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# Development of a Bioorthogonal and Highly Efficient Conjugation Method for Quantum Dots using Tetrazine-Norbornene Cycloaddition

**Hee-Sun Han**[†], **Neal K. Devaraj**[‡], **Jungmin Lee**[†], **Scott A. Hilderbrand**[‡], **Ralph Weissleder**[‡], and **Moungi G. Bawendi**[*,†]

[†]Department of Chemistry, Massachusetts Institute of Technology 77 Massachusetts Ave, Cambridge, MA02139-4307

[‡]Center of Systems Biology, Massachusetts General Hospital, 185 Cambridge Street, Boston, MA02114

In this communication, we present a bioorthogonal and modular conjugation method to efficiently couple organic dyes and bio-molecules to quantum dots (QDs) using a norbornene and tetrazine cycloaddition. The use of non-coordinating functional groups combined with the rapid rate of the cycloaddition leads to highly efficient conjugation. We apply this method to the *in situ* targeting of norbornene coated QDs to live cancer cells that are labeled with tetrazine modified proteins.

Conventional QD conjugation methods typically rely on functional groups such as amines, carboxylic acids, and thiols that are known to interact with the QD surface.[1,2] Surface coordination of functional groups can limit the number of available groups for further coupling, resulting in low conjugation efficiencies.[3] An attractive alternative is to employ coupling chemistry that requires functional groups that do not coordinate to the QD surface. Click chemistries, such as the popular copper catalyzed azide-alkyne cycloaddition, are potential alternative conjugation strategies that have been used with gold nanoparticles.[4–6] The copper catalyst, however, irreversibly quenches QD fluorescence (Figure S1). Additionally, catalyst-free strain-promoted click reactions are limited by poor aqueous solubility of substrates and tedious syntheses.[7,8] Recently, there have been a number of examples of the use of inverse-electron-demand Diels-Alder cycloadditions involving tetrazine and strained alkenes as an alternative bioorthogonal conjugation method.[9–11] This chemistry benefits from sufficiently rapid kinetics that no catalyst is required. Recently, we have developed a tetrazine derivative [3-(4-benzylamino)-1,2,4,5-tetrazine] (BAT) that shows good stability in buffer and serum and high reaction rate when reacted with strained olefins such as norbornene (Scheme 1, 2 $M^{-1}s^{-1}$ at 20 °C)[12] or *trans*-cyclooctene (~6000 $M^{-1}s^{-1}$ at 37 °C)[13].

Utilizing the non-coordinating properties of the substrates and the fast reaction rate, we explored norbornene-tetrazine cycloaddition as a new, efficient conjugation method on QDs. Carboxylic acid modified norbornene (bicyclo[2.2.1]hept-5-en-2-yl acetic acid) was selected for this study as it is commercially available and the carboxylic acid group allows further conjugation to other molecules.

[*] mgb@mit.edu .

Supporting Information Available: Experimental procedures, transmission electron microscopy images, gel filtration chromatograms and additional optical characterization of the conjugates. This material is available free of charge via the Internet at http://pubs.acs.org.

The cycloaddition was achieved by functionalizing QDs with norbornene and reacting with BAT-modified substrates. Polymeric imidazole ligands (PILs) were used to prepare norbornene-coated water soluble QDs. PILs are random copolymers incorporating poly (ethylene) glycol (PEG), amino-$PEG_{11}$, and imidazole groups for water solubilization, functionalization, and QD binding, respectively.[3] The modularity of the polymer and commercial availability of the norbornene allows facile incorporation of norbornene groups on the polymer in gram scale. For this study, poly(amino-$PEG_{11}$)$_{20\%}$-PIL ($NH_2$-PIL), which is composed of 30% poly(ethylene) glycol ($PEG_{12}$), 20% amino-$PEG_{11}$, and 50% imidazole groups, was further modified with n-hydroxysuccinimide(NHS) activated bicyclo[2.2.1] hept-5-en-2-yl acetic acid (norbornene) via amide coupling (Figure 1A). Complete conversion of amines to norbornenes was confirmed by probing free amine in the polymer before and after the conjugation with fluorescamine, an amine-reactive fluorogenic probe (Figure S6). Norbornene-coated QDs were prepared by ligand exchange of natively capped QDs with the norbornene modified PIL (NB-PIL) (Figure 1B). The quantum yield of the QDs after the ligand exchange was ~60% which was maintained after the cycloaddition (Figure S4). To determine conjugation efficiencies of the cycloaddition on QDs, norbornene-coated QDs were conjugated with BAT modified Alexa 594 (Alexa-BAT) (Figure 1B–D, Supporting Information). Coupling yields were determined through knowledge of the extinction coefficients of the dye and QDs and meaurement of the product absorption spectra. The number of Alexa dyes conjugated to the norbornene coated QDs varied depending on the excess of Alexa-BAT (Figure 1C–D). Increasing the dye concentration to 100x excess, led to a saturation coupling yield of ~16 dyes/QD. We believe that this number effectively represents the average number of reactive norbornene molecules on the surface of each QD. One should be able to increase the number of coupled dyes by further increasing the composition of norbornenes in the starting polymer.

To illustrate the utility of the coupling chemistry for live cell imaging with QDs, we labeled epidermal growth factor receptors (EGFRs) overexpressed on the surface of human skin cancer cells. Cellular labeling was achieved either directly through use of preformed QD-EGF conjugates (Figure 2B) or by performing *in situ* conjugation of the norbornene-coated QDs to BAT modified EGFs on the cell surface (Figure 2C). For direct labeling, the norbornene coated QDs were coupled with BAT modified EGF (Figure 2A) and 50 nM of the resulting QD-EGF conjugates were added to A-431 human carcinoma cells at 4 °C for 30 mins (Figure 3B). By using low concentration of the QDs, we were able to observe single QDs which are characterized by their fluorescence intermittency (Figure S5). For *in situ* conjugation, cells were incubated with 200 nM BAT modified EGF (BAT-EGF) at 4 °C for 30 minutes and labeled with 800 nM of norbornene coated QD at 37 °C for 30 mins (Figure 3D). Control experiments using the same procedures but with QDs coated with poly($PEG_{12}$)-PIL, composed of 50 % imidazole and 50% $PEG_{12}$ (without norbornene), are shown in Figure 3A and 3C.

As Figure 3 demonstrates, successful labeling is achieved using either labeling methods. The fast reaction rate in serum and the absence of toxic Cu(I) catalyst allowed for *in situ* conjugation of norbornene-coated QDs to BAT-EGF labeled cells. In addition, this method does not result in an increased QD size and generally works on cells with endogenously expressed receptors.

In summary, we have developed an efficient conjugation method for QDs utilizing the inverse Diels-Alder cycloaddition between tetrazine and norbornene. The absence of toxic Cu(I) catalyst, rapid kinetics, and tolerance of the reaction to functional groups abundant in cells enabled efficient cell labeling *in situ*. The conjugation approach presented here is modular and can be extended to many biological imaging applications, as tetrazine and norbornene functionalities can be easily conjugated to carboxylic acid or amine containing molecules.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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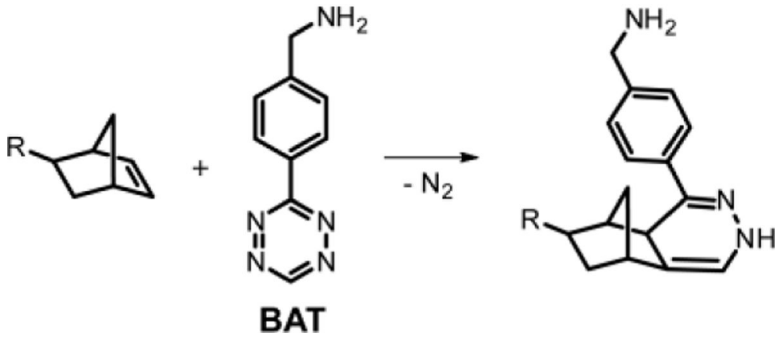


**Scheme 1.**
Click chemistry between BAT and norbornene

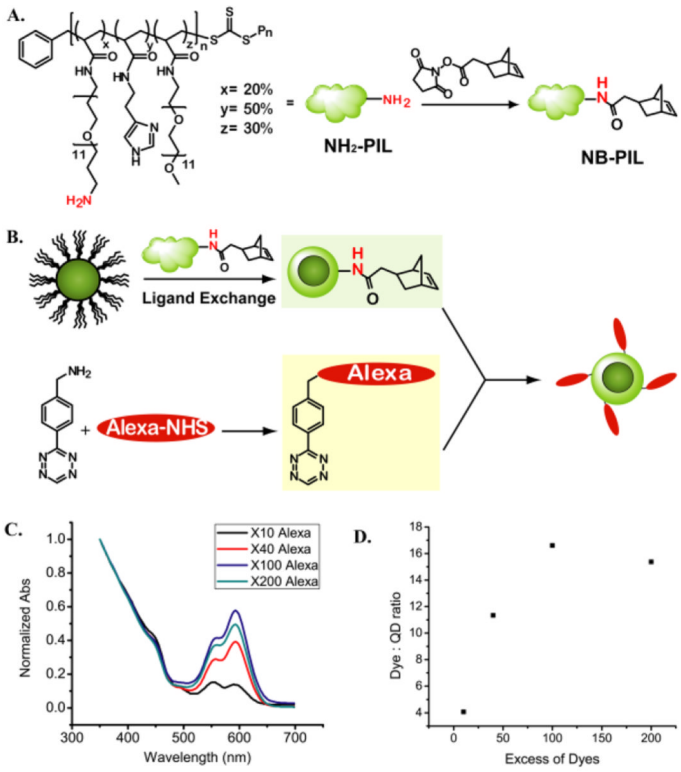


**Figure 1.**

(A) Conjugation of norbornene to 20% NH2-PIL polymer (B) Conjugation of Alexa with QD570 using BAT+norbornene chemistry (C) Absorbance spectra of QD-Alexa conjugates which were prepared by mixing carrying concentrations of the dye (D) Calculated Alexa to QD ratios for the purified conjugates

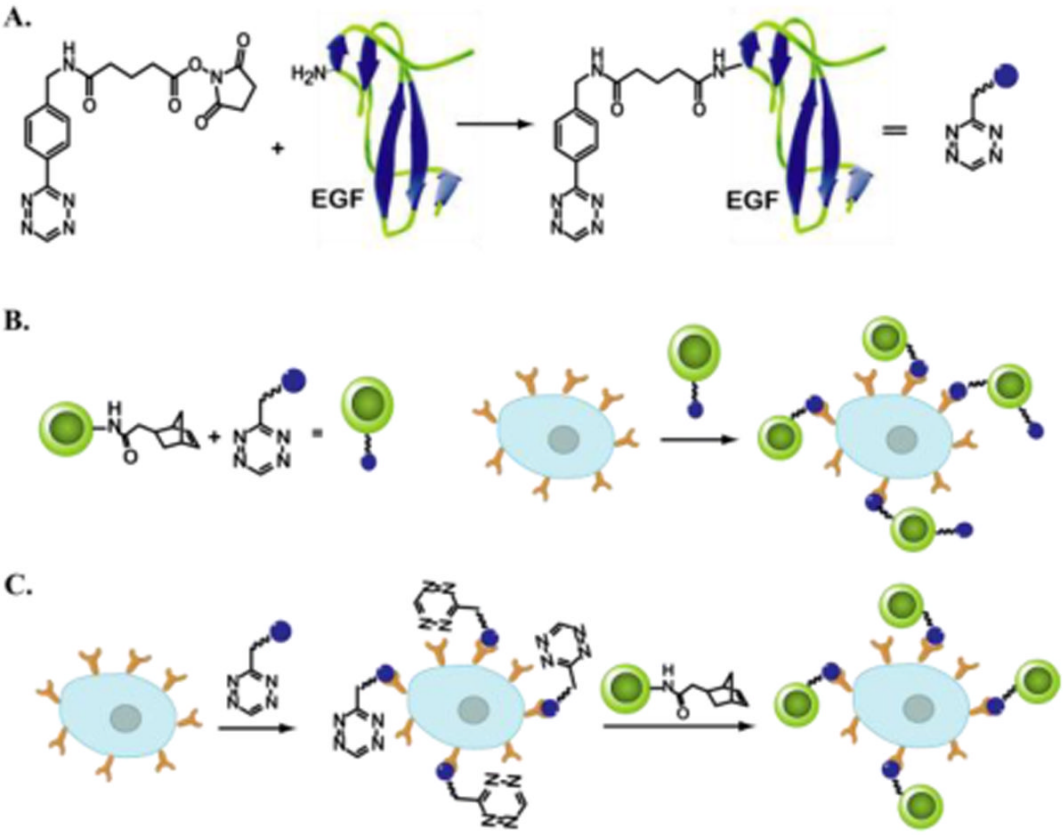


**Figure 2.**
(A) Conjugation of NHS-activated BAT to EGF. (B) Labeling of cells with pre-formed QD-EGF constructs. (C) In situ conjugation of norbornene-functionalized QDs to BAT-EGF on live cells

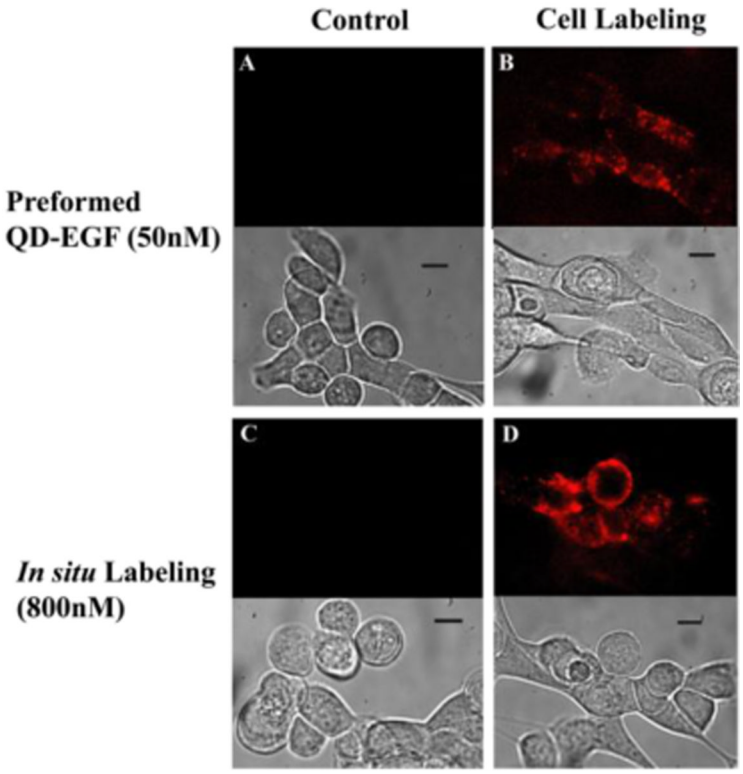


**Figure 3.**

Targeting of QDs to A431 (squamous cancer) cells using norbornene-tetrazine cycloaddition. Top: QD fluorescence at 605 nm with excitation at 488 nm. Bottom: corresponding DIC images (scale bar 10 μm). Cells were targeted either by (B) using preformed QD-EGF complexes (50 nM) for single QD tracking or by (D) performing in situ conjugation using norbornene-functionalized QDs (800 nM) on BATEGF- modified cell surfaces for ensemble labeling. (A) and (C) are control experiment with poly($PEG_{12}$)-PIL QDs (without norbornene).