Quantum Dots Hot Paper

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A Ligand System for the Flexible Functionalization of Quantum Dots via Click Chemistry

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Abstract: *We present a novel ligand, 5-norbornene-2-nonanoic acid, which can be directly added during established quantum dot (QD) syntheses in organic solvents to generate "clickable" QDs at a few hundred nmol scale. This ligand has a carboxyl group at one terminus to bind to the surface of QDs and a norbornene group at the opposite end that enables straightforward phase transfer of QDs into aqueous solutions via efficient norbornene/tetrazine click chemistry. Our ligand system removes the traditional ligand-exchange step and can produce water-soluble QDs with a high quantum yield and a small hydrodynamic diameter of approximately 12 nm at an order of magnitude higher scale than previous methods. We demonstrate the effectiveness of our approach by incubating azido-functionalized CdSe/CdS QDs with 4T1 cancer cells that are metabolically labeled with a dibenzocyclooctyne-bearing unnatural sugar. The QDs exhibit high targeting efficiency and minimal nonspecific binding.*

Quantum dots (QDs) are promising fluorescent probes in biomedical imaging research due to their high quantum yield (QY), good photo-stability, broad absorption, and tunable and narrow photoluminescence (PL) spectra.[1–6] To enable their biological applications, the as-synthesized hydrophobic QDs need to be transferred into aqueous solutions through surface modifications. Previously reported methods to obtain water-soluble QDs include encapsulating QDs in amphiphilic polymers, growing silica shells, and substituting the native hydrophobic ligands with hydrophilic molecules.[7–12] The encapsulation method usually causes minimal loss of QY upon water solubilization owing to preservation of the native hydrophobic ligands and thus little damage to the nanocrystal surface, but a large increase of the hydrodynamic diameter (HD) is inevitable.[7] For example, 4–8 nm QDs were shown to have HDs of 20–40 nm after being coated with amphiphilic polymers.[13] Growing a silica layer produces stable water-soluble QDs whose surfaces can readily be modified with various chemical groups. However, this approach also causes a significant increase in HD.[14,15] The stringent requirement on the small HD of QDs in numerous biological applications has drawn extensive attention to the ligand-substitution method. While replacing native ligands with hydrophilic molecules results in minimal change in HD, significant loss of QY can result from the introduction of surface trap states.[16,17] In addition, phase transfer of QDs using these methods is usually carried out on a small scale (<10 nmol),[18–20] limited by the method itself or by the availability of the replacing ligands.

To obtain water-soluble QDs for biological applications at a larger scale, we evaluated whether incorporation of a clickable moiety at the end of native ligands and further conjugation of a hydrophilic agent would allow phase transfer of QDs while maintaining a high QY and a small HD. We placed emphasis on a ligand system that would not alter the native ligand on the nanocrystal surface post-synthesis in order to maintain high QYs. To minimize the size of the resulting QDs in water, we decided to link hydrophilic molecules to the native hydrophobic ligands via covalent bonds rather than weak Van der Waals forces.[7] Norbornene has been demonstrated to undergo efficient bio-orthogonal click reactions with tetrazine and was thus chosen as the clickable moiety to link the native ligand to a tetrazine bearing hydrophilic molecule.[21,22] The main ligand species for a broad range of QDs are carboxylate ligands,[23–25] as the carboxy group exhibits a strong binding affinity to common QD metals. Considering the cost and availability of reagents, we designed the ligand, 5-norbornene-2-nonanoic acid, later referred to as NB-nonanoic acid (Scheme 1a), as the native ligand to be introduced during QD syntheses.

To demonstrate the effectiveness of our ligand system, NB-nonanoic acid was added to established QD syntheses to generate norbornene functionalized QDs at a few hundred nmol scale. QDs capped with NB-nonanoic acid were successfully transferred into aqueous solutions after clicking with polyethylene glycol (PEG) terminated with a 1,2,4,5-tetrazine group (tetrazine-PEG) (Scheme 1a). Further functionalization of QDs can be achieved through the incorporation of functional groups (azide, maleimide, biotin, thiol,

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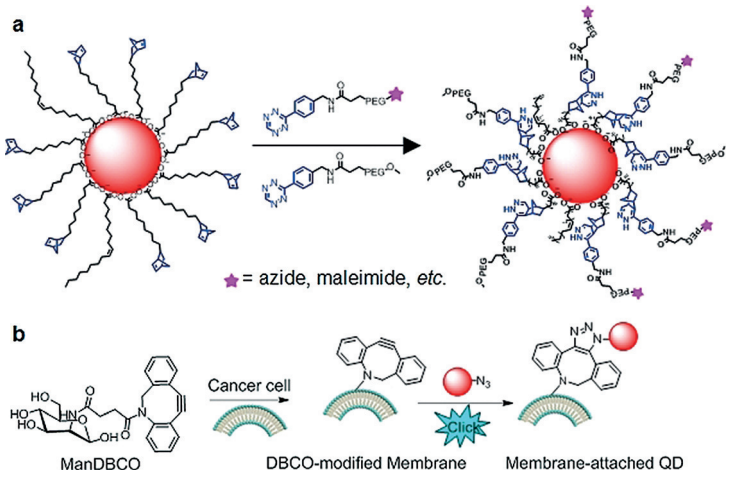

Scheme 1. a) Schematic illustration of water solubilization and flexible functionalization of 5-norbornene-2-nonanoic acid capped QDs via norbornene/tetrazine click chemistry. b) Cancer cells are treated with ManDBCO, which ends up being expressed on cell membranes through cellular metabolic glycosylation pathways. Azido functionalized QDs are then used to label the cells via a N_3-DBCO click reaction.

acrylate, etc.) at the other terminus of the PEG chains. To test the binding efficiency of water-soluble QDs in biological systems, azido functionalized CdSe/CdS QDs (QD-N_3) were incubated with 4T1 breast cancer cells with or without pretreatment with a dibenzocyclooctyne (DBCO) bearing unnatural sugar (ManDBCO, Scheme 1b). Both flow cytometry and confocal imaging showed high targeting efficiency and minimal nonspecific binding of the QDs.

NB-nonanoic acid, designed to be an efficient ligand system for water solubilization and flexible functionalization of QDs, has a carboxylic acid group at one terminus to bind to the metal cation sites on QD surfaces and a norbornene group at the other for conjugation to hydrophilic ligands via norbornene/tetrazine click chemistry. Figure 1a shows the synthetic route to NB-nonanoic acid, which employs readily available starting reagents and which can be readily scaled up. The chemical structure of the intermediate Compound A (Figure S1) and the final product Compound B (Figure 1b, Figure S2) are well characterized by ^{1}H and ^{13}C nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectroscopy. Since QD syntheses are usually conducted at a high temperature, we tested the thermostability of NB-nonanoic acid by heating stepwise and tracking structural changes using ^{1}H NMR spectroscopy. As shown in Figure S3 in the Supporting Information, the ligand is stable up to 200 °C; the norbornene moiety undergoes a retro-Diels–Alder decomposition beyond that point. We applied this ligand to recently described CdSe/CdS QDs, which are synthesized at 310 °C,[1] by adding the NB-nonanoic acid ligand as a final step after cooling the reaction solution to 200 °C, followed by annealing at 200 °C for one hour. The resulting QDs were purified twice by precipitation-redispersion and then by a secondary purification step using a home-built dialysis setup (Figure S4) that can efficiently remove free unbound ligands prior to ^{1}H NMR analysis. The ^{1}H NMR spectrum of the QD-ligand solution shows broadening and loss of resolved splitting of all the resonance peaks of NB-nonanoic acid (Figure 1c inset as an example) as well as loss of the α-proton Peak 3, indicating that all of the NB-nonanoic acid is bound to QD surfaces.[26–28] The final ratio of NB-nonanoic acid to oleic acid on QD surfaces was calculated to be 4:1 by integrating the peaks of double bond protons (NB-nonanoic acid: 5.8–6.2 ppm, oleic acid: 5.3 ppm) on Figure 1c (see also Figure S5). The effect of the annealing temperature on final ligand ratios between 50 °C and 200 °C (Figure S6) was also studied using ^{1}H NMR spectroscopy. The NB-nonanoic acid to oleic acid ratio stays constant at 4:1 when the annealing temperature is above 100 °C (Figure S7). We chose an annealing temperature of 150 °C for all following syntheses.

After demonstrating the synthesis of NB-nonanoic acid capped clickable CdSe/CdS QDs (QD-norbornene), we investigated the subsequent reaction with tetrazine-PEG to yield water-soluble QDs. QD-norbornene in chloroform was added to a mixture of tetrazine-PEG-OMe and tetrazine-PEG-N_3. Upon phase transfer to water, we detected a negligible change in the QD absorption and PL

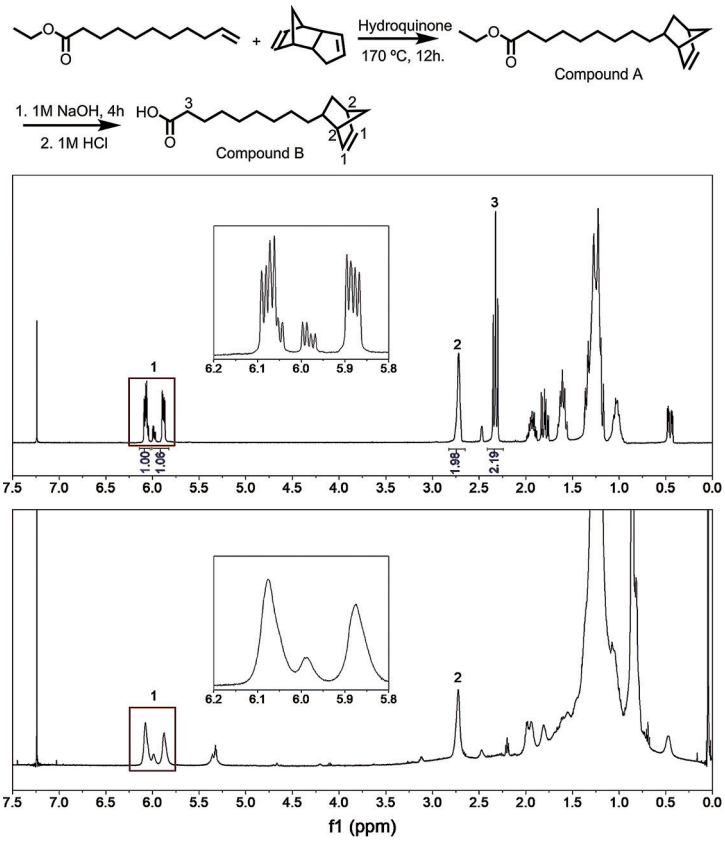

Figure 1. a) Synthetic route to NB-nonanoic acid (compound B). ^{1}H NMR spectrum of b) NB-nonanoic acid and c) NB-nonanoic acid capped CdSe/CdS QDs (insets are expansions of the areas indicated in the red boxes).

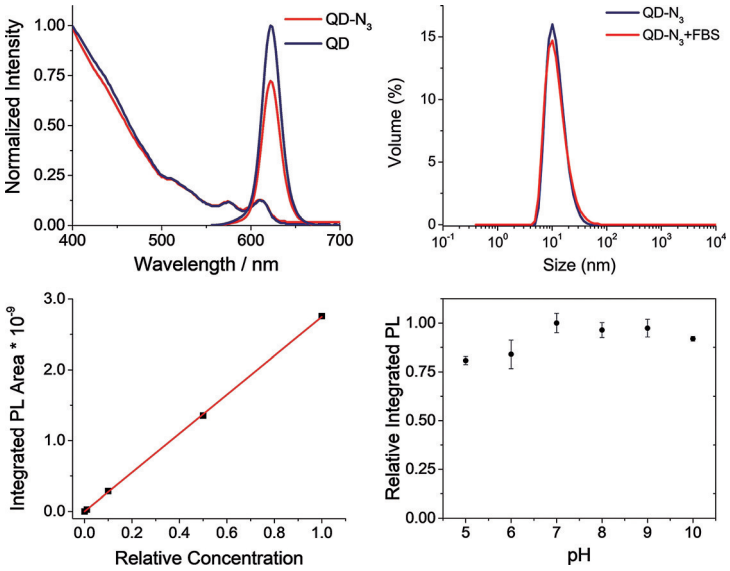

Figure 2. a) Absorption and photoluminescence (PL) spectra of CdSe/CdS QDs before (blue) and after (red) water solubilization. PL intensities were adjusted based on QYs. b) DLS measurement of water-soluble CdSe/CdS QDs before and after incubation with fetal bovine serum at 37 °C for 4 h. c) Linear relationship between integrated PL and relative concentrations of water-soluble CdSe/CdS QDs, inset is an expansion of the area indicated in the blue rectangle. d) Colloidal stability test of water-soluble CdSe/CdS QDs at different pH values (5–10).

spectra and a drop in the PL QY from close to 100 % to about 70 % (Figure 2 a). Our approach preserves approximately 70 % of the original QY, which is comparable to other methods but yields a higher absolute QY value.[8,11,29] Dynamic light scattering (DLS) measurements show a HD of 12 nm (Figure 2 b), which is about 3 nm larger than the inorganic core (Figure S8), a reasonable diameter increase caused by the NB-nonanoic acid and PEG_{12} chains. The HD remains unchanged after incubating with fetal bovine serum at 37 °C for 4 h, indicating minimal nonspecific binding of the QDs to serum proteins. The water-soluble QDs are stable against serial dilution with phosphate buffered saline (PBS), with the integrated emission remaining linear over 3 decades of dilution (Figure 2 c). The dilute QD solution (10 nM) is stable under ambient conditions for at least 15 days (Figure S9). This indicates high colloidal stability that is essential for biological applications where very dilute samples are used. The pH stability study of our QDs shows a slight decrease of the integrated PL intensity with decreasing pH, presumably due to the protonation of NB-nonanoic acid and loss of ligand at lower pH (Figure 2 d). Our characterization shows that the novel ligand system generates water-soluble CdSe/CdS QDs with a small HD, a high QY, and good colloidal stability.

We highlight that our ligand system not only achieves promising results for our recently described CdSe/CdS QDs,[1] but that it can also be readily applied to other carboxylate ligand-stabilized nanocrystal systems, including CdSe/CdS nanorods, CdSe/ZnS QDs, InAs/CdSe/CdS QDs, and PbS QDs. Emission spectra of those water-soluble nanostructures cover a broad wavelength range from the visible to the infrared (Figure S10), and their QYs are well maintained (Table S1). These water-soluble QDs are promising candidates for multiplexed imaging applications, and InAs- and PbS-based QDs, especially, have received recent attention as emissive materials for in vivo imaging in the short-wave infrared.[30,31]

We next demonstrate the cellular binding and uptake of our water-soluble QDs for potential biological applications. In addition to studying endocytosis-mediated cellular uptake, we also describe the targeted uptake of azido-functionalized QDs (QD-N_3) into chemically labeled cells via efficient click chemistry. Metabolic labeling of cells using unnatural sugars has proven to be an effective methodology to introduce artificial chemical receptors to cell surfaces as an alternative to endogenous protein receptors that have shown limitations for cell targeting.[32–34] We explored the use of QD-N_3 probes to target cancer cells that have DBCO groups on the cell surface. Wang et al. recently reported a DBCO-bearing unnatural sugar, Ac_4ManDBCO, that metabolically labels LS174T colon cancer cells with DBCO groups for subsequent targeting by azido-modified nanomedicines.[35,36] To label cells with QD-N_3, we adapted this procedure by using a new unnatural sugar, ManDBCO, a water-soluble de-acetylated analog of Ac_4ManDBCO, for metabolic labeling of 4T1 breast cancer cells (Figure S11). ManDBCO participated in the cellular glycosylation pathways and ended up being expressed on the cell surface in the form of glycoproteins, functioning as artificial receptors.

We investigated whether 4T1 cells treated with ManDBCO express DBCO groups on the cell membranes and can mediate the specific binding of QD-N_3. 4T1 cells were treated with ManDBCO for three days, followed by incubation with QD-N_3 (50 % N_3, Emission: 625 nm) for 2 h. The cells were then washed multiple times to remove free QD-N_3 prior to confocal microscopy and flow cytometry analysis. As shown in Figure 3 a, QD-N_3 shows significantly enhanced cellular uptake in 4T1 cells pretreated with ManDBCO compared to control cells without ManDBCO treatment. We note that the passive cellular uptake of QDs is very low even after 4 h of incubation (Figure 3 b), perhaps because of the slight net negative charge of the QD probe (Figure S12).[37] The covalently conjugated QD-N_3 eventually enter endosomes/lysosomes, as evidenced by confocal images (Figure 3 a,b). Increasing the incubation time from 2 h to 4 h increases the cellular uptake of QD-N_3 from 29 % to 46 % (Figure 3 c), indicating a continuous click reaction-mediated cellular uptake over time. After 4 h of incubation, negligible change of fluorescence intensity in control cells without ManDBCO treatment was observed (Figure 3 d,e), further highlighting the minimal nonspecific cellular uptake of QD-N_3. In contrast, cells pretreated with ManDBCO exhibit an obvious increase in fluorescence intensity overall (Figure 3 f, Figure S13). To exclude the impact of cell-surface DBCO on the permeability of cells, we incubated ManDBCO-pretreated 4T1 cells with QD-OMe under the same conditions, which shows minimal cellular internalization (Figure 3 g). These experiments demonstrate that ManDBCO can metabolically

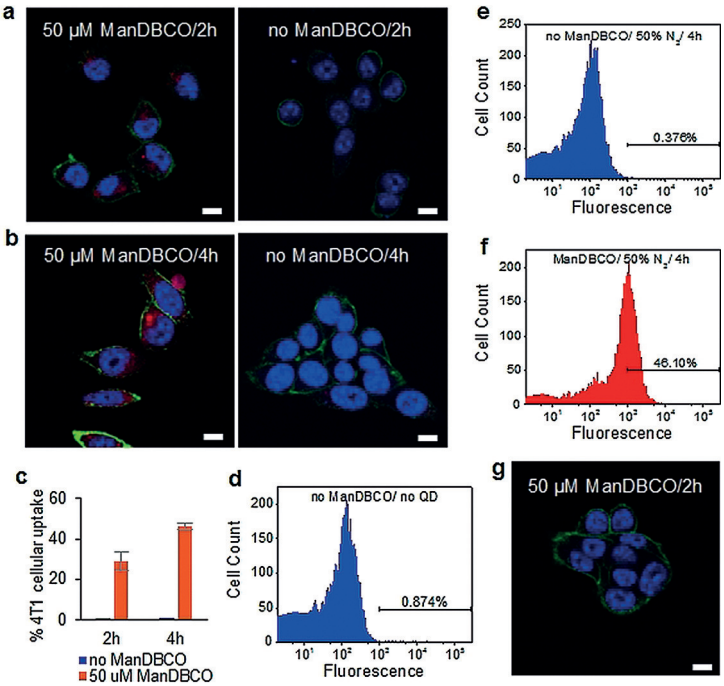

Figure 3. Confocal images of 4T1 cells treated and not treated with 50 μM ManDBCO sugar after incubation with QD-N_3(50%) for a) 2 h and b) 4 h. c) Percent cellular uptake of QD-N_3(50%) by 4T1 cells with (orange) or without (blue) pre-treatment of 50 μM ManDBCO. d)–f) Representative flow cytometry profiles of 4T1 cells after incubation with QD-N_3(50%). The PE-Texas Red channel was used. Cell treatment condition and incubation time information is provided at the top of each Figure. g) Confocal images of 4T1 cells treated with 50 μM ManDBCO and incubated with QD-OMe for 2 h. (For all confocal images: blue: DAPI, green: Alexa Fluor 488 membrane stain, red: QD, scale bar: 10 μm.)

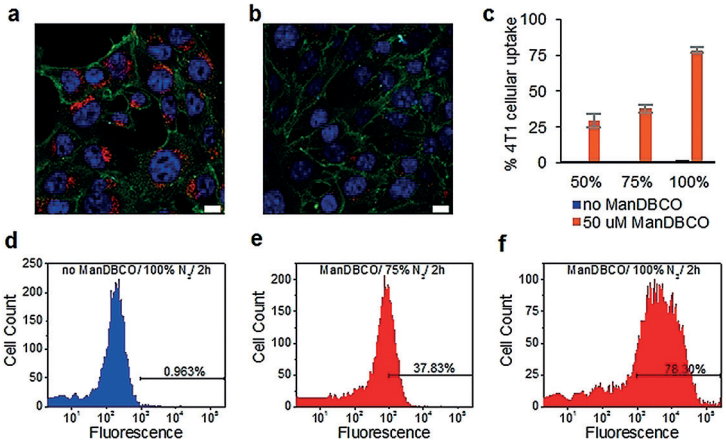

Figure 4. Confocal images of 4T1 cells a) treated with 50 μM ManDBCO, and b) not treated with ManDBCO and incubated with QD-N_3(100%) for 2 h (blue: DAPI, green: Alexa Fluor 488 membrane stain, red: QD, scale bar: 10 μm). c) Percent cellular uptake of QD-N_3(50%), QD-N_3(75%), and QD-N_3(100%) after 2 h incubation with 4T1 cells that were pretreated with 50 μM ManDBCO (orange) or PBS (blue). d)–f) Representative flow cytometry profiles of 4T1 cells after incubation with QD-N_3. The PE-Texas Red channel was used. Cell treatment condition and incubation time information is provided at the top of each graph.

label 4T1 cells with DBCO groups, and this leads to cellular internalization of QD-N_3 via a click reaction.

Given that the click reaction between N_3 and cell surface DBCO groups mediates the cellular uptake of QD-N_3, we further studied whether a higher density of N_3 on the surfaces of QDs would provide better cell targeting efficiency. QD-N_3 with different molar percentages of tetrazine-PEG-N_3 (50%, 75%, and 100%) were prepared. 4T1 cells were treated with ManDBCO for three days, followed by incubation with QD-N_3 (50%), QD-N_3 (75%), and QD-N_3 (100%), respectively, for 2 h. Cells without ManDBCO treatment were used as a control. Confocal images show improved cellular internalization of QD-N_3 (100%) compared with QD-N_3 (50%), while the control cells exhibit minimal QD fluorescence (Figure 4). All formulations show little passive uptake by 4T1 cells (Figure 4d, Figure S13), further highlighting the minimal nonspecific binding of these QD-N_3.

In conclusion, we report a new, versatile ligand system that can be added during QD syntheses and yield clickable QDs of various formulations at the hundred nmol scale. The ligand is characterized by a carboxylic acid group at one end for binding to metal sites on QD surfaces and a norbornene group at the other for flexible functionalization. Upon attaching a tetrazine-PEG molecule via click chemistry, we were able to produce water-soluble QDs with a high quantum yield, a compact hydrodynamic radius, and high colloidal stability. Furthermore, we demonstrated the efficacy and versatility of this ligand system by using azido functionalized CdSe/CdS QDs to label ManDBCO treated 4T1 cancer cells. Our results demonstrate that this ligand system can generate high-quality water-soluble QDs in quantity, which are suitable for biological applications.

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Conflict of interest

The authors declare no conflict of interest.

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