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Sequential and parallel dual labeling of nanoparticles using click chemistry

Hong Zong [a,*], Sascha N. Goonewardena [a,b], Huai-Ning Chang [a], James B. Otis [a], James R. Baker Jr. [a,*]

[a] Michigan Nanotechnology Institute for Medicine and Biological Sciences, United States
[b] Division of Cardiovascular Medicine, Department of Internal Medicine, University of Michigan, United States

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ABSTRACT

Bioorthogonal 'click' reactions have recently emerged as promising tools for chemistry and biological applications. By using a combination of two different 'click' reactions, 'double-click' strategies have been developed to attach multiple labels onto biomacromolecules. These strategies require multi-step modifications of the biomacromolecules that can lead to heterogeneity in the final conjugates. Herein, we report the synthesis and characterization of a set of three trifunctional linkers. The linkers having alkyne and cyclooctyne moieties that are capable of participating in sequential copper(I)-catalyzed and copper-free cycloaddition reactions with azides. We have also prepared a linker comprised of an alkyne and a 1,2,4,5-terazine moiety that allows for simultaneous cycloaddition reactions with azides and *trans*-cyclooctenes, respectively. These linkers can be attached to synthetic or biological macromolecules to create a platform capable of sequential or parallel 'double-click' labeling in biological systems. We show this potential using a generation 5 (G5) polyamidoamine (PAMAM) dendrimer in combination with the clickable linkers. The dendrimers were successfully modified with these linkers and we demonstrate both sequential and parallel 'double-click' labeling with fluorescent reporters. We anticipate that these linkers will have a variety of application including molecular imaging and monitoring of macromolecule interactions in biological systems.

1. Introduction

Selective chemical labeling of biomacromolecules, such as proteins, nucleic acids, and lipids is an important goal in basic research, biotechnology, and clinical medicine. This goal requires chemical reactions with high specificity, efficient conversion, and mild reaction conditions compatible with biological matrices.[1] Bioorthogonal 'click' reactions have provided promising tools to tag biomolecules that overcome many of the existing chemical labeling limitations.[2–6] The most widely used 'click' reaction is the copper(I)-catalyzed azide and alkyne [3+2] cycloaddition (CuAAC).[2] The rarity and inertness of azides and alkynes in biological environments allows for selective labeling without interference from elements in the biological environment. Additionally, Bertozzi et al. have developed a strain-promoted alkyne–azide cycloaddition reaction (SPAAC), which allowed the use of this reaction in living systems because no cytotoxic catalytic Cu(I) is required.[7–12] Another intriguing bioorthogonal reaction using a similar concept, but employing tetrazines and strained *trans*-cyclooctenes (TCO) for inverse electron demand Diels–Alder reaction, has recently emerged. This reaction has gained popularity due to the extremely fast cycloaddition kinetics allowing modifications of biomacromolecules at extremely low concentrations.[13–19]

With the development of multiple bioorthogonal reactions, one could perform two or more reactions in sequentially or in parallel in biological systems. Labeling biomacromolecules often requires introducing multiple labels onto the same molecules.[20–22] For example, modular labeling of DNA sequences with multiple labels is very important for DNA-based molecular diagnostics and for nanotechnology applications.[23–26] Current dual labeling procedures are cumbersome involving multiple coupling reactions, and typically require protection and deprotection steps. Additionally, this strategy frequently suffers from low coupling efficiencies, making the downstream purification process more challenging.[27,28] These difficulties necessitate more efficient labeling protocols, especially when the incorporation of multiple labels is required.

Over the past few years, progress has been made toward this end in 'double-click' labeling of biomacromolecules using a combination of two 'click' reactions SPAAC and CuAAC in a sequential

* Corresponding authors.
E-mail addresses: hzong@med.umich.edu (H. Zong), jbakerjr@med.umich.edu (J.R. Baker Jr.).

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manner.[22,29] Two different labels can be sequentially attached onto biomacromolecules, with the first labeling step being catalyst-free and the second step requiring Cu(I) as a catalyst. Another 'double-click' labeling strategy using a similar concept, but employing different ligation pairs (tetrazine–TCO and azide–cyclooctyne) has also been developed for simultaneous bioorthogonal labeling.[30] These strategies enable observation of multiple targets as well as fluorescence resonance energy transfer (FRET) applications, by using the appropriate pair of fluorescent labels. The 'double-click' approach can also be very advantageous in the fabrication of nanomaterials where the controlled immobilization of multiple ligands is needed.[31,32]

Despite these advances, the 'double-click' strategy still has drawbacks that limit its utility, especially when the conjugation of two or more labels in controlled ratios is required on the same biomacromolecule.[33] The 'double-click' strategy requires two different 'click' ligands to be incorporated into the target biomacromolecules first, followed by 'click' reactions with corresponding ligation partners. Conjugating multiple ligands to macromolecules complicates the synthesis because of the additional synthetic steps and purifications, which in and of itself can be challenging. Furthermore, the traditional multi-step conjugation strategies can lead to heterogenous populations of macromolecules that differ in the number and ratios of ligands leading to inconsistent results.[34,35] Thus, a strategy to introduce multiple labels on to macromolecules with defined ratios preserving the specificity and efficiency of typical click reactions is an important goal.[35–38]

To address these issues, we have used a trivalent triazine molecule as a linker scaffold.[35] Our approach uses a triazine molecule as a trifunctional linker on which two sites are attached with different 'click' ligand pairs (alkyne–cyclooctyne or alkyne–tetrazine) in a 1:1 ratio while a hydroxyl or carboxyl ligand is incorporated to its third functional site. These tri-substituted triazines serve as a small-molecule scaffold modified with 'click' ligands in defined ratios and controlled spatial topology which can then be attached to biomacromolecules through a one-step coupling reaction using the hydroxyl/carboxyl groups creating a platform for sequential or parallel 'double-click' labeling of biomacromolecules.

Herein, we report an efficient synthetic strategy to prepare sequential and parallel 'double-click' trifunctional linkers. Attaching these linkers onto the biomacromolecules will allow dual labeling with fluorescence reporters that can be then used to monitor biomacromolecules trafficking or interactions with other biomacromolecules in biological systems. We used polyamidoamine (PAMAM) dendrimers as model biomacromolecules because of their well-defined structure, similarity to endogenous biomacromolecules, and the possession of a large number of reactive surface functional groups.[39–43] As a proof of concept, we attached the 'double-click' linkers onto a folate-targeted G5 PAMAM dendrimer and demonstrated the feasibility of sequential and parallel dual labeling on the dendrimer using fluorescent reporters. We anticipate that these linkers and this approach will have a variety of application including molecular imaging and monitoring of macromolecule interactions in biological systems.

2. Materials and methods

2.1. General information

^{1}H NMR spectra were obtained using a Varian Inova 500 MHz spectrometer. Matrix-assisted laser desorption ionization time-of-flight mass spectra (MALDI-TOF-MS) were recorded on a PE Biosystems Voyager System 6050, using 2,5-dihydroxybenzoic acid (DHB) as the matrix. Electrospray ionization mass spectra (ESI-MS) was recorded using a Micromass Quattro II Electronic HPLC/MS/MS mass spectrometer. Fluorescence studies were performed on a Jobin Yvon FluoroMax-2 fluorimeter. Analytical ultra-performance liquid chromatography (UPLC) was performed on a Waters Acquity Peptide Mapping System equipped with a photodiode array detector and an Acquity BEH C4 column (100 × 2.1 mm, 1.7 μm) at a flow rate of 0.21 mL/min. Preparative HPLC was performed on a Waters Delta 600 system equipped with a 2996 photodiode array detector, an auto sampler, and an Atlantis Prep T3 column (250 × 10 mm) at a flow rate of 4.0 mL/min.

2.2. Materials

All solvents and chemicals were of reagent grade quality, purchased from Sigma–Aldrich (St. Louis, MO), and used without further purification unless otherwise noted. Cyclooctyne NHS ester was purchased from Berry & Associates (Dexter, MI). *trans*-Cyclooctene amine (TCO–NH_2) was purchased from KeraFAST (Boston, MA). Thin-layer chromatography (TLC) and column chromatography were performed with 25 DC-Plastikfolien Kieselgel 60 F254 (Merck), and Baxter silica gel 60 Å (230–400 mesh), respectively.

2.3. Synthesis

tert-Butyl ((6-methyl-1,2,4,5-tetrazin-3-yl)methyl)carbamate **9**,[44] G5-NHAc-FA **12**,[45] 3-azido-7-hydroxycoumarin **15**,[46] and fluorescein-azide **17**[4] were synthesized according to the literature.

2.3.1. Synthesis of *N*-(but-3-yn-1-yl)-4,6-dichloro-1,3,5-triazin-2-amine (Triazine-Alkyne) (1)

To a solution of cyanuric chloride (5.34 g, 0.029 mol) in acetone (30 mL) in an ice-water bath was added diisopropylethylamine (DIPEA) (3.75 g, 0.029 mol). 1-amino-butyne (1.00 g, 0.014 mol) in acetone (40 mL) was added slowly over 2 h. The reaction was stirred at room temperature overnight. The solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2, washed with water, dried over Na_2SO_4, and rotary evaporated. The resulting residue was purified by column chromatography on silica gel (eluent CH_2Cl_2) to give **1** as a white solid (1.40 g, 45%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.04 (t, J = 7.5 Hz, 1H), 2.53 (td, $J1$ = 6.5 Hz, $J2$ = 2.5 Hz, 2H), 3.36 (q, J = 6.5 Hz, 2H), 6.77 (m, 1H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 19.0, 40.0, 71.0, 80.3, 165.9, 169.9, 171.1; ESI-MS m/z 217.1 ($M+H^+$) calcd for $C_7H_6Cl_2N_4$ 217.0.

2.3.2. Synthesis of 2-((4-(but-3-yn-1-ylamino)-6-chloro-1,3,5-triazin-2-yl)amino)ethanol (Triazine-Alkyne-OH) (2)

Ethanolamine (1.28 g, 20.1 mmol) in acetone (10 mL) was added to a solution of **1** (1.04 g, 4.8 mmol) in acetone (10 mL). The reaction was stirred at room temperature for 48 h. Acetone was removed by rotary evaporation and the residue was suspended in 30 mL H_2O/CH_2Cl_2 (50:50). The mixture was filtered off. The solid collected was washed successively with CH_2Cl_2 and H_2O, and dried under reduced pressure to obtain **2** as a white solid (0.96 g, 83%). The product was sufficiently pure for further reactions: ^{1}H NMR (500 MHz, d_6-DMSO) 2.37 (m, 2H), 2.83 (m, 1H), 3.25–3.37 (m, 4H), 3.46 (m, 2H), 4.68 (m, 1H), 7.67–7.94 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 13.9, 18.4, 43.0, 59.4, 72.3, 82.1, 165.1, 165.4, 167.6; ESI-MS m/z 242.1 ($M+H^+$) calcd for $C_9H_{13}ClN_5O$ 242.1.

2.3.3. Synthesis of *tert*-butyl (2-(2-(2-((4-(but-3-yn-1-ylamino)-6-((2-hydroxyethyl)amino)-1,3,5-triazin-2-yl)amino)ethoxy)-ethoxy)ethyl)carbamate (Triazine-Alkyne-OH-NHBoc) (3)

DIPEA (161 mg, 1.24 mmol), *tert*-butyl 2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate (308 mg, 1.24 mmol), and Triazine-Alkyne-OH **2** (150 mg, 0.62 mmol) were dissolved in THF (5 mL).