Enzyme catalyzed site-specific protein labeling and cell imaging with quantum dots†

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We have developed an efficient method for one-step covalent labeling of cell surface proteins with quantum dots based on enzyme catalyzed site-specific modification of short peptide tags.

Quantum dots (QDs) have recently emerged as powerful fluorescent probes for biological imaging due to their superior optical properties such as high quantum yields, excellent photostability, size-tunable emission spectra and multiplex excitation at a single wavelength.[1] Biologically compatible QDs have been prepared for cell imaging applications by coating the QD surface with amphiphilic polymers, phospholipids and small molecules.[2] QD coatings can be further functionalized with protein ligands or antibodies to label specific cellular proteins.[3] Recently streptavidin-conjugated QDs have been used to label cell surface proteins. This method requires the target proteins to be first labeled with biotin by constructing fusions of the target proteins with a short peptide tag (AP tag) or with an acyl carrier protein (ACP) tag for biotin attachment catalyzed by biotin ligase[4] or phosphopantetheinyl transferase[5] respectively (Fig. 1a).

A covalent linkage between QD and the labeled protein is more desirable for prolonged cell imaging experiments because the QD label would be less likely to dissociate from the target protein inside the cell, which may give rise to imaging artifacts. For this purpose, haloalkane dehalogenase (~33 kDa)[6] or cutinase (~22 kDa)[7] have been fused to the target proteins and specific residues at the enzyme active sites are covalently modified with suicide inhibitors conjugated to the QD particles (Fig. 1b). Thus current methods for QD labeling use large proteins such as antibodies (~150 kDa for IgG), streptavidin (~60 kDa) or enzymes to bridge the linkage between QD and the target protein. This significantly increases the size of the QD label, which may affect endocytosis and distribution of the labeled proteins, and limit the application of QD labeling in the living systems.[4,8]

Here, we report a new method for enzyme catalyzed site-specific conjugation of QDs to the target proteins. In this method, phosphopantetheinyl transferases (PPTases) catalyze the covalent transfer of coenzyme A (CoA) functionalized QDs (QD-CoA) to a specific Ser residue within a short peptide tag fused to the target proteins (Fig. 1c). This method installs a short and flexible phosphopantetheinyl (Ppant) linkage (18 Å in length) between the QD and the labeled protein (Fig. 1d), providing a useful alternative to the large size protein linker implemented in the other methods.

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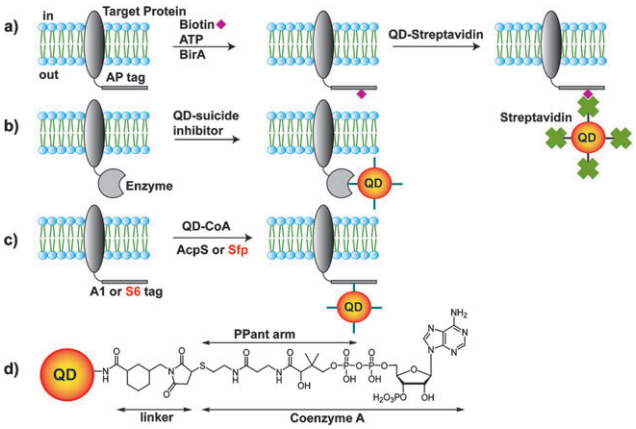

Fig. 1 Methods for site-specific protein labeling on the cell surface with QDs. (a) The target protein is fused to a 15-residue AP tag, which is biotinylated at a specific Lys side chain by biotin ligase, BirA. Subsequently, streptavidin-conjugated QDs are used to label the biotinylated AP-target protein fusion.[4] Similarly, an ACP tag can also be fused to the target proteins and labeled with biotin-CoA conjugates by AcpS for the subsequent binding of streptavidin-QD conjugates;[5] (b) cutinase enzyme is fused to the target protein and labeled with QDs functionalized with a suicide inhibitor of the enzyme. This approach creates a covalent linkage between an active site residue of cutinase and QD;[7] (c) the method for site-specific QD labeling in this study: 12-residue A1 or S6 peptide tags are fused to the target proteins and covalently labeled with QD-CoA in one step catalyzed by PPTase enzymes AcpS or Sfp; (d) the structure of coenzyme A ligand attached to the QD surface.

The native activity of PPTase is to post-translationally modify ACP and peptidyl carrier proteins (PCP) by the covalent attachment of Ppant arm derived from CoA to a conserved Ser residue of the carrier proteins.[9] PPTases exhibit impressive substrate promiscuity for the functional groups attached to the thiol moiety of CoA.[10] This feature has been used to transfer small molecule probes such as biotin, peptides and fluorophores to the target proteins fused to ACP or PCP for site-specific protein labeling.[5,11] We recently identified short peptide tags, A1 and S6, as surrogate substrates of PPTases in order to decrease the size of the ACP and PCP tags (~80 residues).[12] The A1 and S6 tags are only of 12 residues in length, with the A1 tag specifically modified by the *E. coli* PPTase AcpS and the S6 tag by the *B. subtilis* PPTase Sfp, and have been used for orthogonal labeling of distinctive protein receptors on the surface of the same cell.[12]

To label proteins with QDs, we used polymer-coated, amino PEG-functionalized QDs with a fluorescence emission maximum at 655 nm (QD655) due to their high stability and biocompatibility.[2a] Amine groups on the surface of QDs were first reacted with a bifunctional linker with an amine reactive *N*-hydroxysuccinimide ester and a thiol reactive maleimide group.

After the reaction, excess linker molecules were removed and 10 equivalents of CoA with respect to QD were added to attach the free thiol group of CoA to the maleimide functionalities on the QD surface (Fig. S1†). Subsequently, unreacted maleimide groups on the QD surface were capped with β-mercaptoethanol and QD-CoA nanoparticles (Fig. 1d) were purified by buffer exchange. We found that the density of CoA molecules on the surface of QDs can be controlled by adjusting the ratio between CoA and QD during the conjugation reaction. QDs modified with different amounts of CoA can be separated by agarose gel electrophoresis presumably due to the negative charges introduced by the CoA ligands. QDs with a higher density of CoA migrate faster on the agarose gel (Fig. S2†).

We then tested if QD-CoA nanoparticles can be the substrates of AcpS or Sfp for site specific protein labeling. Labeling reactions were set up for Sfp catalyzed QD attachment to N-terminal PCP-tagged maltose binding protein (PCP-MBP) and N-terminal S6-tagged MBP (S6-MBP). Reactions were also set up for AcpS catalyzed QD attachment to N-terminal A1-tagged MBP (A1-MBP) (Fig. 2a). After an hour at 30 °C, amylose resin was added to the reaction mixture to pull-down the MBP fusion proteins.[13] As shown in Fig. 2b, the amylose resin showed intense QD fluorescence in the labeling reactions with PPTase added, suggesting that A1-, S6- and PCP-tagged MBP proteins were all labeled with QDs at a high efficiency and bound to the resin. In contrast, the control reactions without PPTases added showed strong fluorescence in the solution, indicating that the QDs were not attached to MBP in the absence of the enzymes. Taken together, these experiments clearly show that AcpS and Sfp can efficiently label A1-, PCP- and S6-tagged MBP with QDs and the QD modification on the peptide tags does not interfere with the binding of MBP to the amylose resin.

We also used the amylose resin pull-down assay to test the labeling efficiency of the QDs conjugated with different amounts of CoA ligands. QD-CoA nanoparticles prepared by incubating 10 or higher equivalents of CoA with maleimide functionalized QDs gave high labeling efficiency as shown by efficient pull-down of QD labeled A1- and S6-MBP by the amylose resin (Fig. 2b). In contrast, QDs prepared with a CoA : QD ratio below 10 during QD functionalization could not be pulled down as efficiently by the amylose resin after AcpS or Sfp catalyzed MBP labeling (data not shown). This reveals that the density of CoA ligands on the QD surface affects the efficiency of enzyme catalyzed QD labeling. Since high ratios of CoA : QD may potentially induce crosslinking of multiple receptors[14] to the same QD particle, we decided to use QD–CoA conjugates prepared with a CoA : QD ratio of 10 for the subsequent cell labeling experiments. In this way the QD particle would have a relatively low number of CoA ligands on the surface but still function as a good substrate of the PPTase. The exact stoichiometry of CoA ligand on the QD surface was not determined due to technical difficulties. Recently, QDs functionalized with one streptavidin per nanoparticle have been prepared and applied for cell imaging.[15] Other QD functionalization methods produce QDs conjugated with multiple antibody or small molecule ligands.

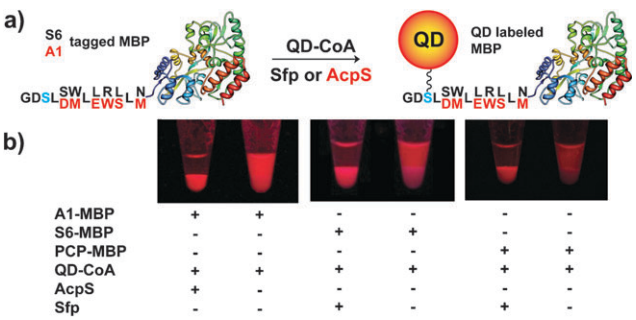

Fig. 2 (a) PPTase catalyzed QD labeling of N-terminal A1- or S6-tagged maltose binding protein (MBP) with QD-CoA; (b) amylose resin pull-down of QD labeled A1-, S6- or PCP-MBP. In the presence of the enzymes, tagged MBP proteins were labeled with QDs, and significant amounts of QDs were pulled down by the amylose resin. In the control reactions where no enzyme was added, QDs were not pulled down by the amylose resin and remained in the solution that was highly fluorescent.

After confirming that the QD–CoA nanoparticles can be used for site-specific protein labeling in solution, we sought to label cell surface receptors with QDs. For this purpose, we expressed A1-tagged transferrin receptor 1 (TfR1) on the surface of TRVb cells,[16] a chinese hamster ovary (CHO) cell line lacking endogenous TfR1. The A1 tag was fused to the C-terminus of TfR1 since the crystal structure of TfR1-transferrin complex shows that the C-terminus of TfR1 is not involved in transferrin binding and would be exposed on the cell surface.[17] TRVb cells expressing TfR1-A1 fusions were labeled with QD-CoA nanoparticles by AcpS and the reaction was allowed to proceed at 30 °C for 20 min. After the labeling reaction, cells were incubated with transferrin ligands conjugated with a small molecule fluorophore Alexa Fluor 555 (Tf-Alexa555) in fresh medium before they were washed with PBS buffer, fixed and imaged by confocal microscopy. As shown in Fig. 3a, fluorescence signals of QD labeled TfR1-A1 and Tf-Alexa555 were colocalized on the surface of TRVb cells suggesting that QD-CoA was site-specifically attached to the short peptide tag fused to the TfR1 receptor and that the QD label did not affect the binding of the receptor to the transferrin ligand. For control reactions, QD-CoA was added to transfected cells in the absence of AcpS (Fig. 3b) or to untransfected cells in the presence of the enzyme. In both cases, minimal background was observed. In addition, unfunctionalized QDs were also incubated with the transfected cells in the presence of AcpS and no labeling of TfR1 receptors was observed (data not shown).

TRVb cells transfected with the TfR1-PCP fusion were also successfully labeled with QD-CoA conjugates in the presence of Sfp enzyme (Fig. S3†).We also tested the labeling of N-terminal S6-tagged epidermal growth factor receptor (S6-EGFR) on the surface of HeLa cells. However, Sfp catalyzed QD labeling of S6-EGFR was observed only with

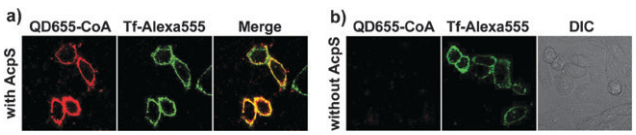

Fig. 3 Confocal fluorescence microscope images of QD labeled representative TRVb cells transfected with TfR1-A1. (a) Cell labeling reactions in which TRVb cells expressing TfR1-A1 were first labeled with QD655-CoA (red) in the presence of AcpS and then incubated with Tf-Alexa555 (green); (b) control reactions in which TRVb cells expressing TfR1-A1 were incubated with QD655-CoA in the absence of AcpS and then incubated with Tf-Alexa555.

QD-CoA conjugates prepared with a higher CoA : QD ratio (> 50) during QD functionalization (Fig. S4†). This implies that the high density of CoA ligands on QDs would increase the labeling efficiency. We also believe that the peptide tags fused to different protein receptors may differ in their accessibility to QD-CoA and PPTases during the labeling reaction. Thus the positions of the peptide tags with respect to the target proteins need to be carefully considered before the construction of protein fusions. Furthermore, it is crucial to optimize cell transfection and target protein expression levels in order to achieve high labeling efficiency of the cell surface proteins.

The orthogonal substrate specificities of Sfp for the S6 tag and AcpS for the A1 tag allowed us to label TfR1-A1 and S6-EGFR receptors with different fluorescent probes in cells cotransfected with the expression constructs encoding the two receptors.[12] As shown in Fig. 4, TfR1-A1 receptors on the Hela cell surface were first labeled with QD-CoA by AcpS. After washing the cells with PBS buffer, S6-EGFR receptors on the surface of the same cells were labeled with CoA conjugated Alexa Fluor 488 fluorophore (Alexa488-CoA) by Sfp. This experiment demonstrates that PPTase catalyzed protein labeling with QDs can be combined with small molecule fluorophores to image distinctive target proteins on cell surfaces.

PPTase catalyzed QD labeling is also compatible with live cell imaging. This was demonstrated by imaging the endocytosis of QD-labeled TfR1 in live TRVb cells (Fig. S5†). After QD labeling of TfR1-A1 receptors on the cell surface, TRVb cells were incubated with Tf-Alexa555 at 22 and 30 °C, respectively, in order to compare the effect of temperature on the endocytosis of TfR1 receptor with Tf ligand bound. When cells were incubated with Tf-Alexa555 at 22 °C for 10 min, not much internalization of QD labeled TfR1 was observed. In contrast, during the same incubation time at 30 °C, a significant amount of TfR1-Tf complex underwent endocytosis as observed by the internalization of the fluorescence signals (Fig. S5†). This result is consistent with the previous literature reporting a sharp increase in the rate of TfR1-Tf complex endocytosis when the temperature was raised above 26 °C.[18]

In summary, we have developed an efficient method for one-step, site-specific protein labeling with QDs *in vitro* and on live cell surfaces. In this method, PPTases Sfp and AcpS covalently attach CoA-functionalized QDs to a short peptide tag fused to the target protein. Our method eliminates the need to functionalize QD surfaces with large size proteins, or to tag the target proteins with large enzymes. We envision that such a method would have the potential to produce functional QDs of small sizes for imaging QD-labeled target proteins in space-restricted subcellular environments.[4,8] Furthermore, the orthogonal reactivities of A1 and S6 tags with different PPTases would make this method applicable for labeling distinctive cellular targets with either QDs or small molecule probes in cell imaging and single molecule studies.

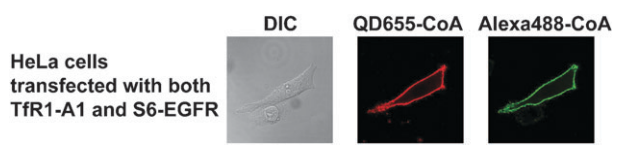

Fig. 4 Orthogonal labeling of TfR1 and EGFR on HeLa cells cotransfected with TfR1-A1 and S6-EGFR. TfR1-A1 receptors on cell surfaces were first labeled with QD655-CoA (red) in the presence of AcpS at 37 °C for 20 min. Cells were then washed with PBS, and incubated with Alexa488-CoA (green) and Sfp to label S6-EGFR.

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