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Site-specific *in vivo* bioorthogonal ligation via chemical modulation

Prof. Heebeom Koo,

Wellman Center for Photomedicine, Massachusetts General Hospital and Harvard Medical School, 65 Landsdowne St., UP-5, Cambridge, MA 02139, USA. Department of Medical Lifescience, College of Medicine, The Catholic University of Korea, Seoul 06591, South Korea

Jeong Heon Lee,

Gordon Center for Medical Imaging, Division of Nuclear Medicine and Molecular Imaging, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114

Kai Bao,

Gordon Center for Medical Imaging, Division of Nuclear Medicine and Molecular Imaging, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114

Dr. Yunshan Wu,

Department of Chemistry, Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA 30303

Prof. Georges El Fakhri,

Gordon Center for Medical Imaging, Division of Nuclear Medicine and Molecular Imaging, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114

Prof. Maged Henary,

Department of Chemistry, Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA 30303

Prof. Seok Hyun Yun,

Wellman Center for Photomedicine, Massachusetts General Hospital and Harvard Medical School, 65 Landsdowne St., UP-5, Cambridge, MA 02139, USA

Prof. Hak Soo Choi, and

Gordon Center for Medical Imaging, Division of Nuclear Medicine and Molecular Imaging, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114

G. Park

Gordon Center for Medical Imaging, Division of Nuclear Medicine and Molecular Imaging, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114

Correspondence to: Maged Henary; Seok Hyun Yun; Hak Soo Choi.

Abstract

A critical limitation of bioorthogonal click chemistry for *in vivo* applications has been its low reaction efficiency due to the pharmacokinetic barriers, such as blood distribution, circulation, and elimination in living organisms. To identify key factors that dominate the efficiency of click chemistry, here we propose a rational design of near-infrared fluorophores containing tetrazine as a click moiety. Using trans-cyclooctene-modified cells in live mice, we found that the *in vivo* click chemistry could be improved by subtle changes in lipophilicity and surface charges of intravenously administered moieties. By controlling pharmacokinetics, biodistribution, and clearance of click moieties, we prove that the chemical structure dominates the fate of *in vivo* click ligation.

Graphical abstract

“Click” in the body: Subtle changes in chemical structures result in large variations in the click efficiency *in vivo*. Physicochemical properties and pharmacokinetics of small molecules dictate the performance of bioorthogonal click chemistry, which was proved using near-infrared fluorescence imaging in live animal models.

Keywords

click chemistry; pharmacokinetics; near-infrared imaging; real-time imaging; targeted contrast agent

1. Introduction

Copper-free click chemistry is ideal for *in vivo* bioorthogonal reactions due to its fast and facile chemical process with little cellular toxicity.^[1] Despite its potential in bioimaging and nanomedicine including optical imaging,^[2] PET,^[3] SPECT,^[4] ultrasound imaging,^[5] and drug delivery,^[6] *in vivo* click chemistry in the past decade has suffered from relatively low reaction efficiency. This problem has been attributed in part to the binding of serum proteins to click components. In addition, the pharmacokinetic barriers, such as blood distribution, circulation, and elimination as well as biodistribution and clearance, make it difficult for the click moieties to reach a target.^[7] To improve the click efficiency *in vivo*, the Weissleder group introduced large molecular weight polymers, which increased the circulation time of click moieties in the blood vessels and eventually improved click reaction efficiency.^[7] Although polymer-modified tetrazine (Tz) components facilitated the cycloaddition reaction with the counterpart *trans*-cyclooctene (TCO) by giving enough reaction time at high

concentration, this approach could result in high background retention and limited transportation through the physiological barriers (e.g., blood-brain/nerve barriers and endothelial/intestinal walls).^[8] Thus, small molecules are still attractive for *in vivo* reactions due to their rapid secretion rates and low potential side effects.^[9] However, the key factors regulating *in vivo* click chemistry of small molecules have not been fully understood.

Previously, we have studied the pharmacokinetics of nanoparticles and small molecules in various animal models, and defined the physicochemical properties such as size, surface charge, and lipophilicity that play a key role in targeting and imaging.^[10] Very recently, we have developed a novel concept of targeted contrast agents (i.e., structure-inherent targeting), where the inherent chemical structure governs the fate of an injected molecule and the ultimate targeting to specific organs such as thyroid/parathyroid glands,^[10] bone,^[11] and cartilage.^[12] Using this concept, we could achieve significantly reduced background tissue retention and nonspecific uptake in the reticuloendothelial system (RES), and thus improve the signal-to-background ratio (SBR) of the target.^[10,13] Armed with this strategy, we hypothesized that the efficacy of bioorthogonal chemistry *in vivo* would be largely dependent on the inherent chemical structure of systemically administered molecules, where biodistribution and pharmacokinetics need to be controlled.

Herein, we describe a series of novel pentamethine near-infrared (NIR) fluorophores composed of three main domains: 1) Tz for click chemistry, 2) pentamethine core for NIR fluorescence imaging, and 3) variable charges for pharmacokinetic modulation (Figure 1). The Tz-containing NIR fluorophores were rationally designed to have assorted side groups (R^1 and R^2) with varying charges by two, resulting in diverse lipophilicity (Supporting Information, Figure S1-S3). We selected these structurally diverse Tz moieties to investigate the reaction efficiency of *in vivo* click chemistry with TCO presenting on the cell surface.

2. Results and discussion

We modified surface charges of the pentamethine core to obtain the final Tz-fluorophores with distinctive physicochemical properties such as logD at pH 7.4 (distribution coefficient), TPSA (topological polar surface area), HBA (hydrogen bond acceptor), and pKa (acid dissociation constant). Compounds **4** with assorted charges were conjugated to amine-functionalized Tz through the conventional *N*-hydroxysuccimide (NHS) chemistry, and the final products were characterized by LC-MS (Supporting Information, Figure S4). Based on the total net charges, we named each fluorophore as Tz-3, Tz-1, Tz+1, and Tz+3 (Figure 1b). The maximum absorption and emission wavelengths are in the NIR window (Supporting Information, Figure S5), where the combined absorption of NIR light and water/hemoglobin is minimal in addition to low autofluorescence.^[14] Physicochemical properties were calculated based on the final chemical structure using MarvinSketch 6.1.4 (ChemAxon), and optical properties were measured by using spectrophotometers (Ocean Optics). As shown in Figure 1c, logD values of Tz-fluorophores increase in the order of Tz+3 < Tz-3 < Tz+1 < Tz-1, representing their lipophilicity under physiological conditions. However, both TPSA and HBA values showed a different trend for Tz+3 < Tz+1 = Tz-1 < Tz-3 because the sulfonate group has a high electron density and multiple sites for hydrogen bonding. Acidic pKa values represent that sulfonates are deprotonated under the

physiological condition. The maximum excitation and emission wavelengths of all 4 Tz-fluorophores were similar in the NIR window. However, the introduction of different surface charges resulted in varying extinction coefficients and quantum yields due to the altered resonance of electrons in the pentamethine core (Figure 1c). To minimize the difference in molecular brightness, we adjusted the imaging exposure time for individual images based on the brightness obtained from *in vitro* and *in vivo* studies.

To validate the click efficiency *in vitro*, we treated B16F10 murine melanoma cells with TCO-PEG₄-NHS resulting in TCO-modified cells by conjugating NHS esters to the amines of proteins on the cell surface.^[15] Then, we treated each Tz-fluorophore to those TCO-modified cells at a concentration of 2 μ M in growth media. After 10 min incubation at 37 °C, all Tz-fluorophores exhibited over 15-fold higher fluorescence signals in TCO-modified cells compared with control cells where no TCO groups exist on the cell surface (Figure 2a).^[16] Although surface charges and lipophilicity cause immediate serum protein binding in cellular media and in the body, positively charged Tz+3 showed the fastest click reaction due to the thermodynamically favorable interactions between the cationic charges of Tz+3 and negatively charged cell surface. On the other hand, Tz-1 representing high lipophilicity (logD at pH 7.4 = 3.0) exhibited a relatively slow Tz-TCO reaction due to the low reaction concentration of fluorophores resulted from microaggregation. Tz-1, however, reached its steady state (V_{max}) similar to the others at 10 min post-incubation, which represents the highly efficient click reactivity of Tz-fluorophores against TCO groups (Figure 2b). Interestingly, the reaction rate of Tz-1 was enhanced in serum-free media because of enriched binding of ligands to the cell surface without serum protein competition (Supporting Information, Figure S6).

Then, we evaluated how the altered chemical structure of fluorophores affects the pharmacokinetics, blood circulation, and biodistribution *in vivo*. 10 nmol of each fluorophore was injected intravenously into C57BL/6J mice while exposing abdominal wall and major organs under the surgical field, and the biodistribution and clearance of each fluorophore were observed by an intraoperative imaging system^[10] for 4 h while collecting blood samples using capillary tubes (Figure 3a). Given the pharmacokinetics of Tz-fluorophores, all molecules distributed rapidly in the bloodstream, but cleared differently from the major organs. As shown in Figure 3b, the distribution half-life ($t_{1/2\alpha}$) of Tz-3 and Tz+3 was about 3-4 fold longer than that of Tz-1 and Tz+1. It shows that blood circulation is closely related to the physicochemical properties of each injected fluorophore, especially the surface charge and logD values as described in Figure 1.^[17,18] The terminal half-life ($t_{1/2\beta}$) of Tz-fluorophores followed a slightly different trend, showing a wide range of values depending on the lipophilicity. Interestingly, the $t_{1/2\beta}$ of Tz-1 is about 10-fold longer than the others, mainly because the lipophilic character promotes spontaneous binding to serum proteins, which drives the dye-protein complex mostly into the hepatobiliary clearance route (Figure 3c and Figure S7 in Supporting Information).^[17] The rest of Tz-fluorophores followed both renal and hepatobiliary excretion as depicted in the biodistribution and clearance data observed by real-time intraoperative fluorescence imaging (Supporting Information, Figure S7). The other physiological and pharmacokinetic factors such as the area under the plasma concentration-time curve (AUC), volume of distribution (V_d), and plasma clearance were dependent on blood half-life values, although their extravasation rates

are considered to be similar.^[19] However, the enhanced AUC and V_d of Tz-1 were resulted from the high logD value (3.0 at pH 7.4), which contributed to tissue penetration, especially to the liver. The logD values of Tz-3 and Tz+3 are similar (-2.7 vs. -3.2 at pH 7.4) and thus showed similar biodistribution, while Tz+3 with cationic charges required a longer elimination time compared to Tz-3 due to the delayed secretion caused by nonspecific ionic adhesion to the negatively charged cell surface.^[20]

We observed a similar trend in the resected tissues from the mice sacrificed at 4 h post-injection of Tz-fluorophores, which was quantified by the SBR of each major organ against surrounding muscle tissue (Figure 3d and Figure S8). Tz-1 accumulated in liver and duodenum resulting in slow elimination from the body, while the rest of molecules majorly excreted by the kidneys and exhibited relatively fast excretion into bladder.

Finally, we determined the effect of the physicochemical properties and pharmacokinetics on the efficiency of click chemistry *in vivo*. We prepared TCO-modified B16F10 cells as described above and administered into syngeneic C57BL/6J mice intravenously to deposit them in lung capillaries, as previously reported.^[21] After 30 min, we injected each Tz-fluorophore to those TCO-presenting mice intravenously. After 4 h post-injection of Tz-fluorophores, animals were sacrificed and lung tissue was harvested to analyze the efficiency of *in vivo* click reaction by comparing fluorescence signals at 700 nm (NIR #1). Since cells were pre-labeled with an 800 nm emitting lipophilic fluorophore CTNF126 (NIR #2), the signals from Tz-fluorophores could be normalized based on the 800 nm emitting cell signals to minimize the potential errors resulting from different cell numbers deposited in the lung. Notably, Tz-fluorophores represented significantly higher signals in the lungs of TCO presenting mice compared to control mice administered with bare cells treated without TCO groups (Figure 4a).

The SBR of Tz-1 and Tz+1 were relatively low (1.42 and 1.09, respectively), which represents less efficient *in vivo* click chemistry (Figure 4b). This could be because of short $t_{1/2\alpha}$ and insufficient contact time of Tz-1 and Tz+1 fluorophores with the TCO-modified cells in lungs, resulted from rapid nonspecific uptake into liver (Tz-1) and kidneys (Tz+1) during the systemic circulation. In contrast, highly hydrophilic Tz-3 and Tz+3 exhibited high SBR (2.53 and 4.24, respectively) in the lung, representing favorably enhanced click reactions *in vivo*. These results emphasize the importance of defining the distribution half-life as a critical pharmacokinetic parameter, which reflects the amount of Tz-fluorophores reaching the target cells in a reliable manner (Figure 4c).

In addition, the reduced lipophilicity and longer circulation contributed significantly to the molecular interactions *in vivo* as reported previously.^[18,22] Thus, we proved that the small molecule click moieties with optimized molecular properties could enhance bioorthogonal chemistry *in vivo* without using a large carrier (i.e., polymers or nanoparticles). Interestingly, Tz+3 outperformed Tz-3 with > 50% higher click reactivity *in vivo*, while exhibiting only 20% longer distribution half-life. This is conceivably because the multiple positive surface charges have a profound effect on cellular accessibility in the luminal and interstitial spaces, which increase the chances for bioorthogonal click reaction *in vivo*.^[23]

This result is in accordance with the previous report by Dearling and Packard where Cu-chelators with different surface charges changed their biodistribution significantly.^[24]

The reaction rate between the two chemicals, especially in the intravenous injection, is important because the contact time is limited by blood flow and/or active secretion processes in the body. The reaction rate constant of original azide-alkyne click chemistry has been reported to be about $10\text{--}100\text{ M}^{-1}\text{s}^{-1}$ (k_{obs}) per $10\text{--}100\text{ }\mu\text{M}$ Cu(I) catalyst (Table 1).^[25] Strained alkynes without copper showed about 1,000-fold slower click reaction ($1\text{--}140 \times 10^{-3}\text{ M}^{-1}\text{s}^{-1}$).^[26] while the Tz-TCO reaction has been reported as fast as $6 \times 10^3\text{ M}^{-1}\text{s}^{-1}$,^[27] which is one of the fastest copper-free click reactions reported in literature. However, considering the association rate constant of biological ligands to their receptors (e.g., Nimotuzumab and epidermal growth factor receptor, $k_{\text{on}} = 5.2 \times 10^4\text{ M}^{-1}\text{s}^{-1}$),^[28] click chemistry is still slower than other biological binding processes.

Therefore, the longer circulation time of click moieties is particularly important to enhance the efficiency of *in vivo* click reactions. In addition, the combination of cationic charges and low logD (non-lipophilicity) of intravenously injected ligands played the critical role to improve surface click chemistry. However, it should be noted that other target models and biological ligands could be dependent on the correlative response to the distribution half-life, where the click moieties need to be re-optimized to fit into the particular model system. For instance, zwitterionic or anionic charged small molecules show relatively short terminal half-life values and high volume of distribution due to the limited tissue penetration, which might be better candidates for peripheral tissue targets with considering other physiological and pharmacokinetic factors preferentially.

3. Conclusion

In summary, we have identified that surface charges and lipophilicity of injected molecules play the key role to achieve high reaction efficiency in bioorthogonal click chemistry. Despite the differences in physicochemical properties, all 4 Tz-fluorophores offered preferentially fast click reactions with TCO-modified cells *in vitro* within 10 min in biological media. However, their pharmacokinetics and biodistribution in animals were dependent upon the chemical structure, which governed the final click efficiency *in vivo*. These newly developed Tz-fluorophores can be used to label various biomaterials including nucleotide, protein, cells, and nanoparticles to control site-specific click reaction. Considering the applicability of copper-free click chemistry, the rationally designed chemical structures to control pharmacokinetics and biodistribution will lay the foundation of new-targeted contrast agents for optical, magnetic resonance, and nuclear imaging.

4. Experimental Section

Synthesis and characterization of Tz-fluorophore

Tz-amine ((4-(1,2,4,5-tetrazin-3-yl)phenyl)methanamine hydrochloride) was purchased from Sigma-Aldrich (St. Louis, MO). TCO-PEG₄-NHS (*trans*-cyclooctene-polyethylene glycol₄-*N*-hydroxysuccinimide ester) was purchased from Clickchemistrytools (Scottsdale, AZ). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA), Sigma-Aldrich

(Saint Louis, MO) or Acros Organics (Morris Plains, NJ). Pentamethine fluorophores with assorted side groups were synthesized first, and they were conjugated with Tz-amine. Briefly, indols and bromides with different side groups were conjugated each other, and they were conjugated with Vilsmeier-Haack reagent to form pentamethine core. Then, 4-(2-carboxyethyl)phenylboronic acid were also conjugated to the intermediate to obtain additional acid group for further modification. Finally, the compounds were conjugated with Tz-amine through the conventional *N*-hydroxysuccinimide (NHS) chemistry. See Supporting Information for detailed chemical syntheses and analysis.

In vitro binding assay of Tz-fluorophores to TCO-modified cells

B16F10 murine melanoma cells were purchased from ATCC (Manassas, VA). Cells were maintained in DMEM (Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) at 37 °C in a humidified 5% CO₂ atmosphere. B16F10 cells were seeded onto 4-chamber glass bottom dish at a density of 1×10^5 cells per chamber in 1 ml full growth media. After overnight incubation, they were incubated with TCO-PEG₄-NHS (20 µM in 1 ml PBS containing Ca²⁺ and Mg²⁺) for 30 min, followed by washing with PBS (pH 7.4) twice, they were further incubated with Tz-fluorophores (2 µM in 1 ml full growth media) and Hoechst 33342 (Invitrogen, Carlsbad, CA) for varied time periods. Cellular images were obtained using a FV1000 multi-photon confocal microscope (Olympus, Tokyo, Japan) equipped with Laser Diode (405 and 633 nm) after washing and fixation with 2% formaldehyde solution for 5 min.

In vivo biodistribution and pharmacokinetics of Tz-fluorophores

Animals were housed in an AAALAC-certified facility and were studied under the supervision of BIDMC IACUC in accordance with the approved institutional protocol (#057-2014). 6 weeks old C57BL/6 mice (male; 20-25 g) were purchased from Charles River Laboratories (Wilmington, MA). Animals were anesthetized with 100 mg kg⁻¹ ketamine and 10 mg kg⁻¹ xylazine intraperitoneally (Webster Veterinary, Fort Devens, MA). 10 nmol of the Tz-fluorophores in saline were administered intravenously, and animals were imaged using our real-time intraoperative dual-NIR channel imaging system.^[10] Herein, 670 nm excitation light (1 mWcm⁻²) and 760 nm excitation light (4 mWcm⁻²) were used with white light (400–650 nm) at 40000 lux. Color and NIR fluorescence images were acquired simultaneously with custom software at rates of up to 15 Hz over a field of view with a diameter of 15 cm. For each experiment, camera exposure time and image normalization was held constant. To quantify the distribution ($t_{1/2\alpha}$) and elimination ($t_{1/2\beta}$) blood half-life values, intermittent sampling from the tail vein was performed over the 4 h period following a single intravenous administration. Approximately 10-20 µL of blood were collected using glass capillary tubes at the following time points: 0, 3, 5, 10, 30, 60, 120, and 240 min. The same intraoperative imaging system was used to measure the fluorescence intensity of each sample, and the concentration was calculated based on the image SBR using a standard curve for each fluorophore. Animals were injected with Tz-fluorophores 4 h prior to imaging, and major organs were resected, imaged, and the signal from each organ/tissue was quantified by measuring fluorescent intensity (photons s⁻¹cm⁻²sr⁻¹). At least 3 animals were

analyzed at each time point. Results were presented as mean $\pm$ s.d. and curve fitting was performed using Prism version 4.0a software (GraphPad, San Diego, CA).

In vivo click chemistry between Tz-fluorophores and TCO-modified cells

TCO-modified B16F10 cells were prepared as above and stained with an 800 nm emitting lipophilic cation CTNFI26 for 30 min incubation. After detachment of labeled cells with cell scraper, the TCO-modified cells (1×10^6 cells per mouse) were injected intravenously into C57BL/6 mice. 10 nmol of each Tz-fluorophore in saline were administered intravenously into the same mice 30 min postinjection of cells. Animals were sacrificed 4 h post-injection of Tz-fluorophores, and lungs were resected, imaged, and analyzed by using our custom-built imaging system.

Quantitative analysis

The fluorescence and background intensity of a region of interest over each tissue was quantified using custom imaging software and ImageJ v1.48 (NIH, Bethesda, MD). The signal-to-background ratio (SBR) was calculated as $SBR = \text{fluorescence}/\text{background}$, where background is the signal intensity of muscle. All NIR fluorescence images for a particular Tz-fluorophore were normalized identically for all conditions of an experiment. A one-way ANOVA followed by Tukey's multiple comparisons test were used to assess the statistical differences between multiple groups. A P value of less than 0.05 was considered significant: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Results are presented as mean $\pm$ standard deviation (s.d.).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Synthesis of Tz-containing NIR fluorophores to analyze the reaction efficiency of *in vivo* click chemistry on the TCO-modified cell surface. a) Composition of Tz-containing pentamethine fluorophores and their subsequent click reactions *in vivo*. b,c) Chemical structures (b) and physicochemical properties (c) of Tz-fluorophores.

Figure 2.

Click reaction between Tz-fluorophores and TCO-modified cells *in vitro*. (a) Confocal images of TCO-modified B16F10 cells post-incubation of Tz-fluorophores. Blue = DAPI; red = 700 nm NIR fluorescence; scale bar = 10 μm . (b) Kinetics of bioorthogonal Tz-TCO click reactions (n = 5). Relative fluorescence intensity (RFU) was normalized by the maximum signal at 10 min post-incubation.

Figure 3.

Pharmacokinetics and biodistribution of Tz-fluorophores in C57BL/6J mice: a,b) Time-concentration curves (a) and pharmacokinetic parameters (b) in blood. c,d) Intraoperative fluorescence images (c) and biodistribution (d) at 4 h post-injection (NIR #1: 700 nm). Abbreviations used are: Bl, bladder; Du, duodenum; He, heart; In, intestine; Ki, kidneys; Li, liver; Lu, lungs; Pa, pancreas; Sp, spleen. Scale bar = 1 cm. (n=3, mean $\pm$ s.d., ** $P < 0.01$, and *** $P < 0.001$).

Figure 4.

In vivo click chemistry of Tz-fluorophores against TCO-modified cells loaded in lungs. a) *Ex vivo* images of lung tissue excised 4 h post-injection of Tz-fluorophores (NIR #1: 700 nm, NIR #2: 800 nm). Scale bar = 1 cm. b,c) Distribution half-life (b) and *in vivo* click efficiency (c) of Tz-fluorophores. SBR represents the fluorescence signal from the lung tissue injected with TCO-modified cells divided by the signal from control cell-injected lung tissue without TCO.

Table 1

Reaction kinetics for typical click chemistry.

Click reaction	Functional groups	2nd order rate constants ($M^{-1}s^{-1}$)	Ref.
Copper-catalyzed azide-alkyne cycloaddition	Alkyne, azide with copper catalyst	10-100 per 10-100 μM Cu(I) catalyst	[25]
Strain-promoted azide-alkyne cycloaddition	Strained alkyne, azide	$1-140 \times 10^{-3}$	[26]
Inverse-demand Diels-Alder reaction	Trans-cyclooctene (TCO), tetrazine (Tz)	6×10^3	[27]