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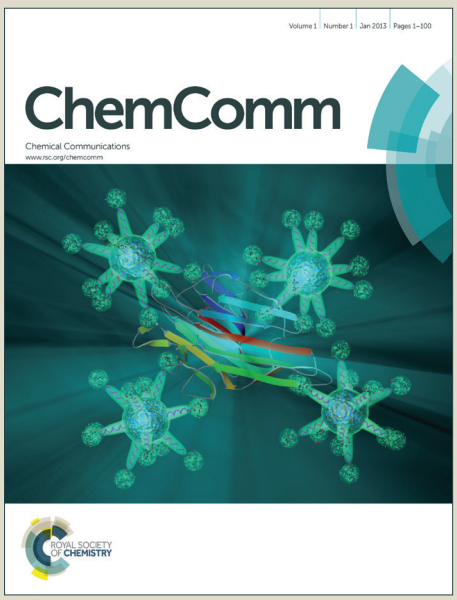

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Graphical abstract

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An in situ labelling chemistry of respiratory syncytial virus by employing the biotinylated host-cell membrane protein for tracking the early stage of virus entry

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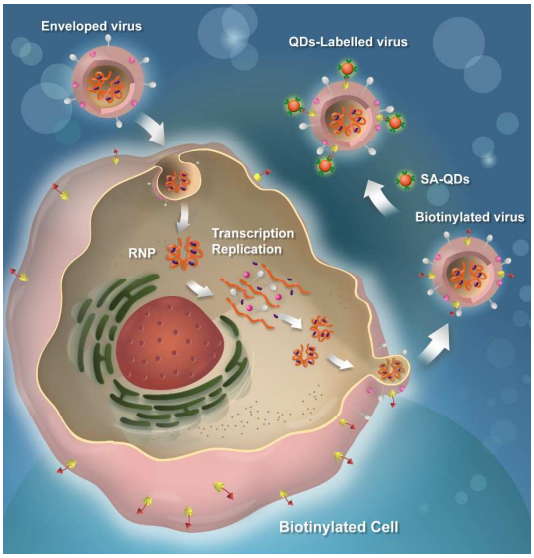

An *in situ* strategy for producing quantum dots-labelled respiratory syncytial viruses by incorporating the biotinylated membrane protein of the host cells into mature virions is reported.

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An *in situ* labelling chemistry of respiratory syncytial virus by employing the biotinylated host-cell membrane protein for tracking the early stage of virus entry

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An *in situ* labelling strategy was proposed to produce quantum dots labelled respiratory syncytial viruses (RSV) by incorporating the biotinylated membrane protein of the host cells into mature virions, followed by conjugating with streptavidin modified quantum dots (SA-QDs), which has the advantages of convenience, efficiency and minor influence to viral infectivity and thus could be successfully applied to track the early stage of virus entry.

Tracking virus infection events in host cells can directly reveal the infection routes and the dynamic interactions between viruses and cells, which is helpful to understand the invasion mechanism of virus and to develop therapies for viral diseases.[1] In such studies, efficient labelling of viruses with fluorophores is the key to successfully track, and the simultaneous labelling of the external and internal components of virus, which allows revealing the intracellular fate of both envelope and genome of virus, is indispensable for understanding of their infection process. Generally, two types of labelling methods have been developed to fluorescently label viral particles. One is dependent on genetic engineering, and the other is chemical labelling.[2] For the case of genetic engineering, fluorescent proteins have to be generally fused to a target viral protein during virus replication, which allows labelling a protein accurately, but still needs complicated design and operation process.[3] Chemical labelling, however, is directly performed by covalently or non-covalently coupling chemical fluorophores with viruses, and recently developed fluorophores with different characteristics enable chemical labelling to be an important method for labelling virus and further investigating the virus infection.[4]

Quantum dots (QDs), which exhibit remarkable photo-stability and brightness, have been widely used as fluorescent tags for tracking virus infection events.[5] The excellent luminescence properties of QDs make it admirable for labelling virus. However, the directly chemical coupling of QDs with viruses, acquired by carbodiimide coupling reaction,[5c] biotin–avidin affinity system,[4b, 5a] and click chemistry,[4a, 6] may occupy the surface recognition sites of viruses, affecting the adsorption of viruses on cell membrane and entry into host cells. In addition, viruses have to go through dialysis, ultrafiltration or gel filtration in order to remove the excessive label reagents. Such treatments would reduce the viral activity of viruses, especially enveloped viruses that are highly sensitive to heat and hydrodynamic. Furthermore, the directly labelling of the surface proteins of virus may result in the overload of QDs and thus affect the inherent infection process of virus. To avoid these problems, some smart *in situ* labelling strategies for enveloped viruses based on the budding process were reported recently.[7] Although successfully avoiding the occupation of the surface recognition sites of viruses, previously reported methods still are greatly limited by the labelling molecules that need to be newly synthesized before labelling. Therefore, it still needs to find a simple method to label the envelope of viruses with high efficiency and minimal influence to viruses.

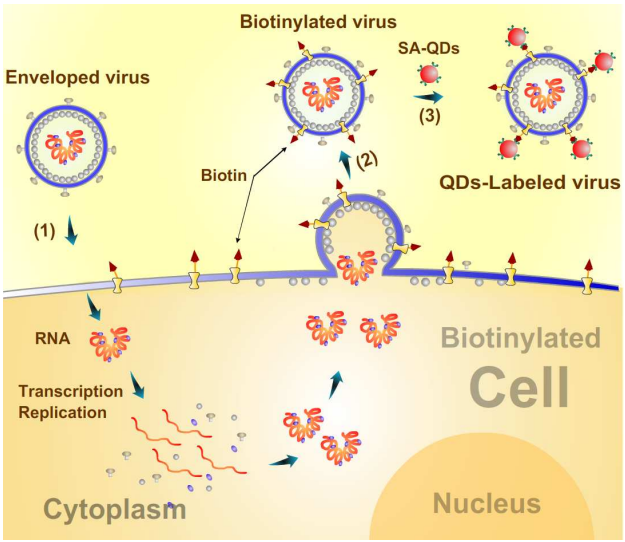

Fig. 1 Scheme of the general strategy for the *in situ* dual-labelling of enveloped virus. The labelling procedure includes (1) host cells were infected with virus and then biotinylated, (2) after 2 days' cultivation, the mature virus was assembled and released from cell surface with biotinylated envelope, (3) further attachment with SA-QDs generated the QDs-labelled virus.

Knowledge is that some cellular proteins can incorporate into mature virions when viruses assemble in host cells,[8] and especially the membrane proteins of host cells are readily taken away by enveloped viruses when they get budding from the cell surface.[8c, 8d, 9] Thus, labelling of the membrane proteins of host cells can indirectly label the viral envelope without occupying any surface recognition sites of viruses. In addition, the biotinylation of cell surface by modifying the membrane proteins of host cells with biotin is a powerful tool to study the proteomics and intracellular transport of membrane protein.[10] Inspired by these facts, we herein designed a smart strategy for *in situ* labelling enveloped viruses with biotin by employing the biotinylated membrane protein of host cells (Fig. 1). RSV as a representative enveloped virus that are extremely sensitive to the environment change are chosen as the model virus. As showed in our designed strategy, the labelling chemistry involves in the following three steps: 1) the biotinylation of host cells after infected with virus; 2) natural incorporation of the biotinylated membrane proteins into the viral envelope after 2 days' cultivation when the mature viruses were assembled and released from biotinylated host cells; 3) conjugation of biotin-labelled virus with SA-QDs to produce QDs-labelled RSV.

The labelling chemistry of the three steps is successful and effective. At first, the biotinylation of membrane proteins of host cells (HEp-2 cells) was achieved by treating cells with biotinylation reagent that contains biotin and amino-reactive NHS.[11] Since many viral glycoproteins would be incorporated into the cell membrane at the late stage of virus proliferation, the biotinylation of HEp-2 cells was performed at the beginning of virus proliferation to avoid labelling the viral components. Thus, fluorescence imaging was carried out to test the biotinylation result. After incubating with SA-QDs, strong fluorescence could be observed in the biotinylated HEp-2 cells but not in normal cells (Fig. S1), suggesting that biotin molecules were successfully and efficiently conjugated to cell surface. Further results showed that the biotinylated cells still could bind abundant QDs even after culturing for 24 h and 48 h (Fig. S2), suggesting that biotin molecules could stay on cell membrane for sufficient time. The cytotoxicity evaluation showed that biotinylation had no noticeable effect on the cell growth and proliferation (Fig. S3). Above results indicated that the biotinylation of cells was effective and mild without disturbing cell proliferation.

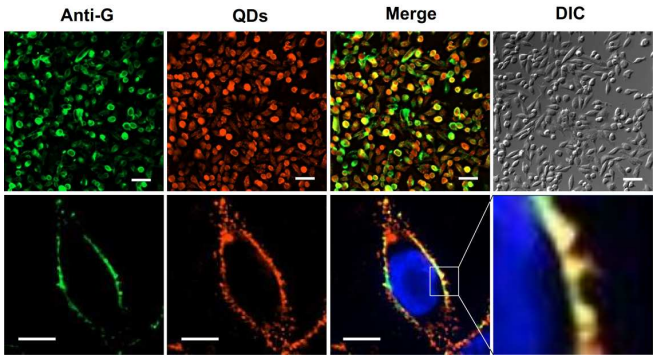

Fig. 2 Incorporation of biotinylated cell membrane to RSV. The cells were infected with RSV and then biotinylated. After 48 h post-infection, the cells were incubated with SA-QDs (red fluorescence), then immunostained with mouse monoclonal antibody against RSV envelope protein G and DyLight 488-conjugated goat anti-mouse IgG (green fluorescence). Images were acquired using a 20× air objective (the first row, scale bars are 50 μm) or a 60× oil immersion objective (the second row, scale bars are 10 μm).

Second, the biotinylated HEp-2 cells were used to propagate RSV as in common viral culture. After 2 days' cultivation, the progeny genome and proteins of RSV were produced abundantly and aggregated closed to the cell membrane, ready for budding from the host cells. The staining pattern of biotin and RSV G protein, one of the major envelope glycoprotein of RSV, were compared to test whether the biotinylated component on cell surface could be incorporated into the viral envelope. The appearance of the green spots that correspond to budding virus[9b] could colocalize with the biotin that labelled with QDs (Fig. 2), suggesting that the budding virions could carry off the biotinylated protein from cell membrane. After collecting the progeny virions released from the biotinylated cells, we tested their infectivity and compared the result with the progeny virions obtained from normal cells (Fig. S4). No significant difference was observed between the RSV harvested from biotinylated cells and normal cells, indicating that the biotinylation of the host cells had little effect on the viral propagation.

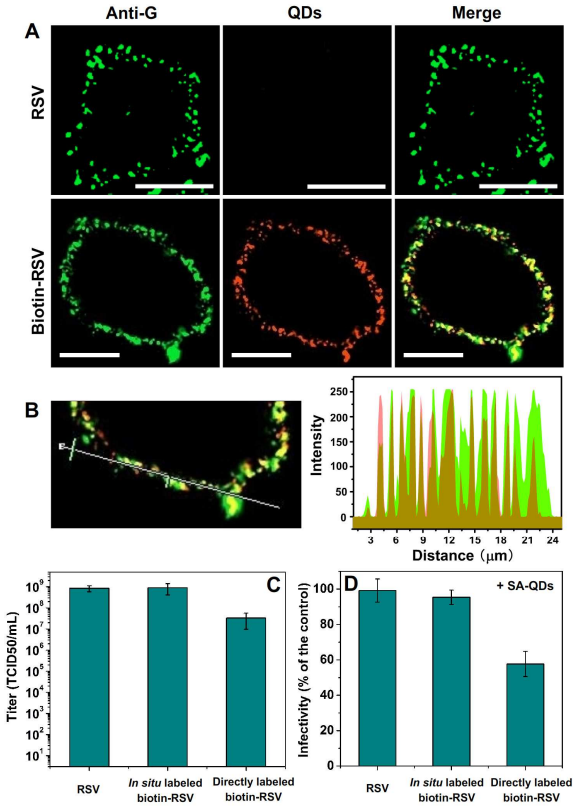

Fig. 3 Characterization of the biotin-labelled and QDs-labelled RSV. (A) Immunofluorescence and QDs fluorescence colocalization imaging of control RSV and biotin-RSV on HEp-2 cells. Scale bar, 20 μm. (B) Line profiles indicating the distribution of signal of DyLight 488 (green area) and QDs (red area) along the line. (C) Titer of the initial RSV, biotinylated virus obtained by *in situ* labelling and directly labelling methods. (D) The influence of binding of SA-QDs to the infectivity of the unlabelled RSV and biotinylated virus obtained from two different methods.

Finally, the harvest biotinylated viruses were incubated with SA-QDs to obtain the QDs-labelled viruses. The specificity and sufficiency of the biotin and QDs labelling of RSV were then evaluated. As shown in Fig. 3A, the fluorescence signals of the QDs distributed all around the HEp-2 cells that inoculated with biotin-labelled virus, whereas no signal could be detected on cells incubated with non-biotinylated RSV, indicating that no obvious

non-specific adsorption occurred between SA-QDs and virus/cells, and the SA-QDs binding was due to the successful labelling of virus with biotin. The fluorescence colocalization assay showed 83.7 ± 4.6% of immunofluorescence signal colocalized with QDs signals (Fig. 3A), illustrating that most virions budding from the biotinylated cells had been modified with biotin. And 86.5 ± 2.7% of QDs signals colocalized with immunofluorescence signals, suggesting that QDs were specific and adequate to exhibit the virus particles. The line profiles of the cell showed that nearly all the intensity peaks of immunofluorescence signals accompanied the appearance of that of QDs signals (Fig. 3B), demonstrating the virus was high-efficiently labelled with QDs.

The influence of biotin- and QDs-labelling on virus infectivity was examined by TCID50 and compared with direct labelling method. As shown in Fig. 3C, the infectivity of direct labelled biotin-RSV was 3.33×10^{7} $TCID_{50}$ mL^{-1}, which was much lower than that of initial virus of 8.52×10^{8} $TCID_{50}$ mL^{-1}. However, the infectivity of the *in situ* labelled biotin-RSV budding from biotinylated cells was 9.15×10^{8} $TCID_{50}$ mL^{-1}, which was even higher than initial virus. This was because the generated progeny viruses usually have highly infectivity than initial virus. After binding with SA-QDs, the infectivity of direct-labelled biotin-RSV and indirect-labelled biotin-RSV reduced to 57.7% and 95.4%, respectively (Fig. 3D). Obviously, QDs binding on direct-labelled RSV had significant effect on their infectivity because of occupying large number of the viral surface proteins that involve in viral infection. However, QDs had little effect on virus infectivity by labelling the protein of host cells that incorporated to virus. Moreover, the cytopathogenic effects (CPE) induced by biotin-RSV obtained by advanced method was similar to native virus (Fig. S5). In the one-step growth curve (Fig. S6), the titer reached a maximum value similar to that of unmodified virus. It could be concluded that our labelling strategy was suitable to generate the QDs-labelled virus with high infectivity.

The viral infection of host cell was composed of multiple steps, such as membrane fusion and genome release for enveloped virus. Thus, simultaneously labelling the viral envelope and the nucleic acid for tracking of these critical events in virus infection is vital for completely understanding the infection mechanisms. Since the biotin could be readily labelled during the virus budding process, simultaneous dual labelling of viruses in host cells by integrating the nucleic acid labelling with the envelope labelling should be feasible. SYTO® RNASelect™ green fluorescent cell stain, a cell-permeant RNA stain, was used to label the viral RNA owing to its nontoxic to cells at low concentration (Fig. S7). After being infected with virus and biotinylated, HEp-2 cells were maintained in medium added with 1.0 μM SYTO RNA stain. After 48 h propagation, the harvest viruses were labelled by SA-QDs and evaluated by fluorescence microscopic imaging. The results showed that 86.3 ± 3.8% of virions had the apparent colocalization of fluorescence of SA-QDs and SYTO RNA stain (Fig. 4A), indicating that progeny virions obtained from the modified cells could be naturally contained both biotinylated envelope and stained nucleic acid.

After establishing the QDs and SYTO RNA dual-labelling of virus, we then tested the potential of the dual-labelled viruses in investigating the virus infection process in living cells. Time-lapse confocal microscopy imaging was carried out to directly observe the entry process of RSV (Fig. 4B). The dual-labelled RSV were inoculated into HEp-2 cells at 4 °C, and then shifted to 37 °C to allow viral entry. In order to avoid the photo-bleaching of the SYTO RNA, the images were recorded every 2 min. At the beginning of viral infection (0 min), the red fluorescence of QDs and green fluorescence of SYTO RNA stain were colocalized and localized at the cell surface. When the temperature shifted from 4 °C to 37 °C quickly, the green spots was separated from the red and gradually vanished as the incubation time prolonged, suggesting the genome released. Since SYTO RNA stain has almost no fluorescence when it does not bind to RNA, the loss of RNA signal was attributed to the detachment of the SYTO RNA stain from viral RNA as a result of the disruption of RNA secondary structure upon externalization.[12] Finally, most of the red fluorescence represent the viral envelope was still close to cell surface, indicating that RNA release occurred at or near the cell surface, consistent with the recent report.[13] Moreover, the viruses were moving a short distance from the original starting point (Or. Dist.) around the cytomembrane in this period, suggested that the early stage of RSV migration in host cells was a slow event. Another imaging procedure that acquired the image of different samples at each time point was also carried out to eliminate the potential effect of photo-damage on the labelled virus and photo-bleaching of the SYTO RNA stain, and the result was similar to time-lapse imaging (Fig. S8).

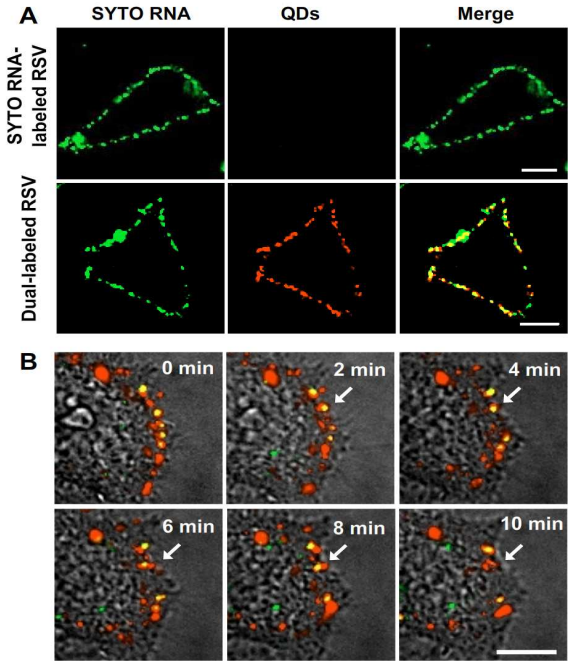

Fig. 4 Dual-labelled RSV for monitoring the RNA release. (A) Fluorescence imaging of HEp-2 cells incubated with SYTO RNA-labelled, QDs-labelled and dual-labelled RSV. SYTO RNA-labelled virus and dual-labelled virus were obtained from normal cells or biotinylated cells, with SYTO RNA was present. Scale bar, 10 μm. (B) Time-lapse live cell microscopy imaging of dual-labelled RSV. Scale bar, 10 μm.

The proposed method employing the membrane proteins at cell surface for enveloped virus labelling is based on the essential assembly process of virus, not dependent on the specific interaction between virus and QDs, thus it can be also used for labelling many other enveloped viruses. However, although most of the enveloped viruses are believed to bud from the cell surface[14], virus budding can also occur on intracellular membranes, such as nuclear envelope,

endoplasmic reticulum and Golgi cisternae[15]. In these cases, our method is not suitable.

In summary, we reported an *in situ* method to label the enveloped virus with QDs, which were used for revealing the early stage of single virus into live cells. The QDs-labelled RSV were prepared by incorporating the biotinylated membrane protein of the host cells into mature virions followed by conjugating with SA-QDs during viral self-assembly process. The dual-labelled RSV was further generated by simultaneously staining the nucleic acids with RNA-binding dye for tracking the viral genome release. Our proposed labelling approach was simple, efficient, and has little effect on the viral infectivity, which is expected to be widely used in further research on the dynamic visualization of the virus infection process.

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Notes and references

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Electronic Supplementary Information (ESI) available: Experimental details and additional figures (Fig. S1-S8). See DOI: 10.1039/c000000x/

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