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Development of a Bioorthogonal and Highly Efficient Conjugation Method for Quantum Dots Using Tetrazine−Norbornene Cycloaddition

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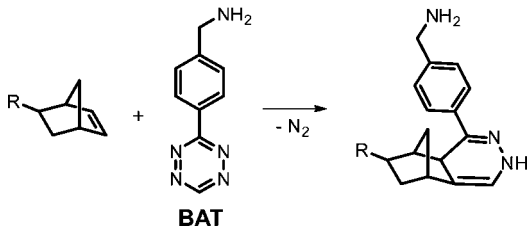
In this communication, we present a bioorthogonal and modular conjugation method for efficient coupling of organic dyes and biomolecules to quantum dots (QDs) using a norbornene−tetrazine cycloaddition. The use of noncoordinating functional groups combined with the rapid rate of the cycloaddition leads to highly efficient conjugation. We have applied this method to the in situ targeting of norbornene-coated QDs to live cancer cells 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 groups available for further coupling, resulting in low conjugation efficiencies.[3] An attractive alternative is to employ coupling chemistry requiring 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 in the Supporting Information). 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 a high reaction rate when reacted with strained olefins such as norbornene (2 M^{-1} s^{-1} at 20 °C; Scheme 1)[12] or *trans*-cyclooctene (∼6000 M^{-1} s^{-1} at 37 °C).[13]

Scheme 1. Click Chemistry between BAT and Norbornene



Utilizing the noncoordinating properties of the substrates and the high 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 because it is commercially available and the carboxylic acid group allows further conjugation to other molecules.

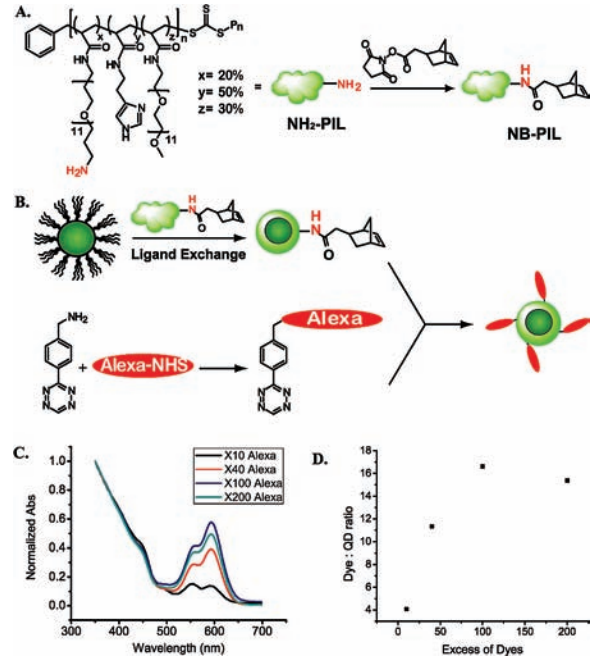


Figure 1. (A) Conjugation of norbornene to 20% NH_2−PIL polymer. (B) Conjugation of Alexa 594 with QDs using BAT−norbornene chemistry. (C) Absorbance spectra of QD−Alexa conjugates prepared by mixing various concentrations of the dye. (D) Calculated Alexa-to-QD ratios for the purified conjugates.

The cycloaddition was achieved by functionalization of QDs with norbornene and subsequent reaction 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 allow facile incorporation of norbornene groups on the polymer on the gram scale. For this study, poly(amino-PEG_{11})$_{20\%}$−PIL (NH_2−PIL), which is composed of 30% 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 the free amine in the polymer before and after the conjugation using 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

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yield of the QDs after the ligand exchange was ~60%, which was maintained after the cycloaddition (Figure S4). To determine the conjugation efficiencies of the cycloaddition on QDs, norbornene-coated QDs were conjugated with BAT-modified Alexa 594 (Alexa-BAT) (Figure 1B−D; also see the Supporting Information). Coupling yields were determined through knowledge of the extinction coefficients of the dye and QDs and measurement of the product absorption spectra. The number of Alexa 594 dye molecules conjugated to the norbornene-coated QDs varied depending on the excess of Alexa-BAT (Figure 1C,D). Increasing the dye concentration to 100-fold excess led to a saturation coupling yield of ~16 dye molecules/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.

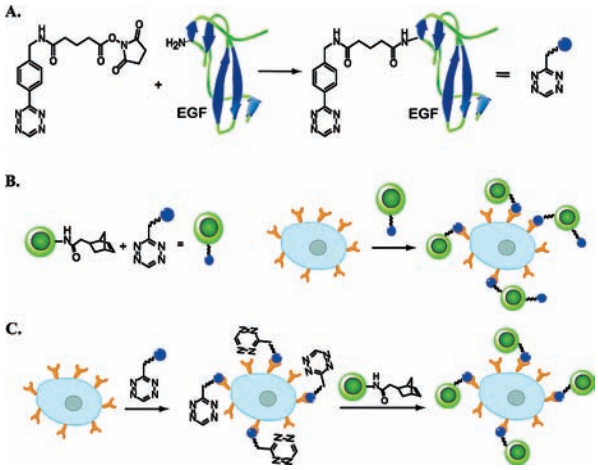

Figure 2. (A) Conjugation of NHS-activated BAT to EGF. (B) Labeling of cells with preformed QD−EGF constructs. (C) In situ conjugation of norbornene-functionalized QDs to BAT−EGF joined to EGFRs on live cells.

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 EGF (BAT−EGF) joined to EGFRs on the cell surface (Figure 2C). For direct labeling, the norbornene-coated QDs were coupled with BAT−EGF (Figure 2A), and the resulting QD−EGF conjugates (50 nM) were added to A431 human carcinoma cells at 4 °C for 30 min (Figure 3B). By using a 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 BAT−EGF (200 nM) at 4 °C for 30 min and then labeled with norbornene-coated QDs (800 nM) at 37 °C for 30 min (Figure 3D). Results of 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,C.

As Figure 3 demonstrates, successful labeling was achieved using either labeling method. The high 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 method for conjugation of 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

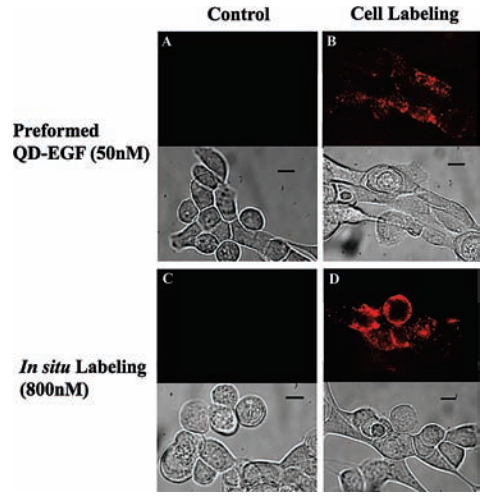

Figure 3. Targeting of QDs to A431 (squamous cancer) cells using norbornene−tetrazine cycloaddition: (top) QD fluorescence at 605 nm with excitation at 488 nm; (bottom) corresponding differential-interference contrast images (scale bar 10 μm). Cells were targeted either by (B) using preformed QD−EGF complexes (50 nM) for single-QD tracking or (D) performing in situ conjugation using norbornene-functionalized QDs (800 nM) on BAT−EGF-modified cell surfaces for ensemble labeling. (A) and (C) show results of control experiments with poly(PEG_{12})-PIL QDs (without norbornene).

groups abundant in cells enables 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.

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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.

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