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The Effect of Quantum Dot Labeling on Virus Activity

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Jie Wang, Xiaoxiao Huang, Rui Zhu, Yijuan Long and Huzhi Zheng*

Key Laboratory of Luminescent and Real-Time Analytical Chemistry (Southwest University), Ministry of Education; School of Chemistry and Chemical Engineering, Southwest University, No. 2 Tiansheng Road, Chongqing, 400715, P. R. China.

In order to evaluate the effect of quantum dots (QD) labeling on virus activity, cell viability and cytokine secretion were measured after host cells were infected by QD-labeled H9N2 and unlabeled H9N2. Our results suggested that in the process of QD labeling, biotin modification (< 1mg/mL) had no obvious effect on virus activity. However, follow-up QD labeling enhanced virus toxicity toward host cells and caused high level of cytokine secretion. And our results indicate that QD-labeling is not suitable for long time tracing of virus.

Introduction

With the development of nanotechonology, a kind of efficient and stable fluorescent nanomaterial, quantum dots (QDs) appeared. Compared with traditional fluorescent reagents, QDs possess many advantages such as broad excitation spectra, narrow and symmetry emission peak, tunable emission from visible to infrared wavelengths by changing the size or composition, strong brightness, photostability, and high resistance to photobleaching.[1-3] With these unique features, QDs have been applied in many biomedical fields over the past decade.

Bird flu is one of the main diseases that threaten the poultry industry and human health, which is widely spread all over the world.[4-7] In order to effectively prevent and control the spread of viral infection, understanding of the infection behaviors of viruses in the host cells and cell response mechanism is particularly important. How virus get into the host cell is an important part of virus infecting, which can facilitate the understanding of pathogenic mechanisms and prevention of viral diseases. Virus infection processes is very complex and involve many steps and interactions between viruses and various cellular structures.[8-10] So far, the main approach of studying the interaction of virus and host cells is mainly based on molecular imaging.[11] QD-labeling technique can be applied in multi-color labeling[12-13] and long-term tracing of virus infection progress, which possess the potential to be the key technology in researching the virus infection.[14-17] However, the QD labeling effect on the virus infection ability has not been provided so far. And the host cells response to QD labeling virus also need to be explored.

Influenza virus commonly has specific host cells, can replicate and spread in natural host cells very well. We adopt low pathogenic influenza virus H9N2 as research object, human lung epithelial cells (A549) and madin-daby canine kidney cells (MDCK) as insensitive and sensitive host cell models, respectively. We investigate the change of cell viability after infected by QD-labeled H9N2 or unlabeled H9N2.

In this paper, RAW 264.7 and A549 cells were used as host cells to delineate the influences of the QD labeling on H9N2 inducing cytokine secretion. Virus-infected cells secrete interferons (IFNs), namely, biologically active substances that induce neighboring uninfected cells to upregulate anti-viral mechanisms. Interferon-gamma (IFN-γ) is a dimerized soluble cytokine that is the only member of the type II class of interferons.[18] The importance of IFN-γ in the immune system stems is in part from its ability to inhibit viral replication, and most importantly from its immunostimulatory and immunomodulatory effects. So we chose IFN-γ as one of cytokines in our research.[19] Interleukin 6 (IL-6) is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine. IL-6 is secreted to stimulate immune response, also plays a role in fighting infection, as IL-6 has been shown in mice to be required for resistance against bacterium *Streptococcus pneumonia*.[20-22] Interleukin 12 (IL-12) is an interleukin that stimulates the production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) from T and natural killer cells, and reduces IL-4 mediated suppression of IFN-γ. IL-12 is also linked with autoimmunity.[23-24] Therefore, productions of IL-6 and IL-12 were also considered to compare the effects of QD-labeled virus with unlabeled virus on cells.

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Experimental

Cell line

Adherent MDCK cells were obtained from Beijing Dingguo Changsheng Biotechnology Co., Ltd. and grown at 37°C in T-flasks (25 cm^2, 20 mL working volume) with DMEM (Gibco, USA) supplemented with glucose (final concentration 5.5 g/l), 10% fetal calf serum (FBS, Gibco, USA), 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate. A549 cells were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. and grown at 37°C in T-flasks (25 cm^2, 20 mL working volume) with F-12 media (Gibco, USA) supplemented with 10% FBS, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate. RAW 264.7 cells (mouse macrophage cell line) were obtain from the American Type Culture Collection (Rockville, MD), and cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 μg/mL), and streptomycin (100 μg/mL) as described previously.[25] The cell lines were incubated at 37°C in a 5% CO_2 incubater and passaged every 2-3 days. The cell density was about 5.6×10^5 cells/cm^2.

Preparation of viruses

Avian influenza A virus (H9N2) strain was produced in the allantoic cavity of 9-11 day old embryonated eggs for 48 h at 37°C. Subsequently, the virions were concentrated by ultra-centrifugation for 90 min at 110, 000g and stored at -70°C. The virus was quantitated by BCA protein quantification method. For infection, growth medium was withdrawn and cells were washed several times with phosphate-buffered saline (PBS) before adding serum-free medium containing virus seed.

Labeling viruses with QDs

To label viruses with QDs, the viruses were incubated with EZ-Link Sulfo-NHS-LC-Biotin (Thermo) for 1h at room temperature firstly. Unbound biotin was removed by gel filtration using a NAPTM-5 column (GE Healthcare). Virus aggregates and bacteria were removed with sterile 0.22 μm filters before labeling. The biotinylated viruses (Bio-H9N2) were labeled with strepavidin modified QDs (SA-QDs, CdSe/ZnS) (Wuhan Jiayuan Quantum Dots Co., Ltd., China) using two-step protocols.[15] The average diameter of SA-QDs is ca. 7.9 nm and the maximum emission is at 615 nm (Supplementary Fig. S1, S2, ESI†). Briefly, cells were cultured in round-bottom 96-well cell culture plate. The Bio-H9N2 were added to cells and then incubated for 10 min at 4ºC, the unbound virus solution was withdrawn and cells were washed several times with PBS. Then SA-QDs (1 nM) solution was added to the cells and incubated for another 10 min. SA-QDs bound with the viruses via biotin-streptavidin interaction. The unbound SA-QDs solution was withdrawn and cells were washed several times with PBS before detection.

Virus infection

MDCK cells and A549 cells were cultured in round-bottom 96-well cell culture plate for 24h. When the cells reached 80-90% confluency, they were washed several times with PBS, and then incubated with H9N2 or QD-labeled H9N2.

Cell viability assay

To study the effect of QD-labeling on virus activity, we investigated the cell viability of host cells infected by QD-labeled virus and unlabeled virus. After incubate for a certain period of time, cell viability was determined using a Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) assay based on 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8).[26-27] Finally, the cellular viability was detected at OD 450 nm by a Multiskan Mk3 microplate reader (Thermo Fisher Scientific Instrument Co., Ltd, Shanghai, China).

Cytokines ELISA assays

After infection, the supernatant was collected from each well and applied to the enzyme-linked immunosorbent assay (ELISA). After stimulated with QDs, H9N2 or QD-labeled H9N2, cytokine levels, including IFN-γ, IL-6 and IL-12 p70 in supernatants from A549 and RAW264.7 cells were determined using ELISA kits (PBL Interferon Source, 131 Ethel Rd West Suite 6, Piscatawayin USA; R&D Systems, Inc. 614 McKinley Place NE, Minneapolis USA) in accordance to the manufacturer's recommendations.

Statistical analysis

Each result in this article is representative of at least three separate experiments. Data are expressed as the mean ± standard deviation of these experiments. Statistical significance was calculated using Student's t-test. P values <0.05 were considered to be significant.

Results and Discussion

Effect of biotin modification on cell viability and cytokine secretion

H9N2 virus can't conjugate with SA-QDs directly. When H9N2 was modified with biotin, it can bind with SA-QDs by bitotin-avidin reaction. Therefore, it's particularly important to explore the biotin modification influence on H9N2 viability. We investigated A549 cell viability infected by H9N2 modified with different concentration of biotin. It was found that there is no obvious different in cell viability when compared with native unmodified H9N2 at the same virus concentration (Fig.S3, ESI†). This meant that biotin modification had no significant effect on virus activity when biotin concentration is lower than 10 mg/mL.

We infected A549 cell with unmodified H9N2 or H9N2 modified with different concentration of biotin, and then, detected the quantities of IFN-γ and IL-6 expression. There was no obvious diference between unmodified H9N2 and biotin-modified H9N2 in cytokine release as the biotin ≤ 1 mg/mL (Fig. S4, ESI†). However, the level of cytokine secretion decreased as the biotin reach 10 mg/mL. Therefore, the concentration of biotin was fixed at 1 mg/mL.

Effect of different concentration of virus on host cell viability

We applied A549 cell and MDCK cell as the insensitive and sensitive host cell model to investigate the cell response to H9N2 and QD-labeled H9N2, so as to investigate the effect of QD labeling on H9N2 activity.

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In order to figure out the QD labeling effect on H9N2 activity, four groups of samples, namely H9N2 (native unmodified H9N2), Bio-H9N2 (biotin modified H9N2), Bio-H9N2-QDs (QDs labeled H9N2), QDs (pure SA-QDs) were used to infect A549 cell. After infected by different concentration of samples, cell viability of A549 cell and MDCK cell were determined. Fig. 1 showed that when the concentration of H9N2 is 0.8×10^{-4} and 0.8×10^{-3} mg/mL, the A549 cell viability infected by H9N2 was lower than that of Bio-H9N2, while the cell viability infected by Bio-H9N2-QDs was lower than that of H9N2. The above results indicate that, under certain conditions, biotin modification can reduce H9N2 activity. However, further QD labeling can enhance activity of H9N2. At the same time, when the concentration of H9N2 is high enough (0.08 and 0.8 mg/mL), biotin modified H9N2 and subsequent QD labeling does not affect the activity of the H9N2. And as a negative control, QDs don't reduce cell viability.

The MDCK cells were infected by five groups of virus, namely H9N2, H9N2-QDs (native unmodified H9N2 and SA-QDs mixture, without interaction), Bio-H9N2, Bio-H9N2-QDs, QDs. As Fig. S5(a) (ESI†) showed that when the H9N2 concentration was 0.8×10^{-3} to 0.8×10^{-1} mg/mL, the MDCK cell viability infected by Bio-H9N2-QDs was lower that of H9N2 after 24h ($P < 0.05$). It also indicates that the QD-lableling can enhace activity of H9N2. This result is similar to the result obtained using A549 cells as host cells. When the infection time was 48 h, there was no obvious difference in activity between H9N2, H9N2-QDs, Bio-H9N2 and Bio-H9N2-QDs (Fig. S5(b), , ESI†). This result indicates that, for a long-time infection, QD-labeling has no effect on the activity of virus.

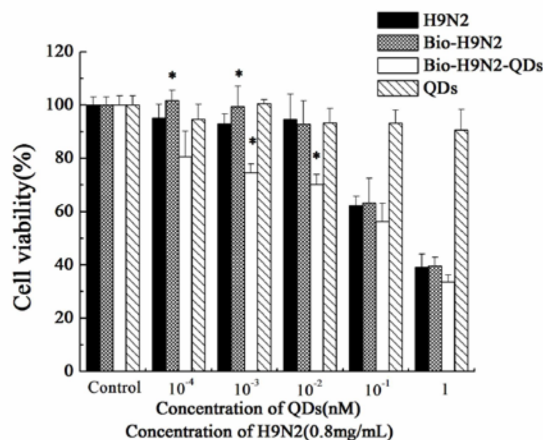

Fig. 1 The cell viability of A549 cells incubated with different concentration of H9N2 virus after 24 h. Asterisks denote a statistically significant difference in cell viability compared to native unmodified H9N2 (*, $P < 0.05$).

Effect of different concentration of virus on host cytokine secretion

Releasing cytokines such as IFN-γ, IL-6 and IL-12 p70 was one mechanism that cells regulating the immune response. We studied the expression of IFN-γ, IL-6, IL-12 p70 from A549 and Raw 264.7 cells after they are infected by H9N2 and QD-labeled H9N2. Cells were infected with H9N2, Bio-H9N2, H9N2-QDs, Bio-H9N2-QDs and QDs at different concentrations. After 24 h or 48 h, the supernatant was collected from each well of both two cell lines and applied to the ELISA assays. As showed in Fig. 2 and S6-S8 (ESI†), the cytokine levels increased with the increasing of virus concentrations. And there is no obvious difference between H9N2 and Bio-H9N2 in cytokine release of infected cells ($P < 0.05$). This result suggests that modifying H9N2 with biotin has no effect on the activity of virus. Compared with unlabeled H9N2, QD-labeled H9N2 cause more serious cytokine releasing (Fig. 2, and Fig. S6-S8, ESI†). It indicates that QD-labeling can enhance the virus activity.

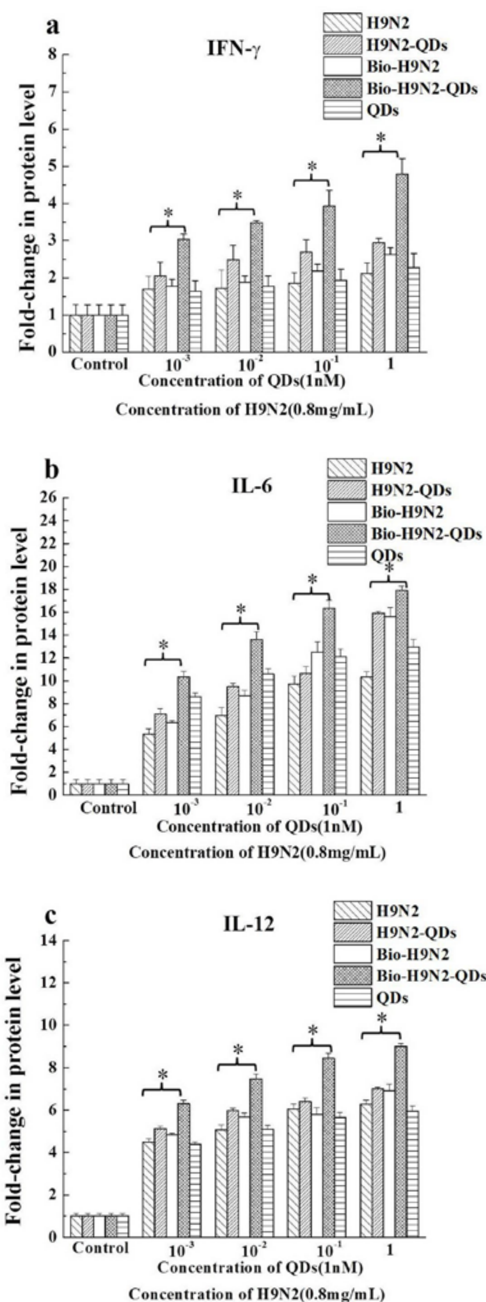

Fig. 2 The cytokine secretion of A549 cells incubated with different concentration of virus after 24h. Asterisks denote a statistically significant difference in cell viability compared to native unmodified H9N2 (*, $P < 0.05$).

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Effect of different incubation time of H9N2 on host cell viability

We also investigated the effect of five different samples, namely H9N2, H9N2-QDs, bio-H9N2, Bio-H9N2-QDs, QDs, on these two host cells for different incubation time. Fig. 3 and Fig. S9 (ESI†) showed that the cell viability decreased gradually in turns of QDs, bio-H9N2, H9N2, Bio-H9N2-QDs. For short-time incubation, the viability of QD-labeled virus (Bio-H9N2-QDs) treated cell was close to native unmodified H9N2. As the extension of incubation time, the difference in cell viability between QD-labed virus treated cells and unlabeled H9N2 treated cells increased gradually, which indicated that QDs labeling is not suitable for long time tracing.

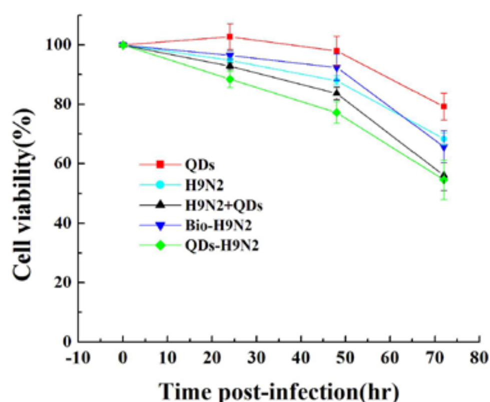

Fig. 3 The cell viability of A549 cells incubated with H9N2 virus for different time. Concentration of H9N2 was 0.08 mg/mL; concentration of QDs was 0.1 nM.

Effect of different incubation time of H9N2 on host cytokine secretion

Cells were infected with QDs, H9N2, Bio-H9N2-QDs or PBS (control) at a concentration of 0.08 mg/mL. Medium supernatant was collected at 0, 6, 12, 24, 36, 48, 72, 96 h after infection, and all samples were stored at -70°C until cytokine determinations. As shown in Fig. S10, S11 (ESI†), levels of three kinds of cytokine increased gradually with the increasing of infection times until reached the maximum. And there is a common tendency that QD-labelded virus (Bio-H9N2-QDs) caused more serious cytokine secretion than other samples after 12 h. However, for a short incubation, there was no obvious difference between H9N2 group and Bio-H9N2-QDs group (Fig. S10, S11, ESI†). These results indicate that "two-step" QD-labeling strategy is suitable for short time tagging (less than 12 h).

Conclusion

In this study we have investigated the feasibility of using QDs to label H9N2 underlying the differences in H9N2 infected cells. We adopted A549 cells and MDCK cells as sensitive and insensitive model to H9N2 virus and evaluated the feasibility of "two-step" quantum dots labeling strategy via cell viability assay and cytokine secretion measurement. Our results suggest that in the process of quantum dot labeling, biotin modification have no obvious effect on virus activity while it affect IFN-γ, IL-6 and IL-12 secretion. We found that QDs had no effect on the viability of H9N2 infected A549 and MDCK, but follow-up quantum dots labeling enhanced virus toxicity to host cells. And QD labeling increased the releasing of cytokines obviously in both A549 and RAW 264.7 after infected 24h or 48h. There are significant differences of IFN-γ, IL-6, IL-12 releasing between cells infected with labeled and unlabeled H9N2. It demonstrated that QDs labeling promote the infectivity and toxicity of virus in long-term tagging, so it only can be used in short time tagging to present the immune responses of cells to infected virus.

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Notes

The authors declare no competing financial interest.

* Corresponding Authors. Email address: zhenghz@swu.edu.cn. Tel: +86 23 68252360; Fax: +86 23 68254000.

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