“Click Chip” Conjugation of Bifunctional Chelators to Biomolecules
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ABSTRACT: There is a growing demand for diagnostic procedures including in vivo tumor imaging. Radiometal-based imaging agents are advantageous for tumor imaging because radioisotopes (i) have a wide range of half-lives and (ii) are easily incorporated into imaging probes via a mild, rapid chelation event with a bifunctional chelator (BFC). Microfluidic platforms hold promise for synthesis of radiotracers because they can easily handle minute volumes, reduce consumption of expensive reagents, and minimize personnel exposure to radioactive compounds. Here we demonstrate the use of a “click chip” with an immobilized Cu(I) catalyst to facilitate the “click chemistry” conjugation of BFCs to biomolecules (BMs); a key step in the synthesis of radiometal-based imaging probes. The “click chip” was used to synthesize three different BM-BFC conjugates with minimal amounts of copper present in reaction solutions (~20 ppm), which reduces or obviates the need for a copper removal step. These initial results are promising for future endeavors of synthesizing radiometal-based imaging agents completely on chip.

INTRODUCTION

Molecular imaging, the noninvasive visualization of biochemical processes on the subcellular and cellular levels, is a powerful technique for early detection of diseases and drug discovery.1 Positron emission tomography (PET) and single-photon emission computed tomography (SPECT), are currently two widely used clinical molecular imaging modalities. PET and SPECT are based on detection of radioisotopes, typically attached to tumor-targeting molecules, which are known as “radiotracers”.2 Currently, for clinical oncology, cardiology, and neurology, one of the most widely utilized radiotracers is the PET tracer [18F]fluorodeoxyglucose ([18F]FDG).3 Yet, [18F]FDG and other 18F-based radiotracers have multiple shortcomings including a short half-life and lengthy and harsh reaction conditions.4 Clinically relevant radioisotopes, including 68Ga, 64Cu, and 89Zr, have half-lives ranging from roughly 1 h to a few days. A wide range of available half-lives enables production of radiotracers where the half-life of the radioisotope matches the in vivo pharmacokinetics of the radiotracer. For example, antibody-based radiotracers may require days to achieve appropriate signal-to-noise ratios for imaging, and therefore require a radioisotope with a long half-life. Radiometal incorporation into radiotracers is usually accomplished by covalently bonding a BFC to the targeting BM and chelating a radiometal to the BFC. This radiometalation event is typically rapid and accomplished under mild conditions.5 Covalent attachment of BFCs is usually accomplished via amide, thiourea, and thioether bonds.6 However, biomolecules tend to have multiple free primary amines and thiols making site-specific BFC attachment a challenge.7

The advent of “click chemistry”, defined as selective and rapid reactions that require mild conditions,8 has provided a new bioorthogonal approach to site-specific BFC attachment to BMs.9 A wide variety of “click reactions”, including Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC),10 strain-induced azide–alkyne cycloaddition,11 and Michael addition of thiols to maleimides12 have been utilized to link BFCs to BMs. CuAAC is particularly advantageous because the reaction is compatible with many functional groups13 and the formed triazole heterocycle mimics amide bonds that can also assist with metal chelation.13,14 However, key challenges for CuAAC chelator conjugation reactions are (i) the Cu(I) catalyst is cytotoxic,9 (ii) Cu(I) may chelate with BFCs, and (iii) copper ions combined with sodium ascorbate, typically used to maintain the copper catalyst in the Cu(I) oxidation state, form reactive oxygen species and byproducts that can damage biomolecules, particularly proteins.15–17 One convenient method to potentially avoid these complications is to immobilize the Cu(I) catalyst on a solid support.

In this paper we describe a microfluidic approach for using immobilized Cu(I) catalyst for CuAAC bifunctional chelator conjugation reactions with biomolecules. Only a minute amount (often nanogram levels) of radiotracer is required
for imaging in vivo, but traditionally radiotracers are synthesized on relatively large-scale automated synthesis modules that require at least 400 μL of solution for easy handling. Microreactors are promising platforms for radiotracer synthesis because they (i) enable handling of small volumes, (ii) provide precise control over reaction conditions, (iii) reduce consumption of expensive precursors, and (iv) minimize radiation shielding size and radiation exposure to personnel. To date, though, most microfluidic radiotracer research has focused on 18F-based probes, in particular, the optimization of fluorine incorporation. In prior work we and others have shown the advantages of microfluidics for radiolabeling peptides and antibodies with radiometals, and a recent report indicated the benefits of using microfluidics for radiotracer quality control analysis.

The microfluidic approach for conjugating a biomolecule to a bifunctional chelator developed here utilizes a thinner version of the “click chip” developed previously that features an immobilized Cu(I)-ligand complex. This unique approach integrates the advantages of microfluidics (e.g., reduced reagent consumption) and “click chemistry” (e.g., site-specific attachment) to yield BM-BFC conjugates. Additionally, an immobilized Cu(I)-ligand complex reduces complications associated with Cu(I) catalyst dissolved in solution. The platform was validated by synthesizing three different BM-BFC conjugates. Two of the conjugates consisted of the azide-modified peptide cyclo[Arg-Gly-Asp-D-Phe-Lys] (cRGDK-azide) conjugated to an alkyne derivative of 1,4,7,10-tetrazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) (Figure 1A) or 1,4,7-triazacyclononane-1,4-diacetic acid (NO2A) (Figure 1B). The third conjugate was comprised of the nucleoside 5-ethyl-2′-deoxyuridine (EdU) conjugated to an azide derivative of DOTA (Figure 1C).

RESULTS AND DISCUSSION

In this work, we use a thin “click chip” for conjugating BFCs and BMs as an elegant approach to eventually enable the multistep synthesis of radiotracers on chip starting from initial reagents. To clarify terminology used in the paper we utilized both Cu(I) and Cu(II) ions. Cu(I) is the active catalyst for CuAAC bioconjugation reactions. In this case, the Cu(I) catalyst was bound to a water-soluble Cu(I) ligand to form a Cu(I)-ligand complex. The Cu(I) ligand not only immobilizes Cu(I) catalyst, but maintains copper in the +1 oxidation state critical for CuAAC reactions. Note we also utilized Cu(II) ions in this work to chelate copper to BFCs in an effort to reduce loss of immobilized Cu(I) catalyst discussed in greater detail in subsequent sections.

Design and Fabrication of Thin “Click Chips”. The “click chip” for dye conjugation we reported previously was comprised of reservoirs containing posts to reduce reagent diffusion times to catalyst sites located on the inner chip walls. Chip features were patterned in polydimethylsiloxane (PDMS) using traditional soft lithography techniques and subsequently bonded to glass. PDMS and glass were used for the “click chip” fabrication because of the availability of well-established fabrication procedures and facile methods to immobilize compounds onto inner channel surfaces. In this work we utilize the same design, five reservoirs with posts, to provide ample solution volume for analysis. However, most nontherapeutic radiotracers are given at nanogram levels. Here, we demonstrate the synthesis of BFC-BM conjugates at roughly microgram levels (~100s μM concentrations) for accurate analysis utilizing a LC-MS setup with a UV detector. In addition to reducing reagent concentrations, the number of reservoirs can easily be adjusted to tune reactor volume without changing the immobilization process to produce the desired nanogram levels of radiotracer with little loss of expensive reagents.

However, to use the “click chip” approach with immobilized Cu(I) catalyst for the synthesis of radiotracers we needed to address the challenge of solvent loss resulting from long incubation times in traditional, thick (~3–5 mm) PDMS chips. The previous “click chip” was well suited for short reactions (~30–50 min), but “click” conjugation of BFCs to BMs at or near room temperature require longer incubation times (~12–18 h). Our approach to alleviating this solvent loss was to create a much thinner chip (Figure 2A) comprised of three layers, glass, PDMS, and cyclic olefin copolymer (COC) (Figure 2B). PDMS thickness was reduced from approximately 4 mm to 400 μm to minimize solvent loss into the bulk PDMS and through the sides of the microreactor. A 100-μm-thick layer of water impermeable COC was bonded onto the PDMS to minimize water loss through the top of the reactor. Thick PDMS was only present at inlets and outlets to support tubing used to inject fluids into the “click chip”.

Control experiments were performed to confirm that the solvent loss in thin COC/PDMS/glass chips was lower compared to traditional thick PDMS/glass chips. Both thin and thick microreactors were filled with water and incubated in humidity chambers at room temperature, 37 °C, or 47 °C for 12 h. Very little solvent loss occurred in either reactor when incubated at room temperature. However, thin chips lost less solvent (~6.58% ± 1.12%) compared to thick chips (19.7% ± 4.12%).
BFC and BM Conjugation Reactions. General Method for Conjugation Reactions. Prior to the reaction, a reagent mixture was created by mixing stock solutions of the appropriate BFC and BM together in a phosphate buffer. Then, the “click chip” with immobilized Cu(I) was rinsed and a portion of the BFC and BM solution was injected into the microreactor. The microreactor was placed in a preheated humidity chamber for the specified incubation time, and reaction samples were collected for LC-MS analysis. Following sample collection the chip was rinsed thoroughly with methanol and water. Then, more BFC and BM solution was injected into the microreactor and incubated in a humidity chamber again. The process of adding the reagent mixture, incubating, and collecting a sample was repeated until three samples were collected from each “click chip”. The Cu(I) solution (CuSO₄ and sodium ascorbate) was only injected prior to the first reaction. All conjugation reactions were performed on two separate “click chips”, so a total of six samples were collected for each set of conjugation reaction parameters.

LC-MS was utilized to determine the yields of the bioconjugation reactions. No further workup of reaction samples was performed prior to LC-MS. All yields discussed here were determined from crude reaction samples. Samples were collected from the chip and directly injected into the LC-MS system along with multiple dilutions of the initial reagents to establish a standard curve. Yields were calculated by comparing peak areas of the initial reagents before and after the reaction.

Unexpected tert-Butyl Protecting Group Loss. The initial “click chip” conjugation reaction tested was between propargyl-DOTA-tris(tert-butyl) ester and cRGDK-azide. The reagent mixture was incubated for 12 h at 47 °C. DOTA-cRGD product formed but two unexpected results occurred; (i) DOTA-alkyne lost tert-butyl (tBu) protecting groups and (ii) DOTA-alkyne chelated copper by removing Cu(I) from the Cu(I)-ligand complex. These two observations were true for both unreacted DOTA-alkyne and DOTA in the DOTA-cRGD product. Divalent cations have been utilized before to bind near esters in non-BFC compounds to catalyze ester hydrolysis, and the rate of hydrolysis was likely related to binding affinities for the divalent cation. Previous reports regarding macrocycle BFCs indicated tBu protected DOTA-alkyne species will chelate Cu, but reaction conditions varied widely (e.g., temperature, catalyst concentrations) and no previous work used an immobilized Cu(I) catalyst. We previously demonstrated that DOTA-alkyne without tBu protecting groups removes and chelates copper from a non-immobilized Cu(I)-ligand complex. Here, we determined that even when the Cu(I)-ligand complex is immobilized and the BFC reagent (DOTA-alkyne) contains tBu protecting groups, tBu protected DOTA-alkyne still (i) loses tBu protecting groups and (ii) removes Cu(I) catalyst from the Cu(I)-ligand complex via chelation.

To further investigate tBu protecting group loss and metal chelation, tBu protected BFCs were incubated with metal ions. A recent review mentioned that little systematic data is available on how other protected chelators, besides DOTA-alkyne derivatives, behave during CuAAC. Therefore, three different protected chelators, including propargyl-DOTA-tris(tBu) ester, were incubated with CuSO₄ at 47 °C for 4 h to determine the effect of copper ions on different tBu protected chelators. Protected DOTA-alkyne displayed complete conversion to Cu-DOTA-alkyne with 2 t-butyl groups, similar to the initial click reaction tested. However, when an azido-DOTA-tris(tBu) ester

Previously described method. Briefly, the channel surfaces were activated with an acidic hydrogen peroxide solution followed by injection of a neat “silane” solution. The water-soluble Cu(I) ligand was connected to the silane functionalized surface using an aza-Michael reaction catalyzed by a borax catalyst. To this end a ligand solution consisting of the Cu(I) ligand and borax was then injected into the chip. Finally, a Cu(I) solution comprised of an aqueous mixture of CuSO₄ and sodium ascorbate was injected into the microreactor to form an immobilized Cu(I)-ligand complex. Excess nonchelated Cu(I) and other ions were removed via rinsing. We previously quantified the amount of chelated Cu(I) catalyst present in microreactors (1136 ± 272 nmol) using ⁶⁴Cu. In the next subsection we describe how the thin “click chip”, hereafter simply referred to as “click chip”, was used for BFC and BM conjugation reactions.

Figure 2. Photograph (A) and side-view schematic (B) of a thin “click chip” with five reservoirs filled with posts; the red line in (A) is the location of the side-view in (B). PDMS thickness was reduced and a gas impermeable COC layer was added to minimize solvent loss. A thicker layer of PDMS was used at inlets and outlets to support tubing.

Figure 3. Schematic illustration of the water-soluble analog of the commonly used Cu(I) chelator tris(benzyltriazolylmethyl)amine (TBTA) used here to immobilize Cu(I) catalyst.
compound was incubated under the same conditions, a mixture of Cu-DOTA-azide compounds with 2 and 3 tBu protecting groups formed (Figure S3 in SI). When incubating copper(II) with a different chelator, propargyl-NO$_2$A-bis(tBu) ester, the incubation also yielded a mixture of Cu-NO$_2$A compounds with either 0 or 1 tBu groups.

Additionally, Ni(II) and Fe(II) were incubated with protected DOTA-alkyne and NO$_2$A-alkyne to determine the effects of other metals on tBu protected chelators. Ni(II) and Fe(II) were selected because both are potential contaminants of $^{64}$Cu solutions. While Ni(II) caused partial or complete loss of t-butyl groups similar to Cu(II), incubating DOTA- or NO$_2$A-alkyne with Fe(II) caused no substantial loss of t-butyl groups (Figures S4 and S5 in SI). Therefore, our results indicate that other tert-butyl protected bifunctional chelators, NO$_2$A-alkyne and DOTA-azide, are also susceptible to (i) tBu protecting group loss and (ii) metal ion chelation. Furthermore, our results indicate the number of tBu groups lost depends on the chelator and the metal ion present.

**“Click Chip” Characterization and Testing.** In light of the tBu loss and chelation issues of tBu protected BFCs in the presence of metal ions, even when the metal ion was immobilized, we modified the BM and BFC conjugation reaction process. Specifically, BFCs were reacted with Cu(II) ions prior to “click” reactions in an effort to reduce loss of Cu(I) catalyst from the immobilized Cu(I)-ligand complex. Therefore, all reagent mixtures consisted of the appropriate Cu-BFC and BM mixed in a phosphate buffer. Control reactions were performed by incubating a solution of Cu-DOTA-alkyne and cRGDfK-azide in a chip without immobilized silane or water-soluble Cu(I) ligand. The Cu(I) solution (CuSO$_4$ and sodium ascorbate) was still injected into the microreactors identically to “click chips” but no immobilized water-soluble Cu(I) ligand was present. Only a small amount of product (8% ± 4%) formed (Figure S6 in SI), demonstrating the importance of the immobilized TBTA analog to first capture the Cu(I) catalyst and then support the Cu(I) oxidation state critical for CuAAC reactions.

Next, we systematically tested the conjugation reaction of Cu-DOTA-alkyne and cRGDfK-azide at different temperatures (Figure 4A) and incubation times (Figure 4B) to determine their effects on yields. Figure 4 displays the yields for each...
individual reaction for “click chip” 1 (red columns) and “click chip” 2 (blue columns), and also the cumulative mean yield of all six reactions ± one standard deviation (yellow columns) for each condition. Some Cu-DOTA-cRGDK yields slightly exceeded 100% due to difficulty in subtracting background levels of the cRGDK-azide reagent in LC-MS chromatograms (Figure S7 in SI). As expected, yields were higher when operating at 47 °C compared to 19 °C (Figure 4A), especially for the first incubation (left-most red and blue columns).

Incubation time also affected yields with a general increase in yields with increasing incubation time. Yields for “click chip” 2 were considerably lower after 1 h incubations at 47 °C. In our previous report, we determined the total amount of immobilized Cu(I) catalyst was 1136 ± 272 nmol. Therefore, the difference in yields is likely due to less Cu(I) on “click chip” 2, which is further corroborated by Cu(I) loss experiments (Figure 7) discussed in detail later. However, yields for both chips after 6 and 12 h incubations were nearly identical, indicating the chip-to-chip variation in Cu(I) catalyst loading had negligible impact on yields for longer incubations.

The “click chips” successfully facilitated the conjugation of BFC (Cu-DOTA-alkyne) and BM (cRGDK-azide). Complete conversion of reagents was observed after 6 h incubations at 47 °C for the first incubations on both microreactors. These are promising results considering many traditional BM and BFC CuAAC conjugation reactions require either harsh reaction conditions, including high temperatures and microwave irradiation, or long reaction times (∼18 h) at room temperature. Additionally, the same two “click chips” were utilized for all reactions and similar yields for both microreactors for 6 and 12 h incubations indicate “click chip” fabrication is reproducible.

Figure 4A,B clearly indicates decreasing yields for successive reactions. The likely cause of the decline in yield was active catalyst loss during incubations and rinse steps. To determine the extent of Cu(I) catalyst loss, a solution without BFC or BM was incubated in the “click chips” identical to conjugation reactions. Incubation samples were collected similarly to conjugation reactions, but methanol and water used to rinse the microreactors after each reaction were also collected. The amount of copper (Figure 5A) in collected solutions was quantified by inductively coupled plasma optical emission spectrometry (ICP-OES). Because ICP-OES requires a few milliliters of sample, incubations and rinse solutions were combined in separate vials for each chip. Therefore, the results in Figure 5A are cumulative amounts of copper for each chip after three incubations.

Despite losing Cu(I) catalyst, most Cu(I) was lost during the rinsing step and both catalytic activity and yields were easily recovered by injecting more Cu(I) ions into the microreactor. Copper concentration in samples was low (∼20 ppm), which is much lower than typical CuAAC “click” conjugation reactions of BFCs and BMs which can easily have copper concentrations in the range of 100s to 1000s of ppm if not higher. The reduced copper concentration in samples mitigates or obviates purification requirements typically required for CuAAC reactions. Additionally, Figure 5B indicates yields are nearly identical after multiple uses, where one use is defined as injection of Cu(I) ions followed by three simultaneous incubations without the addition of fresh Cu(I) catalyst in between reactions. The same two microreactors were used for conjugating BFCs and BMs under a variety of conditions, but the yields after seven uses (21 reactions) were nearly the same as the first use (initial three reactions). Figure 5B demonstrates the robustness of the “click chips” after multiple uses and indicates the microreactors can be reused for at least 20 reactions.

Versatility for Other Substrates. After establishing ideal “click chip” operation conditions with the conjugation reaction between Cu-DOTA-alkyne and cRGDK-azide we tested additional conjugation reactions to demonstrate synthesis of different BM-BFC conjugates on chip. Peptides are attractive biomolecules for radiotracers because of their rapid clearance and facile synthesis, but DNA or RNA based imaging probes are receiving increased attention because of their low immunogenicity, high affinity, and stable structures, among other traits. Therefore, we tested conjugation reactions between Cu-DOTA-azide and EdU, a commercially available nucleoside, to demonstrate a conjugation reaction with a nonpeptide BM. There are also a wide variety of BFCs besides DOTA utilized in radiotracers. The choice of chelator depends on the radionuclide utilized, and different chelators can greatly affect biodistribution of radiotracers. NO2A derivatives are another widely used BFC, and here we tested the conjugation reaction of Cu-NO2A-alkyne and cRGDK-azide to test conjugation of different BFCs to BMs using “click chips”.

The “click chip” successfully synthesized both NO2A-cRG and DOTA-EdU conjugates. When Cu-DOTA-azide and EdU were incubated at 47 °C for 12 h in “click chips”, the CuAAC conjugation reaction proceeded with similar yields to the Cu-DOTA-alkyne and cRGDK-azide conjugation reaction with near complete conversion and ∼80% average yield (Figure 6).

Figure 6. Yields for three different CuAAC conjugation reactions on chip, incubated at 47 °C for 12 h.

When the Cu-NO2A-alkyne and cRGDK-azide reaction was performed on chip using the same conditions, the yields (∼45% average yield) were lower than those of the other two conjugation reactions. This indicates that longer incubations are likely required for some conjugation reactions (Figure 6). Ultimately, the two additional conjugation reactions demonstrate the versatility of the “click chips” to facilitate conjugation reactions between multiple BMs and BFCs. This versatility is crucial when synthesizing custom radiotracers to meet the needs of individual patients.
CONCLUSION

Here we developed a thin “click chip” with an immobilized Cu(I) catalyst suitable for CuAAC conjugation of biomolecules and bifunctional chelators. The microreactor design was improved from our previous “click chip” to reduce solvent loss for longer reactions by reducing PDMS thickness and bonding a more gas impermeable COC layer on top of the PDMS. We demonstrated the formation of BM-BFC conjugates using either a peptide, which are easily synthesized and have ideal in vivo clearance characteristics, or a nucleoside, representative of the recent interest in DNA or RNA based imaging agents because of their low immunogenicity and stable structure. Additionally, a wide variety of BFCs are utilized in radiotracer synthesis largely depending on the desired radio-metal. Here, we utilized three different BFC derivatives of DOTA or NO2A, two widely used bifunctional chelators. The ability to synthesize three different BM-BFC conjugates, without potentially harmful microwave irradiation or high temperatures, demonstrates the flexibility of the “click chips” to facilitate conjugation reactions under mild conditions. Cu(I) catalyst loss was observed but the concentration of copper in reaction samples was minute (~20 ppm), reducing or obviating the need to remove copper from reaction solutions, and enabling direct injection of crude reaction samples into LC-MS systems. Thus, enabling rapid purification of BFC-BM conjugates.

We also further explored tBu loss from protected BFCs. Some previous work has mentioned hydrolysis of tBu esters from protected BFCs, but the few papers that do discuss tBu loss are mostly DOTA-alkyne species. No systematic data exists on how other protected chelators respond to CuAAC reaction conditions. Here we demonstrated that tert-butyl ester hydrolysis occurs for BFCs besides tBu protected DOTA-alkyne in the presence of metal ions, and that the quantity of tBu protecting groups lost depends on the BFC and metal ion. Further work determining the effects of other parameters (e.g., temperature) were not studied, but are worthy of further research.

Due to tBu ester hydrolysis by copper, even when Cu(I) catalyst was immobilized, the BFCs chelated Cu(I) catalyst. Therefore, BFC complexes were prechelated with Cu(II) ions to reduce loss of immobilized Cu(I) catalyst. Here, we did not remove the copper ions from the Cu-BFC-BM product. Two general radiometal-based radiotracer production methods using “click” chemistry can be used: either chelate then “click” or vice versa. The “click chip” described here will likely be useful for chelate then “click” schemes where radiometal chelation occurs prior to conjugation to the appropriate BM. This nullifies the need to remove copper ions from the Cu-BFC-BM product because 64Cu or the desired radiometal is already chelated.

EXPERIMENTAL PROCEDURES

General. All solvents and chemical reagents were purchased from Thermo Fisher Scientific (Waltham, MA), Sigma-Aldrich (St. Louis, MO), or Avantor (Center Valley, PA) and were used as received unless otherwise specified. Water was purified in-house using a Barnstead E-Pure filtration system. Syglard 184 PDMS from Dow Corning (Midland, MI), a G3P-8 Spin Coater from Specialty Coating Systems (Indianapolis, IN), silicon wafers from University Wafer (Boston, MA), 4 mil (101.6 μm) thick COC from TOPAS Advanced Polymers (Florence, KY), glass microscope slides from Thermo Fisher Scientific (Waltham, MA), and a PDC-001 Harrick Scientific Plasma Cleaner (Ossining, NY) were utilized for chip fabrication. DOTA-alkyne was purchased from CheMatech (Dijon, France), and NO2A-alkyne and DOTA-azole were from Macrocyclics (Plano, TX). The peptide crGDKF-azole was synthesized as described previously.23 See Figure S8 in the SI for a schematic illustration of the BM and BFC structural formulas. Thirty gauge PTFE tubing from Cole-Parmer (Vernon Hills, IL), glass syringes from Hamilton Company (Reno, NV), or plastic syringes from Henke-Sass Wolf (Dudley, MA) and Becton, Dickinson and Company (Franklin Lakes, NJ), and microliter and milliliter syringe pump modules from Harvard Apparatus (Holliston, MA) were used for injecting liquids into chips. Cu(I) catalyst loss was determined by a PerkinElmer Optima 2000 DV ICP-ES (Waltham, MA). LC-MS was performed on an Agilent LC-MS (HPLC: 1100, MS: Trap XCT Plus) (Santa Clara, CA) using a Luna C18(2) column (5 μm, 100 Å, 150 × 4.60 mm) from Phenomenex (Torrance, CA). The same LC-MS method was used for all samples. The flow rate was 400 μL/min with the mobile phase of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The linear gradient used was 0% B (0–2 min), 0% → 10% B (2–10 min), 10% → 15% B (15–25 min), 15% → 45% B (25–35 min), 45% → 100% B (35–36 min), 100% B (36–51 min), 100% → 0% B (51–51.5 min), 0% B (51.5–76.5 min). MS (ESI) used a nebulizer pressure of 35.00 psi, a dry gas flow rate of 8.00 L/min, a dry temperature of 350 °C, and a capillary voltage of 4.5 kV.

Thin “Click Chip” Fabrication. Silicon wafers used for PDMS molds were etched as described previously.27 PDMS was combined in a 10:1 mass ratio (base/curing agent), mixed, and degassed. Then PDMS was poured on top of the silicon wafer and spin-coated at 200 rpm for 60 s. The PDMS was cured in an oven for 1 h at 65 °C. Thick interconnects were prepared by pouring PDMS on a silicon wafer and curing for 2 h at 65 °C. COC was cut to size and placed in a plasma cleaner along with the spin-coated PDMS and PDMS interconnects. All components were treated with an oxygen plasma for 1 min. The PDMS interconnects were permanently attached to the spin-coated PDMS followed by the COC, and then the entire assembly was placed in a 65 °C oven for 30 min. The PDMS was cut along the edge of the COC, carefully removed from the silicon, covered with low-tack Scotch tape, and cut to size. Through holes were punched at interface ports (inlets and outlets). A 50 × 45 × 1 mm glass slide was scrubbed clean with an aqueous Alconox solution, rinsed with water, and dried with N2. The glass slide and bottom side (feature side) of the spin-coated PDMS were treated with an oxygen plasma for 1 min, bonded together, and incubated overnight in a 65 °C oven.

Quantifying Solvent Loss in Thin and Thick Chips. Thick PDMS/glass chips were fabricated as described previously. Methanol was injected into thin (COC/PDMS/glass) and thick (PDMS/glass) chips to eliminate bubbles, and then water was injected for 10 min to replace methanol as the solvent. Double-sided Scotch tape was applied onto small pieces of glass (~10 × 10 × 1 mm), which were then placed on top of the interface ports of each chip. Microfluidic chips were placed in preheated humidity chambers consisting of 150-mm-diameter glass Petri dishes with lids and two aluminum weight boats filled with water. After placing the chips in the glass dishes the lid was sealed with Parafilm. Chips were incubated at the appropriate temperature (23 °C, 37 °C, or 47 °C) for 12 h.
Water loss was determined by comparing chip mass before and after heating. Chips were allowed to cool for at least 10 min prior to determining the mass after incubation.

**Ligand Immobilization Process.** The water-soluble Cu(I) ligand, an analog of TBTA, was synthesized as described previously. The immobilization process was performed similarly to our previous protocol, and a detailed description of the process is available in the SI.

**Incubating BFCs with Metal Ions.** Propargyl-DOTA-tris(tert-buty) ester (11.61 mg, 15.36 μmol) or NO2A-butyne-bis(tert-buty) ester (11.11 mg, 23.81 μmol) in DMSO, was combined with water (2:3 DMSO/water) to create 3.00 mM solutions. Separate 50 mM metal stock solutions were prepared by dissolving CuSO4 (0.12569 g, 0.50340 mmol), NiSO4 (0.13216 g, 0.50282 mmol), or FeSO4 (0.14034 g, 0.50480 mmol) in 10 mL of water. In separate amber glass vials the appropriate 3.00 mM tBu protected BFC solution (700 μL), 180 mM potassium phosphate buffer pH 7.20 (175 μL), appropriate 50 mM metal solution (63 μL), and water (112 μL) were combined and stirred on a 47 °C hot plate for 4 h along with a control vial of tBu protected BFC with additional water added (63 μL) instead of metal solution. A 3.00 mM solution of azido-monoamide-DOTA-tris(tert-buty) ester (17.94 mg, 24.38 μmol) was prepared identically to DOTA-alkyne and NO2A-alkyne and incubated in a similar fashion with a CuSO4 solution so the final Cu concentration was 3.00 mM. The solution was incubated for 4 h at 47 °C. Samples were analyzed by LC-MS. Multiple products with different Cu concentrations may reduce Cu concentration below detection limits. Solvent for each sample was evaporated under vacuum then placed in a vacuum oven overnight to ensure removal of all methanol. Nitric acid solution (3 mL, 100 mM) was added to each sample. Samples were transferred between different vials during processing so a portion of the nitric acid solution was aliquoted to previously used vials, vortex mixed, and then transferred back to the sample container to reduce Cu loss from adsorption to container walls. Samples were diluted with an additional 2 mL of water and Cu concentration was quantified for each sample by ICP-OES. The 100 mM nitric acid solution was also analyzed and confirmed minimal amounts of Cu (<0.005 ppm) present in this solution compared to incubation and rinse samples with Cu concentrations between roughly 0.5 and 5 ppm.

**ASSOCIATED CONTENT**

* Supporting Information

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**Notes**

The authors declare no competing financial interest.

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assistance with LC-MS experiments and the Microanalysis Laboratory for analyzing ICP-OES samples.

**ABBREVIATIONS**

BFC, bifunctional chelator; BM, biomolecule; PET, positron emission tomography; SPECT, single-photon emission computed tomography; [18F]FDG, [18F]fluorodeoxyglucose; CuAAC, Cu(I)-catalyzed azide–alkyne cycloaddition; cRGDK-azide, cyclo-[Arg-Gly-Asp-D-Phe-Lys]; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; NOA2, 1,4,7-triazacyclononane-1,4,7-diacetic acid; EdU, 5′-bromodeoxyuridine; PDMS, polydimethylsiloxane; COC, cyclic olefin copolymer; LC-MS, liquid chromatography–mass spectrometry; tBu, tert-butyly; ICP-OES, inductively coupled plasma optical emission spectrometry; ESI, electrospray ionization; TMSPA, 3-(trimethoxysilyl)propyl acrylate; TBTA, tris(benzyltriazolylmethyl)amine.

**REFERENCES**


