

Bioconjugated nanoparticle detection of respiratory syncytial virus infection

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Abstract: The integration of nanotechnology with biology has produced major advances in molecular diagnostics, therapeutics, and bioengineering. Recent advances have led to the development of functionalized nanoparticles (NPs) that are covalently linked to biological molecules such as antibodies, peptides, proteins, and nucleic acids. These functionalized NPs allow for development of novel diagnostic tools and methods, particularly for pathogens, as rapid and sensitive diagnostics are essential for defining the emergence of infection, determining the period that preventive measures should be applied, for evaluating drug and vaccine efficacy, and for controlling epidemics. In this study, we show that functionalized NPs conjugated to monoclonal antibodies can be used to rapidly and specifically detect respiratory syncytial virus in vitro and in vivo. These results suggest that functionalized NPs can provide direct, rapid, and sensitive detection of viruses and thereby bridge the gap between current cumbersome virus detection assays and the burgeoning need for more rapid and sensitive detection of viral agents.

Keywords: RSV, virus, nanoparticle, bioconjugated, functionalized

Introduction

Semiconductor quantum dots are excellent fluorescent nanoprobe for biological and biomedical applications because of their unique size-dependent optical and electronic properties (Penn et al 2003; Alivisatos et al 2005; Hotz 2005). These nanoparticles (NPs) possess unique optical properties in comparison with traditional organic dyes including size- and composition-tunable emission, narrow emission spectra, and excellent photostability (Hotz 2005). Recent improvements in surface chemistry have augmented the ability to make functionalized NPs to create bioconjugated NPs that allow for specific targeting or signal enhancement (Farokhzad et al 2006; Fujiwara et al 2006; Goodman et al 2006; Ipe et al 2006; Suk et al 2006). Bioconjugated NPs, coupled with spectroscopy or other spectral imaging tools, eg, flow cytometry and histological techniques, provides a novel and powerful platform for mapping the molecular and disease profiles associated with infection by different pathogens (Zhao et al 2004; Driskell et al 2005; Yao et al 2006; McVeigh 2006). Another advantage of bioconjugated NPs over conventional pathogen detection methods is that the NPs may be conjugated with many biomolecules thereby enhancing the signal and the binding avidity by mitigating a multivalency effect. This is particularly important for targeting NPs and for increasing the delivery or efficacy of bioconjugated molecules. However, only limited reports are available which address the utility of NPs for detection or diagnosis virus infection (Agrawal et al 2005, 2006; Bentzen et al 2005; Driskell et al 2005; Li, Cu, et al 2005; Li, Liu, et al 2005; Wabuye and Vo-Dinh 2005; Wan et al 2005; Fuentes et al 2006; Liu, Cao, et al 2006; Souza et al 2006).

Respiratory syncytial virus (RSV) is a single-stranded negative sense RNA virus in the *Paramyxoviridae* family that is the primary cause of morbidity and life-threat-

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ening lower respiratory tract disease in infants and young children worldwide, as well as an important pathogen of the elderly and immune compromised (Bader and McKinsey 2005; Ebbert and Limper 2005; Mejias et al 2005) The disease burden associated with RSV infection is considerable as RSV is a leading cause of hospitalization for infants and young children worldwide having infection rates approaching 70%–80% in the first year of life with many patients requiring hospitalization (McCarthy and Hall 2003; Leung et al 2005). Unfortunately, despite five decades of research, no safe and effective RSV vaccine is available and few effective prophylactic or therapeutic treatments are available. Thus, there is a critical need for the development of novel tools and platforms to augment virus diagnosis and foundational studies required for development of disease intervention strategies.

The surface topography of viruses lends to detection by antibodies, a feature that has been successfully used for bioconjugated NP detection of RSV (Agrawal et al 2005, 2006; Bentzen et al 2005). RSV has two major surface proteins, ie, G and F proteins, that are primarily responsible for virus attachment and fusion, respectively (Tripp 2005). Both G and F proteins are recognized by the immune system as neutralizing antigens; thus a majority of antibodies are directed toward these proteins (Tripp 2005). The RSV F protein is more conserved than the G protein among RSV strains (Tripp 2005), thus antibodies reactive to the F protein are ideal target developing bioconjugated nanoparticles.

Taking advantage of the composition-tunable emission of NPs and their multivalent specificity when conjugated to monoclonal antibodies, we tested the ability of these bioconjugated NPs to detect RSV infection *in vitro* and *in vivo* using in a single-step format. We show that bioconjugated NPs can be used to rapidly and sensitively detect RSV infection in both cell lines and mice, and that the detection can be done in a single step format. This single-step format dramatically improves virus detection time, potentially saves costs, and reduces background staining that is currently associated with conventional virus detection methods.

Experimental procedures

Bioconjugated nanoparticles

To develop bioconjugated nanoparticles to detect RSV infection, semiconductor cadmium telluride (CdTe) quantum dots (QDs) were synthesized as previously described (Wang et al 2002; Liu, Chen, et al 2006). QDs have been reported to be an excellent quantum particle for use in biological systems based on very high photoluminescence

quantum efficiencies, and the ability to cover the whole visible spectral range depending on particle size (Liu, Chen, et al 2006). Thus, two different sized CdTe QDs with emission peaks at 585 nm and 540 nm were prepared (Figure 1) and used in the preparation of bioconjugated nanoparticles (NPs) for detection of RSV infection. These particles were prepared for bioconjugation as previously described (Wang et al 2002), and conjugated to monoclonal antibodies reactive to RSV F protein, clone 131-2A. Briefly, thioglycolate (TGA)-coated CdTe particles were mixed 1:1 with RSV

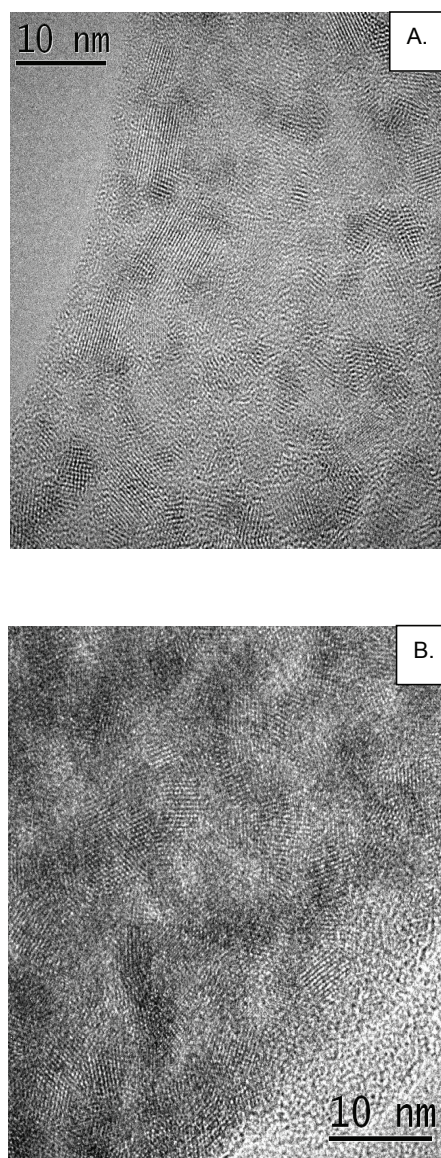


Figure 1 HRTEM images of green and orange CdTe nanoparticles showing an average size of 3 nm (A) and 5 nm (B), respectively.
Abbreviations: CdTe, cadmium telluride; HRTEM, high resolution transmission electron microscopy.

anti-F protein monoclonal antibody (4 mg/ml) and the solution was stabilized with 10X phosphate-buffered saline (PBS), pH 7.2 to a final 1 X PBS concentration. EDC/NHS (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl/N-Hydroxysulfosuccinimide) was added to bring to QD solution to a final concentration of 50 mM/5mM, respectively. The reaction was incubated for 2 h at room temperature, then overnight at 4 °C. Precipitated bioconjugated NP aggregates were separated by centrifugation to remove unwanted aggregates from single, bioconjugated nanoparticles. The final concentration of the purified NP-antibody conjugate stock solution was 0.625 mM.

Vero cell and virus propagation

Vero cells (African green monkey kidney cells) were grown in Dulbecco's Modified Eagle's Media (DMEM) containing 10% fetal bovine sera (DMEM-10%). For some experiments involving NPs, Vero cells were propagated on Lab-Tek chamber slides to 80%–90% confluency using DMEM-10%.

Respiratory syncytial virus strain A2 (RSV) was propagated in Vero cells as previously described (Tripp et al 1999). Briefly, semi-confluent Vero cells were prepared and washed with PBS. RSV was diluted in DMEM and the cells infected at a multiplicity of infection (MOI) of 1. The virus was allowed to adsorb for 2 h at 37 °C after which DMEM-10% was added and the cells incubated at 37 °C for 4 days. At day 4 post-infection (pi), the virus was recovered by removing the cell culture supernatant, freeze-thawing the infected cells, and centrifuging the cell lysate to remove debris and recover the virus from the cell lysate supernatant.

Detection of RSV infection in vitro

The bioconjugated nanoparticles conjugated to anti-RSV F protein monoclonal antibody (RSV-NPs), ie, both 585 nm and 540 nm QDs, were tested for their ability to detect RSV infection of Vero cells. RSV-infected Vero cells were propagated on Lab-Tek chamber slides to 80%–90% confluency and subsequently infected with RSV at a MOI = 1 as previously described (Tripp et al 1999). At day 4 pi, the media from the cells was removed, the cells were washed gently with PBS, fixed with acetone:methanol (60:40), and air dried. RSV-NPs (diluted 1:10,000) were added to the cells and incubated for 1 hour at 37 °C, followed by three washes with PBS/0.05% Tween 20. Cells were visualized by fluorescent microscopy (40X) on an Olympus CKX41 inverted microscope (Olympus, Center Valley, PA), and photographed with an Olympus

Q-Color3 digital camera. Images were analyzed using the Olympus Q-Capture software.

The most widely used assay to quantitate virus titers in both the laboratory and clinical setting is the virus plaque assay. Virus plaques on the cell lawn are enumerated by immunostaining to specifically detect virus plaques due to confounding background staining that is often associated with the counterstain procedure. RSV-NPs were evaluated for detection of RSV infection using a modification of an immunostaining plaque assay as previously described (Tripp et al 1999). Briefly, the modification involved substituting RSV-NPs in place of the primary (anti-RSV monoclonal antibody: clone 131-2A), secondary (anti-mouse whole molecule immunoglobulin G (IgG)-alkaline phosphatase conjugated), and detection substrate, eg, Vector black alkaline phosphatase, to generate a single-step nanoparticle-based assay. In this modified assay, Vero cells were plated onto 24-well flat-bottom plates 24-h prior to RSV infection. Subsequently, the cell supernatants were removed and RSV diluted in DMEM was added to the cells at a MOI = 1. The virus was allowed to adsorb for 1 h at 37 °C after which the cells were overlaid with 2% methylcellulose in DMEM-10% as previously described (Tripp et al 1999), and cells incubated for 6 days at 37 °C, 5% CO₂. At day 6 pi, the overlays were removed, and cells were fixed with acetone: methanol (60:40), and allowed to air dry. Cells were blocked for 30 minutes with Powerblock (10% casein in PBS) at room temperature. RSV-NPs, either 540 nm or 585 nm bioconjugated QDs, were diluted 1:1,000 from 0.625 mM stock solution in PBS. For positive control wells, anti-RSV monoclonal antibody (clone 131-2A) was diluted 1:500 in PBS containing Powerblock, added to the appropriate wells, and incubated 1 hour for RSV-NPs, or 2 hours for the primary monoclonal antibody at 37 °C. Cells were washed 3 times with PBS/0.05% Tween. For standard immunostaining plaque assay, secondary antibody (anti-mouse IgG-alkaline phosphatase conjugated) was diluted 1:500 in Powerblock, added to cells, incubated for 1 hour at 37 °C, washed twice with PBS, and developed using a Vector black alkaline phosphatase substrate kit according to the manufacturer's protocol. For RSV-NPs, the plates were directly scanned for virus plaques using an Amersham Biosciences Typhoon 9210 scanner with a fluorescence excitation (532 nm) and emission (526 SP) filter. The virus plaques were enumerated and compared to plaque numbers obtained by standard counting using an Olympus SZ dissecting scope.

Detection of RSV infection in vivo

To determine the ability of RSV-NPs to detect RSV infection in lung tissue, the natural site of RSV infection and replication (Tripp 2005), 6–8 week old female BALB/c mice were intranasally infected with 10^6 plaque-forming units (pfu) of RSV as previously described (Tripp et al 1999). At day 4 pi, RSV-infected or naïve BALB/c mice were intravenously administered by tail vein 0.05 ml of RSV-NPs, either 540 nm or 585 nm bioconjugated QDs, diluted 1:1000 in PBS from a 0.625 mM stock solution. At day 5 pi, the lungs from RSV-NP-treated or PBS carrier-treated mice were removed and prepared for thin sectioning and immunohistochemistry (IHC) analysis as previously described (Li, Sarmiento, et al 2005). Briefly, lung tissue was frozen as tissue blocks and sectioned (20 μ m) on a cryostat. The frozen sections were placed on slides which were immersed in acetone at 4 °C for 15 min, air dried for 30s, and then stored in PBS until immunostaining was performed. Briefly, slides were stained with anti-RSV monoclonal antibody (clone 131-2A) diluted 1:500 in PBS and incubated for 2 h at 37 °C. The slides were washed 2 times with PBS, and a secondary antibody (FITC conjugated anti-mouse IgG) diluted 1:500 in PBS was added to slides and incubated for 1 h at 37 °C, and washed twice with PBS. The lung sections from RSV-NP-treated mice and untreated mice that were immunostained as noted above were directly visualized using a fluorescent microscope.

Results and discussion

Current antibody-based methods used for virus detection are cumbersome, have limited sensitivity due to substantial background staining, and are costly as they often require multiple antibodies and enzyme substrate for detection. In an effort to ameliorate these issues, and reduce the time for virus detection, we synthesized semiconductor CdTe QDs with emission at either 540 nm or 585 nm to be conjugated to monoclonal antibodies to detect RSV-infected cells. These bioconjugated NPs (RSV-NPs) that are specific to RSV F protein were tested for their ability to detect RSV infection of cells both in vitro and in vivo. As expected, no differences in virus detection were observed using either 540 or 585 nm NPs.

As shown in the high resolution transmission electron microscopy (HRTEM) images in Figure 1, the green emission CdTe NPs had an average size of approximately 3 nm (A) and the orange CdTe NPs of 5 nm (B). Given the reported difficulties conjugating proteins to CdTe NPs (Tan et al 2004; Gao et al 2005; McVeigh 2006), the CdTe NPs

were produced with a thioglycolate coating to allow direct anti-RSV F protein monoclonal antibody conjugation via an EDC/Sulfo-NHS conjugation reaction. These bioconjugated NPs (RSV-NPs) were stable for >1 year at 4 °C, and did not precipitate in the PBS diluent at room temperature or at 4 °C.

To determine the ability of the RSV-NPs to detect RSV infection, in vitro studies were performed using RSV-infected Vero cells. In these studies, we compared RSV detection using conventional antibody staining methods, ie, primary and secondary antibody staining plus enzyme substrate, to that of a single-step detection method using RSV-NPs. Conventional antibody staining methods produced substantial background staining in mock-infected Vero cells (Figure 2A), however did detect RSV plaque formation in the Vero cell lawn (Figure 2B). In contrast, RSV-NPs used in a single-step method produced little background (Figure 2C), and readily detected RSV plaques (Figure 2D). These results show that RSV-NPs can be used to detect RSV in a single-step fashion, and that this method can be used to greatly reduce the amount of time and reagent needed for RSV detection compared to conventional immunostaining procedures (Tripp et al 1999).

Extending the in vitro detection findings (Figure 2), we determined if RSV-NPs could be used to quantitate RSV titers in a single step fashion, thereby shortening the detection time needed to quantitate virus titers by plaque assay (Figure 3). In these studies, we compared a conventional immunostaining plaque assay which requires 5 or 6 days of in vitro culture post-RSV infection (Tripp et al 1999) to rapid single-step detection by RSV-NPs. For comparison of the different plaque assay methods, virus titers were assessed at days 2, 3, 5, or 6 pi. The results showed that at days 5 or 6 pi, conventional immunostaining (Figure 3A) and the single-step RSV-NP detection method (Figure 3B) were similarly sensitive for detecting RSV plaques in the Vero cell lawn. However, RSV-NPs could detect RSV plaques earlier (days 2 or 3 pi) compared with conventional immunostaining which was comparatively ineffective (Figure 4), indicating that the single-step RSV-NPs procedure was more sensitive at detecting limiting levels of RSV. This result is not unexpected given the multivalent properties of RSV-NPs.

To evaluate the efficacy of RSV-NPs to detect RSV infection in vivo, BALB/c were either intranasally infected with 10^6 pfu RSV or mock-treated, and at day 4 pi, RSV-infected or mock-treated mice were intravenously administered 0.05 ml of RSV-NPs from the stock solution

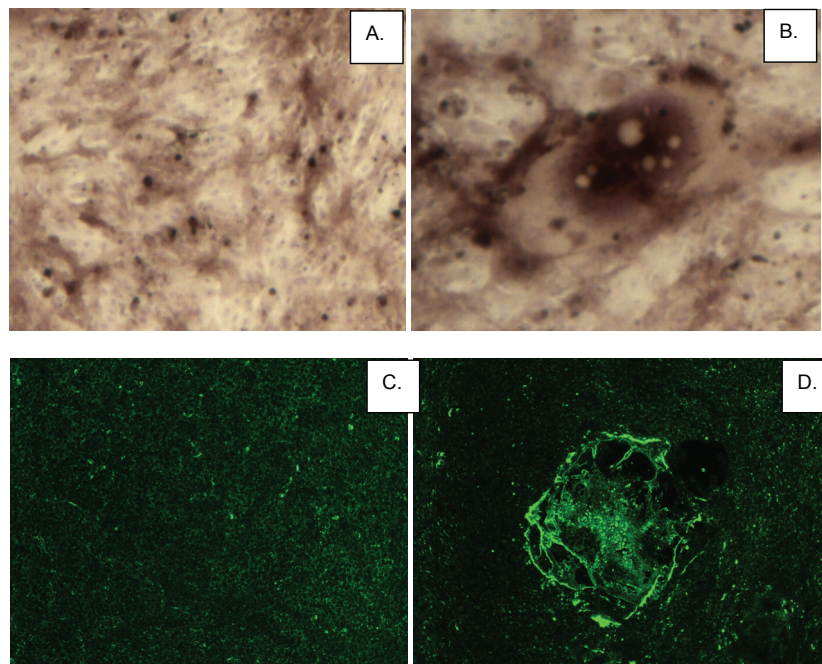


Figure 2 RSV-NP detection of RSV-infected Vero cells. Vero cells were mock-infected (A, C) or RSV-infected at a MOI = 1 (B, D) and fixed with acetone: methanol (60:40). Cells were immunostained with by conventional methods (A, B) or by RSV-NPs (C, D), and analyzed using an immunofluorescence microscopy (40X; A, C or 100X; B, D). **Abbreviations:** MOI, multiplicity of infection; RSV-NP, respiratory syncytial virus-nanoparticles.

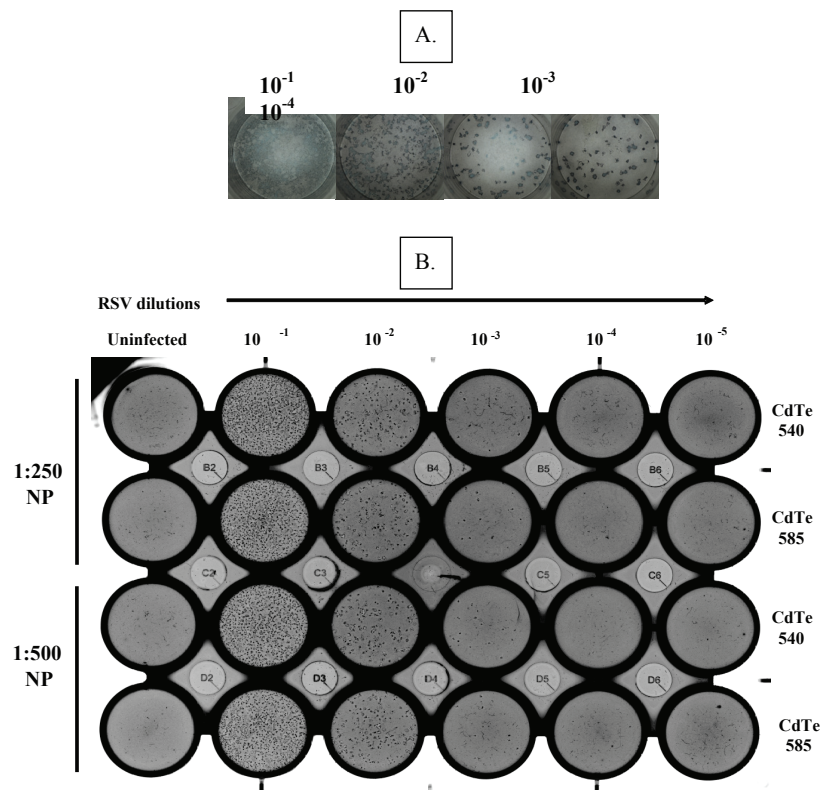


Figure 3 RSV-NP virus plaque assay. RSV was serially diluted ten-fold and the dilutions used to infect Vero cells for determination of virus titers. The viral titer determined from a conventional immunostaining plaque assay (A), were compared with RSV-NPs detection (B). Both 540 nm and 585 nm QDs were evaluated as RSV-NPs (B). RSV plaques were enumerated using an Amersham Biosciences Typhoon 9210 scanner.

Abbreviations: RSV-NP, respiratory syncytial virus-nanoparticles; QDs, quantum dots.

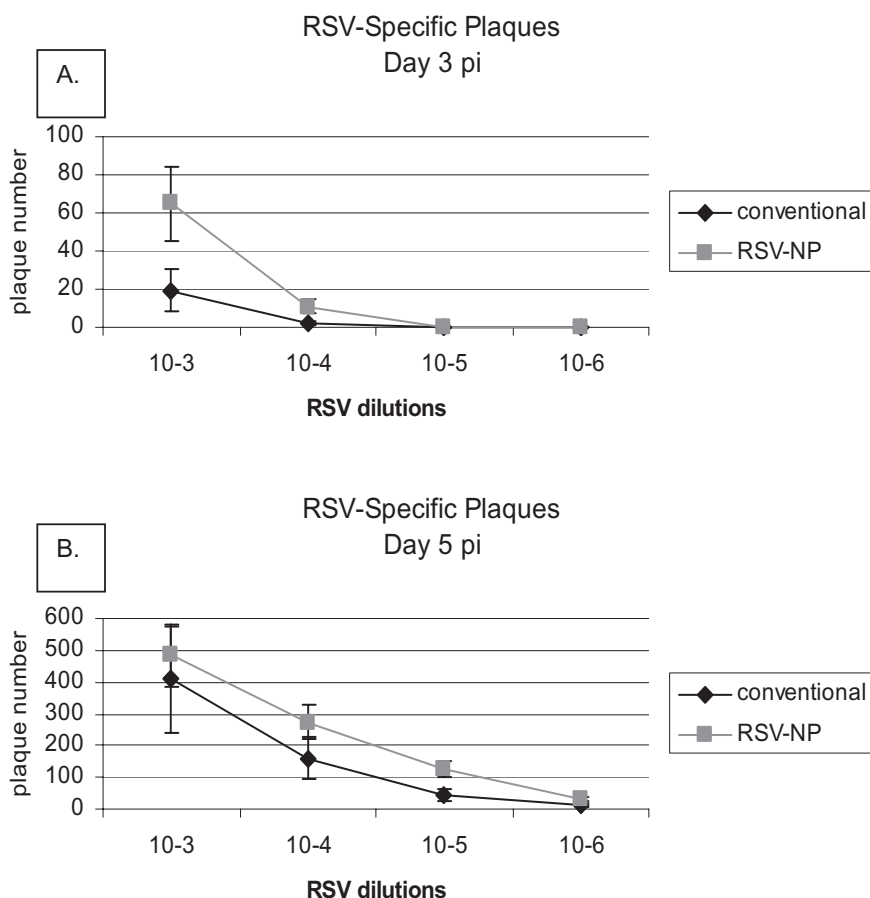


Figure 4 Dynamic range and sensitivity of detection of RSV-NP plaque assay compared with a conventional plaque assay. The viral titer of RSV-infected Vero cells was determined at day 3 pi (A) and day 5 pi (B) using conventional immunostaining plaque assay or single-step detection using 1:250 dilution of RSV-NPs derived from 585 nm QDs. RSV plaques were enumerated using an Amersham Biosciences Typhoon 9210 scanner. **Abbreviations:** RSV-NP, respiratory syncytial virus-nanoparticles; QDs, quantum dots.

(0.625 mM) by tail vein. At day 5 pi, the lungs from RSV-NP-treated or mock-treated mice were removed, frozen, and prepared for thin sectioning and IHC analysis using conventional methods, i.e. primary and secondary antibody staining, or directly visualized by fluorescence microscopy in the case of RSV-NP treatment. Treatment with RSV-NPs provided clear and rapid detection of RSV-infected lung tissue (Figure 5A) with very limited background staining as assessed in lung tissue from mock-treated mice similarly administered RSV-NPs (Figure 5B). Importantly, the magnitude and location of RSV-NPs staining along the epithelial cells of the alveoli in the lungs was similar to that of conventional IHC staining (Figure 5C), and the background staining for RSV-NPs was lower in naïve mice compared to conventional IHC staining (Figure 5D). The selective staining by RSV-NPs of only RSV-infected lung epithelial cells suggests antibody-mediated specific targeting to sites of infection. These results suggest that

RSV-NPs may be useful to determine the progression of RSV infection in the lungs, and as a result, aid our understanding of sites of infection, and help to define disease intervention strategies that employ bioconjugated NP targeting strategies.

Conclusions

The results from this study show that RSV-NPs can be used to detect RSV infection both *in vitro* and *in vivo* and suggest that beyond diagnostics, bioconjugated NPs may be useful for multiplexed detection of viral and/or host cell antigens, as well as for targeting sites of virus infection with antiviral agents that may be linked to the NPs. Indeed, bioconjugated NPs have been used to identify the presence and progression of RSV infection in a human epithelial cell line, i.e. HEP-2 cells (Bentzen et al 2005), as well as used in a variety of intracellular tracking and related studies (Alivisatos et al 2005; Bruchez 2005; Gao et al 2005; Hotz 2005; Shenoy et al 2006; McVeigh 2006).

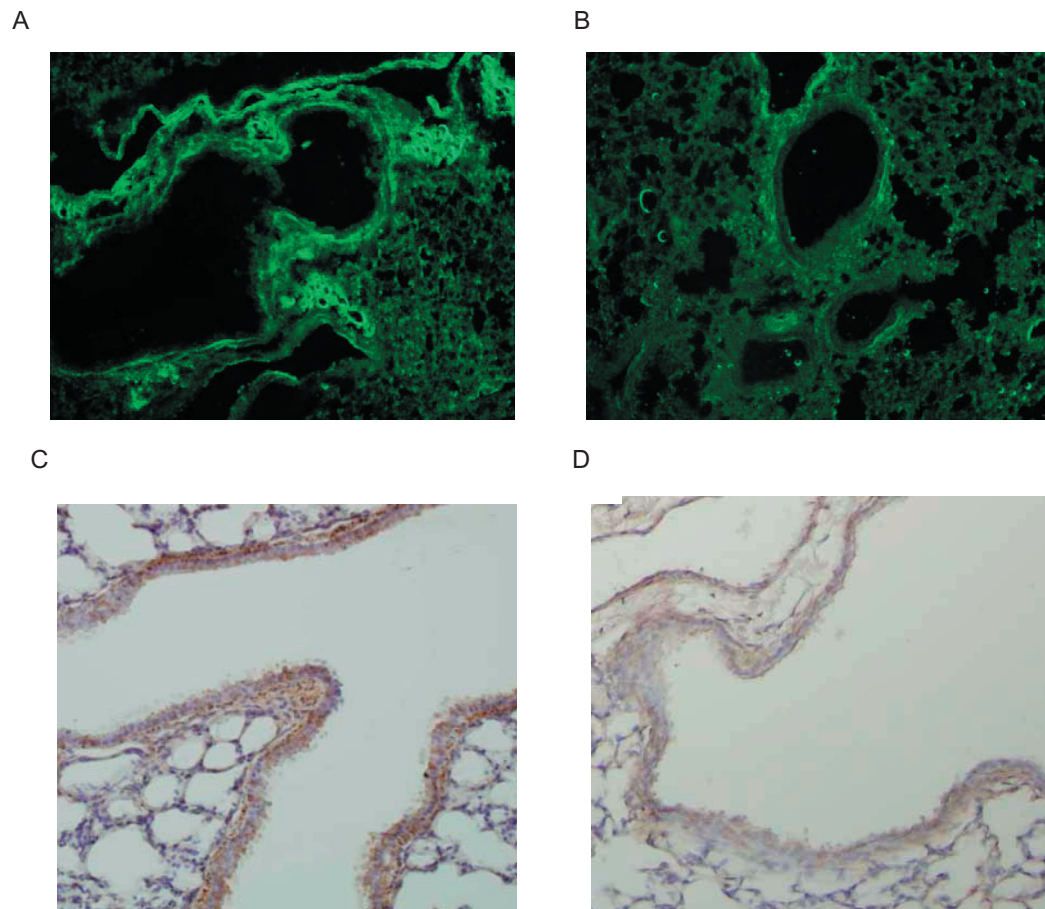


Figure 5 RSV-NPs detection of RSV-infected lung tissue. Lung tissue sections from RSV-infected (A, C) or naive, mock-treated BALB/c mice (B, D) were stained by IHC using RSV-NPs (A, B), or by conventional IHC (C, D), and analyzed using an immunofluorescence microscopy.

Abbreviations: IHC, immunohistochemistry; RSV-NP, respiratory syncytial virus-nanoparticles.

Importantly, we show here that RSV-NPs can be applied as tools to enhance virus detection by increasing sensitivity of virus detection while decreasing the detection time. Application of RSV-NPs for virus detection in animal models can provide a path to assist our ability to dissect important parameters of RSV disease pathogenesis such as determining sites of virus infection and virus load in the lungs of infected animals. This understanding is critical to move the field forward in antiviral disease intervention strategies.

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