.; Shweky, I.; Banin, U. *J. Phys. Chem. B* **2005**, *109* (49), 23715−23719.

(21) Li, F. W.; Xue, M. Q.; Ma, X. L.; Zhang, M. N.; Cao, T. B. *Anal. Chem.* **2011**, *83* (16), 6426−6430.

(22) Cai, L. P.; Zhan, R. Y.; Pu, K. Y.; Qi, X. Y.; Zhang, H.; Huang, W.; Liu, B. *Anal. Chem.* **2011**, *83* (20), 7849−7855.

(23) Du, D.; Wang, L. M.; Shao, Y. Y.; Wang, J.; Engelhard, M. H.; Lin, Y. H. *Anal. Chem.* **2011**, *83* (3), 746−752.

(24) Zarrin, H.; Higgins, D.; Jun, Y.; Chen, Z. W.; Fowler, M. *J. Phys. Chem. C* **2011**, *115* (42), 20774−20781.

(25) Kranbuehl, D. E.; Cai, M.; Glover, A. J.; Schniepp, H. C. *J. Appl. Polym. Sci.* **2011**, *122* (6), 3740−3744.

(26) Deng, M.; Yang, X.; Silke, M.; Qiu, W. M.; Xu, M. S.; Borghs, G.; Chen, H. Z. *Sens. Actuators, B: Chem.* **2011**, *158* (1), 176−184.

(27) De, M.; Chou, S. S.; Dravid, V. P. *J. Am. Chem. Soc.* **2011**, *133* (44), 17524−17527.

(28) Li, W. W.; Geng, X. M.; Guo, Y. F.; Rong, J. Z.; Gong, Y. P.; Wu, L. Q.; Zhang, X. M.; Li, P.; Xu, J. B.; Cheng, G. S.; Sun, M. T.; Liu, L. W. *ACS Nano* **2011**, *5* (9), 6955−6961.

(29) Cui, P.; Seo, S.; Lee, J.; Wang, L.; Lee, E.; Min, M.; Lee, H. *ACS Nano* **2011**, *5* (9), 6826−6833.

(30) Liu, M.; Zhao, H. M.; Chen, S.; Yu, H. T.; Zhang, Y. B.; Quan, X. *Biosens. Bioelectron.* **2011**, *26* (10), 4111−4116.

(31) Chandra, V.; Kim, K. S. *Chem. Commun.* **2011**, *47* (13), 3942−3944.

(32) Chen, L.; Zhang, X. W.; Zhang, C. L.; Zhou, G. H.; Zhang, W. P.; Xiang, D. S.; He, Z. K.; Wang, H. Z. *Anal. Chem.* **2011**, *83* (19), 7316−7322.

(33) Song, J. L.; Yin, Z. Y.; Yang, Z. J.; Amaladass, P.; Wu, S. X.; Ye, J.; Zhao, Y.; Deng, W. Q.; Zhang, H.; Liu, X. W. *Chem.—Eur. J.* **2011**, *17* (39), 10832−10837.

(34) Swathi, R. S.; Sebastian, K. L. *J. Chem. Phys.* **2009**, *130* (8), 1−3.

(35) Dong, H. F.; Gao, W. C.; Yan, F.; Ji, H. X.; Ju, H. X. *Anal. Chem.* **2010**, *82* (13), 5511−5517.

(36) Jiang, T.; Liu, J.; Deng, Y. Q.; Xu, L. J.; Li, X. F.; Han, J. F.; Cao, R. Y.; Qin, E. D.; Qin, C. F. *J. Clin. Microbiol.* **2011**, *49* (3), 870−874.

(37) Chen, T. C.; Chen, G. W.; Hsiug, C. A.; Yang, J. Y.; Shih, S. R.; Lai, Y. K.; Juang, J. L. *J. Clin. Microbiol.* **2006**, *44* (6), 2212−2219.