Molecular beacon–quantum dot–Au nanoparticle hybrid nanoprobes for visualizing virus replication in living cells†

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Here we describe a new hybrid fluorescent nanoprobe composed of a nuclease-resistant molecular beacon (MB) backbone, CdSe–ZnS core–shell quantum dots (QDs) as donors, and gold nanoparticles (Au NPs) as quenchers, for the real-time visualization of viral replication in living cells. By using a Au NP–MB to QD ratio of 6 : 1, a 7.3-fold increase in fluorescent signal was achieved upon target binding. For living cell experiments, a hexahistidine-appended Tat peptide was self-assembled onto the QD surface to provide nearly 100% non-invasive delivery of the QD–MB–Au NP probes within 2 h. By directly visualizing the fluorescent complexes formed with the newly synthesized viral RNA, this QD–MB–Au NP probe provided sensitive and real-time detection of infectious viruses as well as the real-time visualization of cell-to-cell virus spreading.

The ability to provide real-time, intracellular monitoring of gene expression is becoming increasingly important for different biomedical applications.1–5 In particular, real-time detection of viral genomes in living cells is essential to gain insights into the mechanisms involved in the viral reproductive cycle (e.g., entry, replication, and egress), the cell-to-cell spread of progeny virions, and for rapid clinical diagnostics. Among the various methods reported to date, molecular beacons (MBs) are considered one of the most promising technologies. MBs are single-stranded oligonucleotides with a hairpin structure that fluoresce upon hybridizing to the target sequence.6,7 Although MBs have been used for intracellular detection of gene expression, their applications for viral detection in infected cells have only been recently reported.8 The major challenges in using the conventional MB for in vivo viral detection are their modest half-life (∼50 min) due to cytoplasmic nuclease degradation and a lack of non-invasive intracellular delivery.9,10 Recently, we reported the use of nuclease-resistant MBs for the real-time detection of Coxackievirus replication in living cells via Tat peptide delivery.11 The enhanced stability and the non-invasive delivery also enabled real-time monitoring of cell-to-cell spreading of viral infection.

However, one major limitation of conventional MBs is their use of organic fluorophores, which have low resistance to photodegradation, rendering them ineffective for long-term monitoring.12 Semiconductor quantum dots (QDs) are inorganic fluorophores that are brighter and more resistant to photodegradation (up to 100 times) than organic fluorophores,13 making them excellent for single-molecule observation over an extended period of time.14 Their broad absorption spectra and narrow emission peaks allow the simultaneous excitation of different QDs at a single wavelength for multiplex detection.15 These advantages make QDs attractive for in vivo imaging in living cells and live animals.16–19

QD-based MBs using organic quenchers such as the black hole quencher BHQ2 or Cy5 have recently been reported.20,21 In both cases, less than a 100% increase in the QD fluorescence was observed upon hybridization. This relatively modest increase is a result of inefficient quenching afforded by BHQ2 and Cy5. It has been shown that the quenching efficiency can be over an order of magnitude better for gold nanoparticles (Au NPs)22–25 than organic quenchers, and QD–Au NP nano assemblies with a quenching efficiency close to 100% have been reported.24 One can envision that the use of QD and Au NP as the FRET pair for MBs will significantly improve not only long-term monitoring of gene expression in live cells or animals, but also the sensitivity due to the improved signal-to-noise ratio.

In this study, we report the development of nuclease-resistant MBs using QD and Au NP as the FRET pair for real-time in vivo viral detection via Tat peptide delivery. The cell-penetrating Tat peptide is derived from the Human Immunodeficiency Virus type 1, HIV-1, Tat protein and has been shown to enable the intracellular delivery of a variety of cargos, from proteins to nucleic acids.25 We chose Coxackievirus B6 (CVB6) as our virus model considering its importance in waterborne diseases. A nuclease-resistant MB targeting an 18 bp non-coding region of the CVB6 genome was designed similar to that reported previously,11 except for the inclusion of a thiol group at the 5′ end and an amino group at the 3′ end. A 12 bp linker sequence was inserted at the 3′ end to act as a spacer between the MB and the Au NP. To synthesize the QD–MB–Au NP probes (Fig. 1), a maleimide-modified hexahistidine (His6) peptide linker was first conjugated with the free 5′ thiol group of the MB to form a stable thioether. The presence of mono-sulfo-NHS esters on the surface of Au NPs enabled their facile attachment to the peptide–MB conjugates via the 3′ amino group on the MB. Finally, self-assembly of the Au NP–MB conjugates onto DHLA-capped QDs was accomplished via the strong metal-affinity coordination between the ZnS shell and the (His)6 tag.26

The QD emission at 540 nm was measured to follow the QD–MB–Au NP conjugation (Fig. 2A). The emission peak
before DHLA-capping was detected at 540 nm and was red shifted to 548 nm after DHLA replacement as reported previously.26 The effect of QD quenching was investigated by incubating a fixed concentration of QD (0.1 μM) with an increasing molar ratio of Au NP-labeled MB from 1 to 6. Nearly a 40% loss in QD emission was achieved even with an Au NP/QD ratio of 1. This result confirms the correct assembly of Au NP–MB conjugates onto QDs based on the His6 interaction, resulting in the efficient quenching of the QD via donor–quencher FRET. A sequential increase in the QD quenching efficiency from 37% to 91% was observed when the Au NP–MB to QD molar ratio was increased from 1 : 1 to 6 : 1. Since only a marginal increase in quenching was observed beyond the ratio of 6 : 1, this particular Au NP–MB–QD preparation was chosen for subsequent characterization and in vivo experiments.

To ensure that the modifications with QD and Au NP had no effect on the hybridization kinetics, an excess amount of complementary oligos was added. Fig. 2B shows the time-course recovery of QD emission in the presence of complementary oligos. The QD fluorescence intensity upon target binding was enhanced up to 7.3 times within 50 min, which is significantly higher than the 2–3 fold increase reported previously for other QD-based MB conjugates.23 This improvement in the signal to background ratio is likely a combination of using Au NP as the quencher and the insertion of a 12 bp linker, which provides better spacing between Au NPs from the QD surface upon hybridization.

After confirming the expected properties of the QD–MB–Au NP probes, Tat peptides were appended to the QD surface via coordination with the His6-tag at a ratio of 10 : 1. To investigate the intracellular delivery efficiency, QD–MB–Au NP probes appended with Tat peptides were first hybridized with an excess amount of complementary oligos before being added to a monolayer of Buffalo green monkey (BGMK) cells. As depicted in Fig. 3, intracellular delivery occurred within 1 h and the level of intracellular fluorescence continued to increase with time. Since the extracellular fluorescence signal continued to decrease within the same duration, this result indicates that the MB-target hybrids were retained inside the cells after delivery. In the absence of Tat peptide conjugation, there was no significant fluorescence detected inside the cells (Fig. S1, ESI†).

To demonstrate the ability of the Tat-modified QD–MB–Au NP probes to monitor the infection state of individual cells, a confluent monolayer of BGMK cells was first incubated with 50 nM probes for 3 h before being infected with 0 to 103 plaque forming unit (PFU) of CVB6. The number of fluorescent cells was followed by fluorescence microscopy after 4 h of infection. As shown in Fig. 4, a significantly higher number of fluorescent cells was detected with increasing infection dosages, while the uninfected cultures (0 PFU) showed a negligible amount of fluorescence (background) (Fig. 4). A calibration curve was obtained over the range of 1–200 PFU (Fig. S2, ESI†), indicating that this QD-based MB assay can be used to provide rapid quantitative

Fig. 1 A schematic representation of the QD–MB–Au NP probe with or without presence of the complementary viral RNA.

Fig. 2 Characterization of the QD–MB–Au NP nanoprobe. (A) Fluorescence spectra of the QD–MB–Au NP complex at a ratio of MB–Au NP conjugate from 1 to 6. (B) The time profile of QD emission in 10-fold molar excess of a complementary oligo.

Fig. 3 Intracellular delivery of QD–MB–Au NP probes. BGMK cells were incubated with the QD–MB–Au NP conjugates (50 nM) for 12 h, and fluorescent images were captured at different time points.
Detection of infectious viruses by QD-MB-Au NP probes. Fluorescent images of cells infected with 0, 1, 10² or 10³ PFU per well at 4 h post infection (p.i.).

Real-time detection of viral spreading. BGMK cells were first incubated with 50 nM QD-MB-Au NP probes, infected with CVB6 at an M.O.I. of 0.1 PFU per cell, and monitored using a fluorescence microscope. After 1 h infection (Fig. 5), several fluorescent spots were already observable under the microscope. As time proceeded, the fluorescent intensity inside these infected cells became more intense, indicating that continual RNA synthesis and virus assembly was occurring. The further outward spread of fluorescent cells from 6 to 12 h p.i. indicated secondary infection caused by the progeny virions.

In summary, we demonstrated the utilization of nuclease-resistant QD-MB-Au NP probes to examine the viral replication cycle in living host cells via Tat peptide delivery. Our findings indicate that this new MB probe design can be used to explore many real-time molecular mechanisms that are critical for understanding virus-host interactions and viral pathogenesis.

Notes and references