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ARTICLE TYPE

Photoreleasable Thiol Chemistry for Facile and Efficient Bioconjugation

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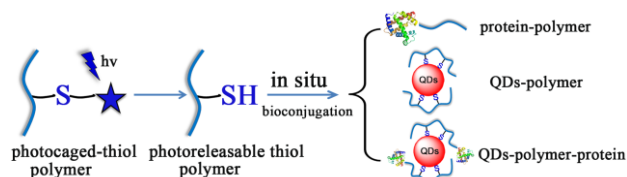
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5 A facile methodology of light-triggered release of thiols on mild condition is presented, which can be utilized for *in situ* bioconjugation with protein and quantum dots nanoparticles (QDs) efficiently.

Recently, a set of click orthogonal chemical conjugations has been widely applied ranging from polymer modification to bio-related materials.¹ Although the most prominent technique was initially the azide-alkyne cycloaddition reactions, its generally required use of copper as a catalyst has led to some drawbacks, especially the biological toxicity and the irreversibly quenching ability to fluorescent substances.² Efforts have been made to the application of suitable metal-free click reactions.³ Notably, the metal-free thiol-maleimide click reaction as its high reaction efficiency at ambient or physiological temperature in aqueous media has shown to be very powerful in various fields of applications.⁴ Besides the chemical conjugation to maleimide-contained organic matrixes, thiol groups are also able to strongly bind to a wide range of metallic surfaces (QDs, Au, Ag).⁵ However, free thiols can react very efficiently with themselves to form disulfide bridges because of air oxidation, and thus severely limit the storage and the extent of conjugation reactions or coordination binding, especially for the multithiols-contained molecules.⁶ Consequently, active-thiol groups need to be protected when they are initially present. Protecting group chemistry has been employed to obtain free thiols by hydrolysis,⁷ aminolysis,⁸ or metal hydride reduction.⁹ However, these transformations require severe conditions, especially limit to biomaterial applications. Compared to the former traditional protecting chemistry, the phototriggered release of chemical groups is very attractive as it allows not only spatiotemporal control of the released reaction but also proceeding in mild conditions,^{6b,6c,10} which is particularly suitable for bio-related applications.

Our aim was to demonstrate proof-of-concept in the application of photoreleasable thiols for facile and efficient bioconjugate in mild conditions. Herein, we synthesized a series of phototrigger caged thiol-contained hydrophilic polymers in which the active thiol groups could be released efficiently under light irradiation and then *in situ* bioconjugated with protein, and QDs independently (protein-polymer and QDs-Polymer) and simultaneously (QDs-polymer-protein), to construct biofunctional materials (scheme 1). Here, QDs was selected as model nanoparticles for its efficient luminescence that can be used for tracking the process, although it has uncertain cytotoxicity.



50 **Scheme 1** Schematic representation of photoreleasable thiol and efficient bioconjugation.

As known, PEGylation of biological relevant protein is a strategy that improves protein stability, prolongs therapeutic half-life, reduces immunogenicity and increases solubility.¹¹ In this work, a 7-amino-coumarin-caged thiocarbonate modified PEG (M_n= 5 KDa) (CTP) was utilized to photorelease free thiols to conjugate with maleimide-activated protein *in situ* upon 420 nm irradiation, to finally obtain protein-polymer conjugates (Fig. 1a). CTP polymer was synthesized through a simple PyBOP coupling approach and characterized by ¹H NMR (ESI†, Fig. S1-S2). Avidin (M_n= 65 KDa) and Transferrin (Tf, M_n= 80 KDa) were selected as the model protein and modified with maleimide by the crosslinker intermediate (ESI†, Fig. S3) to finally obtain activated proteins Mal-avidin and Mal-Tf, respectively, that were ready for the conjugation with the photoreleased thiols. Before investigating the conjugation, the photochemical properties of CTP were analyzed. It exhibited a long-wavelength absorption from 330 nm to 450 nm ($\lambda_{\text{max}}= 380 \text{ nm}$) with a high extinction coefficient ($\epsilon_{\text{max}}= 1.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). Upon 420 nm irradiation, the absorption of CTP at 380 nm was decreased accompanying

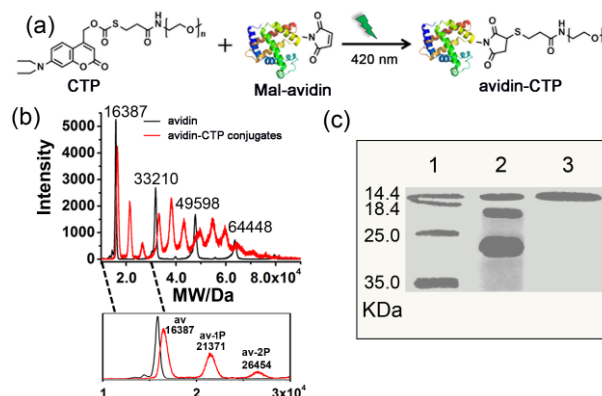


Fig. 1 (a) Bioconjugation between maleimide-activated protein and CTP under the excitation of 420 nm light. (b) MALDI-TOF mass spectrum of avidin and avidin-CTP conjugates. (c) SDS-PAGE pictures of (1) marker; (2) the mixture of CTP and Mal-avidin upon light irradiation; (3) avidin.

with a blue-shift (ESI†, Fig. S4), which indicated the successful photolysis of coumarin phototrigger. The release of the free thiols was detected by Ellman test and the final efficiency of photoreleased thiols was determined at 70%.

The phototriggered conjugation between Mal-protein and CTP was proceeded by dissolving CTP and Mal-protein in PBS (pH=7.4) directly upon 420 nm irradiation (10 mW cm⁻²). The excess salt was removed by dialysis and the residue was lyophilized to obtain the protein-polymers for further analysis. To confirm the successful conjugation, MALDI-TOF mass spectrometry (Fig. 1b) and gel electrophoresis (Fig. 1c) were explored respectively. In the MALDI-TOF mass spectrometry, avidin showed four peaks of its monomer, dimer, trimer and tetramer with M_w at 16387, 33210, 49598, and 64448 Da, respectively. After conjugation with CTP, there were two series of new peaks appeared with the value of augment of 5 and 10 K, respectively. It matched with one and two CTP molecular weight and indicated that the number of PEGylation to avidin was 0, 1 and 2 correspondingly. For example, the M_w of monomer avidin was at 16387 Da, while the conjugated avidin-CTP exhibited another two peaks at 21371 and 26454 Da that attributed to avidin conjugated with one CTP (av-1P) and two CTP (av-2p). While the number of CTP bound to Tf was 0, 1, 2 and 3 correspondingly. (ESI†, Fig. S5). In the SDS-PAGE pictures of Gel electrophoresis experiments (Fig. 1c and Fig. S5b), new spots with higher apparent molecular weight further confirmed the successful conjugation between CTP and proteins upon light irradiation. All above results underlines the effective photorelease of 7-amino-coumarin-uncaged thiols and the following facile thiol-maleimide conjugation with proteins.

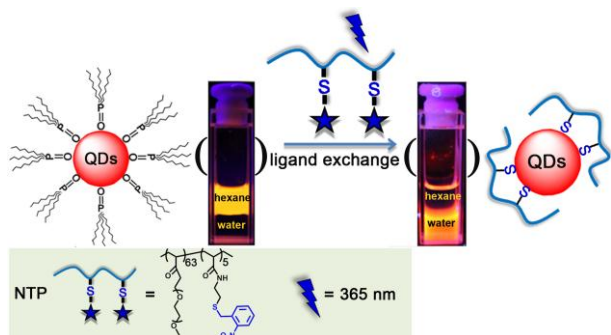


Fig. 2 Schematic representation of NTP transferring QDs from oil-phase to water-phase by ligand exchange upon 365 nm irradiation.

As known, thiol groups exhibit stronger affinity to semiconductor nanocrystal surfaces. Especially, ligands with multiple thiol anchoring groups greatly enhance the QDs stability over a wide range of biological conditions compared to monothiol-contained ligands.¹² However, multithiol-contained ligands are difficult to be prepared and stored because of the tendency of disulfide bonds formation. Thus, the *in situ* photorelease of multithiols would solve this problem. Herein, o-nitrobenzyl-caged multithiols polymer (NTP, M_n= 21636 g mol⁻¹, PDI= 1.67) was synthesized based on the copolymerization of o-nitrobenzyl thioether methylacrylamide and PEG-MA (M_n= 300 g mol⁻¹) monomers with the molar ratio of 1:12.6. It was used to capture TOPO-capped QDs from the oil-phase with the *in situ* photoreleased multithiols ligand upon irradiation, to finally obtained QDs-polymer conjugates (as shown in Fig. 2). The

efficient photolysis of o-nitrobenzyl phototrigger and release of active thiols were confirmed by the decreased and even disappeared absorption of NTP at 260 nm (ESI†, Fig. S6) and Ellman test with the number of the photoreleasable multithiols in one NTP is about 4.1. In the experiment, the capture of QDs from hexane to water was completed in 5 min upon 365 nm irradiation with no visible QDs orange fluorescence in hexane. The final obtained QDs in water phase was quite stable over a broad pH range and in the presence of a large excess of NaCl, and the quantum yield was comparable to the original TOPO-QDs (ESI†).

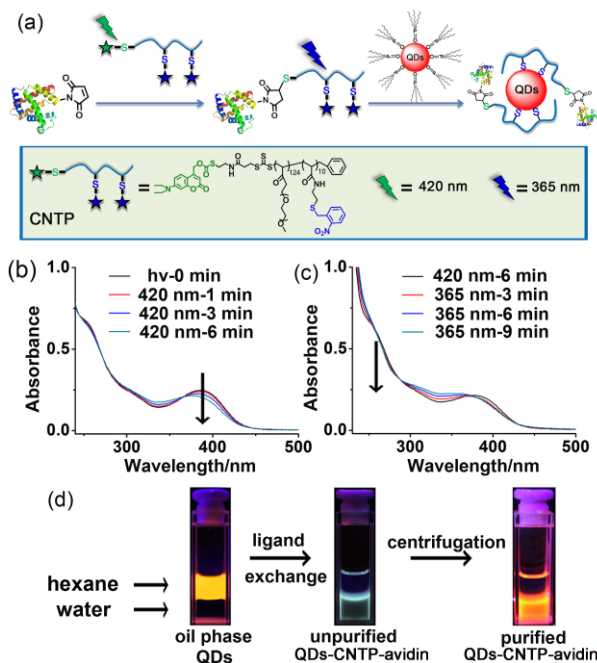


Fig. 3 (a) Schematic representation of sequential photorelease of thiols and bioconjugations with protein and QDs in one-pot process. (b-c) UV-vis spectra of CNTP (PBS, pH=7.4) upon 420 nm irradiation and followed 365 nm irradiation. (d) Pictures are TOPO-QDs, unpurified QDs-CNTP-avidin and purified QDs-CNTP-avidin after centrifugation (from left to right).

Moreover, the different excitation of above phototriggers, coumarin at 420 nm and nitrobenzyl at 365 nm respectively, offered a possibility to realize wavelength-selective photodeprotection, thus generated different thiols to bioconjugate with different functional materials in one-pot process. Here, Mal-avidin and QDs were selected to continue to be the samples for the sequential conjugations for facile preparation of QDs-polymer-protein conjugates. As shown in Fig. 3a, a well-defined block hydrophilic polymer (CNTP, M_n= 41832 g mol⁻¹, PDI= 1.15) was designed in which 7-amino-coumarin and o-nitrobenzyl phototriggers were used to protect the end- and pendant-thiols correspondingly. In order to demonstrate the wavelength-selective photodeprotection between 7-amino-coumarin and o-nitrobenzyl phototriggers in CNTP, UV-vis spectroscopy was utilized to monitor its photolytic process. As shown in Fig. 3b, when excited at 420 nm, the absorption of 7-amino-coumarin phototrigger was decreased and generated a blue shift whereas the absorption of o-nitrobenzyl phototrigger at 260 nm was remained. It indicated that 7-amino-coumarin phototrigger occurred photocleavage upon 420 nm irradiation, while all o-nitrobenzyl

cages kept intact for its weak absorption in this wavelength region. Sequentially excited at 365 nm, the absorption at 260 nm became decreased even disappeared (Fig. 3c), suggesting the allowable photocleavage of o-nitrobenzyl phototrigger.

This excellent wavelength-controlled photocleavage of CNTP provided the prerequisite for selective liberation of the end- and pendant-thiol groups. Upon 420 nm irradiation, CNTP photoreleased the end-thiol to conjugate with Mal-avidin firstly, followed by the sequentially photorelease of pendant-multithiols upon 365 nm irradiation to capture QDs from the oil phase by ligand exchange. All the procedures for QDs-polymer-protein (QDs-CNTP-avidin) bioconjugate were simple and in one-pot process without any additional purification. The final obtained QDs-CNTP-avidin in water phase showed blue emission as shown in Fig. 3d, attributing to the photolysis side-product hydroxyl-coumarin, unreacted raft polymer, and so on. After purification by centrifugation, the water-phase QDs finally recovered pure orange fluorescence with comparable quantum yield (ESI†).

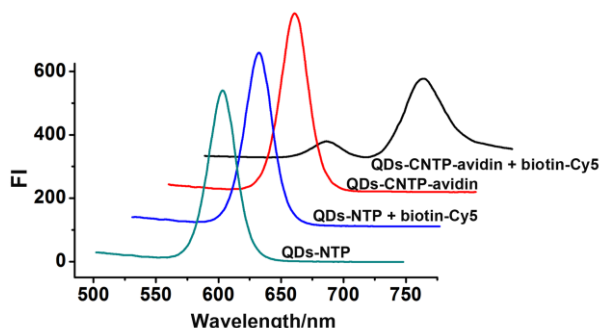


Fig. 4 Activity detection of the labelled avidin in QDs-CNTP-avidin bioconjugate using fluorescence spectrum in PBS.

To verify the successful bioconjugation, the activity of the labelled protein in QDs-CNTP-avidin conjugate was further explored by fluorescence resonance energy transfer (FRET) induced fluorescence quenching between QDs-CNTP-avidin and biotin-Cy5 because of strong biotin-avidin association constant.¹³ As shown in Fig. 4, only the fluorescence spectra of QDs-CNTP-avidin with the addition of biotin-Cy5 showed quenched emission of QDs and strong emission of Cy5 induced by FRET mechanism between QDs and Cy5 upon excitation at 440 nm. This hinted the successful bioconjugation of avidin with QDs and the mild operation condition of our photoreleasable thiol chemistry kept the bioactivity of the protein effectively.

In summary, we have demonstrated a facile method to photorelease free active-thiols *in situ* by using the well-known 7-amino-coumarin and o-nitrobenzyl phototriggers. We not only demonstrated the single photodeprotection of thiols to conjugate with protein and QDs, respectively, but also achieved selective liberation of different thiols to conjugate QDs and protein simultaneously by wavelength-selective photolysis. We envision this photoreleasable thiol chemistry may lead to a broad spectrum of studies which can be applied in variety thiol-modified systems.

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† Electronic Supplementary Information (ESI) available: Experimental details and characterization, See DOI: 10.1039/b000000x/

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