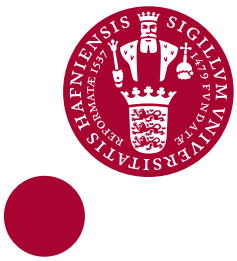

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Multifunctional Clickable Reagents for Rapid Bioorthogonal Astatination and Radio-Crosslinking

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In the past decade, several developments have expanded the chemical toolbox for astatination and the preparation of ^{211}At-labeled radiopharmaceuticals. However, there is still a need for advanced methods for the synthesis of astatinated (bio) molecules to address challenges such as limited in vivo stability. Herein, we report the development of multifunctional ^{211}At-labeled reagents that can be prepared by applying a modular and versatile click approach for rapid assembly. The introduction of tetrazines as bioorthogonal tags enables rapid radiolabeling and radio-crosslinking, which is demonstrated by steric shielding of ^{211}At to significantly increase label stability in human blood plasma.

Astatine-211 is considered as one of the most promising α-particle emitting radionuclides for therapeutic application.[1–3] Its favorable physical properties include (i) a half-life time of 7.2 h compatible with the pharmacokinetics of potential targeting carriers, (ii) an α-particle emission yield of 100%, (iii) no long-lived α-emitting decay products, and (iv) a high linear energy deposition enabling effective cell-killing.[4–8] These features motivated the development and evaluation of therapeutic strategies applying a variety of ^{211}At-labeled radio-pharmaceuticals such as monoclonal antibodies,[6,8–10] antibody fragments,[11] nanobodies,[12] diabodies,[13] and other carriers.[14–15] Despite the potential of ^{211}At for medical use, its application has been impeded mainly by the low *in vivo* stability of labeled compounds.[1–2] Several labeling strategies and reagents have been developed including (i) conjugation with astatobenzoates, or (ii) radiohalogenation of boron cage moieties,[1,4,7,16–21] or (iii) copper-catalyzed astatination of boronic esters.[22] However, additional studies and methodologies are required to enable further advancement in the field of ^{211}At-based radiotherapy. In this regard, researchers are facing several challenges related to astatine, which is not only one of the rarest naturally occurring elements, but also has no stable isotope. Consequently, the chemistry of astatine is yet not fully understood and many properties have only been extrapolated.[2]

Astatine-211 is produced by irradiation of stable bismuth with 28 MeV α-particles in the ^{209}Bi(α,2n)^{211}At reaction followed by isolation from the target material and used for subsequent labeling.[23] As there is no existing stable isotope, non-radioactive astatine-labeled compounds cannot be prepared and used as reference material, e.g. to enable identification of ^{211}At-labeled compounds by HPLC after radiosynthesis. Therefore, iodine-labeled analogs are often used as reference compounds, not only for analytics, but also to study *in vivo* deastatination by comparison with structurally equivalent ^{211}At-labeled compounds, although significant differences of ^{211}At and iodine have been reported.[1,16] Another drawback of ^{211}At-radiopharmaceuticals is that several manual steps are often required in the production process.[23] Thus, further advancements rely on the development of efficient and rapid methods for the synthesis of astatinated (bio)molecules.

In order to circumvent problems related to astatine chemistry and to simplify and accelerate the preparation of ^{211}At-labeled compounds, we aimed to develop multifunctional reagents that can be used for radiolabeling and radio-crosslinking applying bioorthogonal chemistry, also focusing on the potential effect of steric shielding (Figure 1).

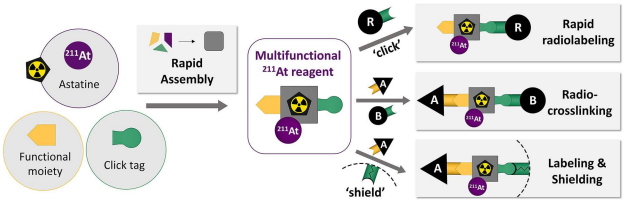

Figure 1. Rapid assembly of multifunctional ^{211}At-reagents applicable for rapid bioorthogonal radiolabeling, crosslinking and/or steric shielding to increase label stability.

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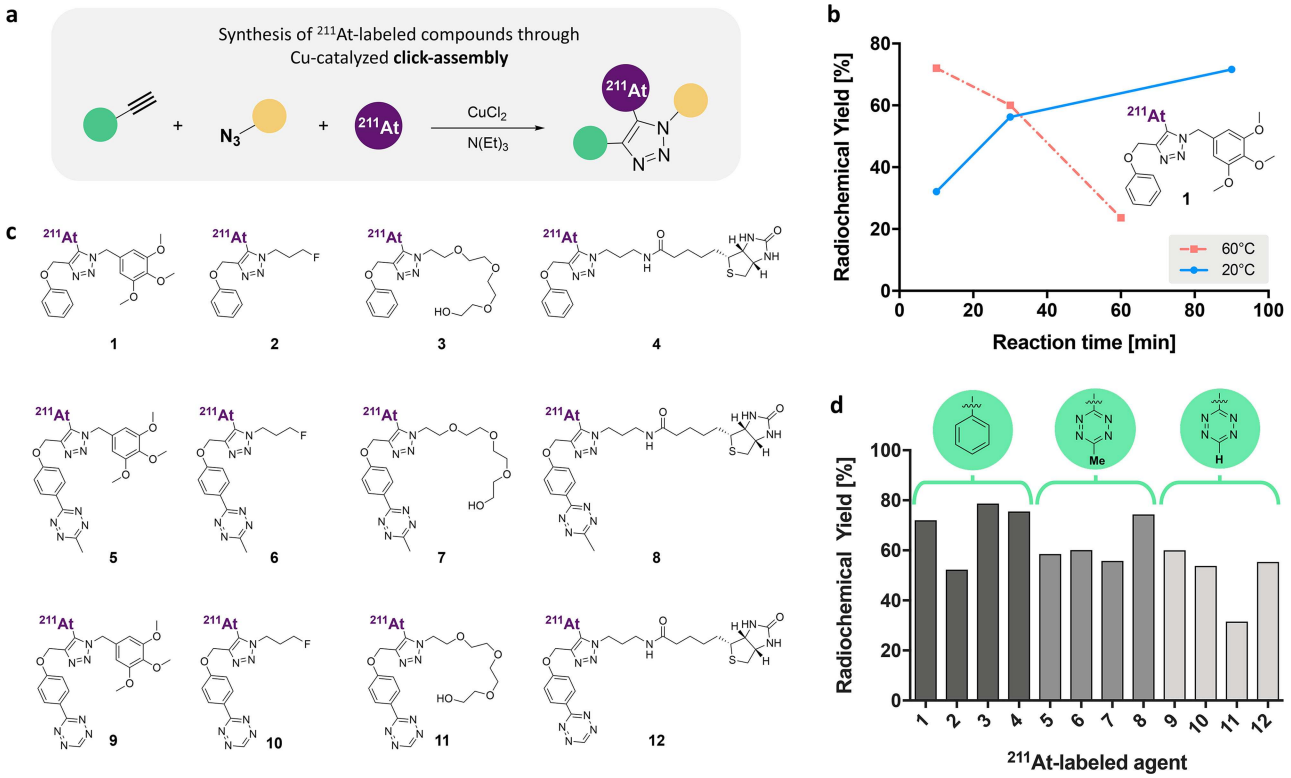

Figure 2. (a) Rapid and modular assembly of ^{211}At-labeled compounds applying a three-component copper-catalyzed click reaction. (b) The radiochemical yield of click-astatination depends on reaction time and temperature as shown for the synthesis of **1**. (c) Synthesized ^{211}At-labeled compounds **1–12** including methyl-substituted tetrazines, more reactive H-tetrazines and the two multifunctional biotinylated ^{211}At-tetrazines **8** and **12**. (d) Radiochemical yields of compounds **1–12** (10 min reaction time, 60 °C, 2.4–2.6 MBq ^{211}At) as determined by radio-HPLC (see Supporting Information; n = 1).

The development of rapid, modular and highly selective bioorthogonal ligations has led to numerous applications of click reactions in radiochemistry. In particular, tetrazine ligations – the fastest bioorthogonal reactions described so far[24] – have had a significant impact by enabling strategies for (i) rapid radiolabeling using prosthetic groups, (ii) site-specific radiolabeling, and (iii) pretargeting approaches based on *in vivo* chemistry.[25–27] To this end, various radiolabeled 1,2,4,5-tetrazines (Tz) have been developed as clickable tools for PET and SPECT imaging (^{18}F, ^{11}C, ^{111}In, ^{64}Cu, ^{123}I, ^{125}I),[28–33] and targeted radiotherapy (^{177}Lu, ^{67}Cu)[34,35] making use of the fast bioorthogonal ligation with *trans*-cyclooctenes (TCO).

Recently, the first Tz labeled with α-emitting radionuclides (i.e. ^{212}Pb and ^{225}Ac) have been reported aiming at the advantages of α-emitters over β^--emitting radionuclides such as the delivery of a higher radiation dose within a shorter path length.[36,37]

Considering the potential and capability of both, ^{211}At-based radiotherapy and bioorthogonal chemistry, we focused on the synthesis of ^{211}At-labeled clickable reagents inspired by the pioneering study of Årstad and coworkers describing a modular three-component reaction for the synthesis of ^{125}I-labeled compounds.[38] Based on this approach we have been successful to access ^{211}At-labeled compounds by copper-catalyzed click-assembly of terminal alkynes, azido-functionalized molecules and astatine-211 (2.4-2.6 MBq) (Figure 2a). This method was further investigated and optimized with emphasis on reaction temperature and time to reach radiochemical yields >70% in 10 min (Figure 2b). Using this procedure, we were able to prepare a variety of ^{211}At-labeled compounds (**1-12**, Figure 2c) showing compatibility with different functional groups including moieties such as biotin (**4**, **8**, **12**) and tetrazines (**5**–**12**). Even more reactive and thus less stable mono-substituted tetrazines (H-tetrazines, H–Tz) were used for the synthesis of bioorthogonal ^{211}At-labeled agents (**9**–**12**) in radiochemical yields of >50% (except compound **11**) similar to more stable methyl-tetrazines (**5**–**8**) (Figure 2d).

To test for compatibility with higher activities, compound **1** was prepared using 33 MBq of astatine-211 in a radiochemical yield of 87% (see Supplementary Information). Moreover, high radiochemical purities were achieved in most cases already prior to further purification and impurities could readily be removed by preparative radio-HPLC (see Supporting Information).

To verify correct click-assembly as shown in Figure 1a and bioorthogonal reactivity, we used the multifunctional biotinylated ^{211}At-tetrazine **12** in a scavenging experiment exploiting the orthogonal reactivity of both functional moieties (Tz/TCO vs. biotin/streptavidin). Magnetic beads functionalized with either TCO or streptavidin were used to remove **12** from solution through rapid ligation to the beads followed by magnetic separation (Figure 3). Compound **3** was used in control experiments to test and correct for unspecific binding to the beads (as partly observed with TCO-beads). In this experiment we could

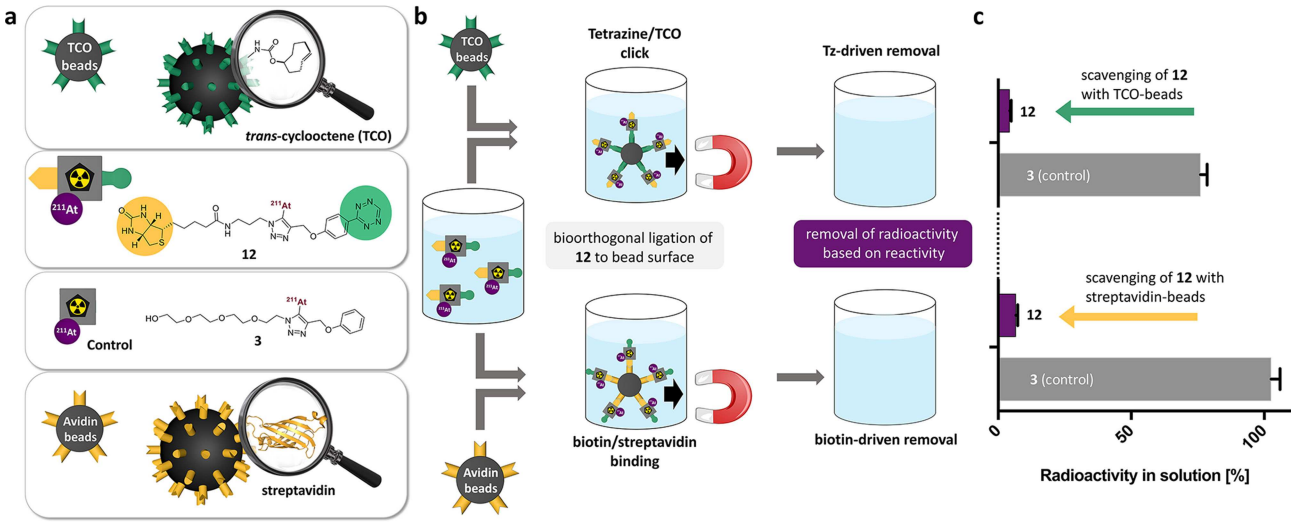

Figure 3. (a) Structure Verification of ^{211}At-labeled compounds using multifunctional compound **12**, non-reactive compound **3** as a control, and functionalized magnetic beads. (b) Compound **12** was reacted with either TCO- or streptavidin-functionalized beads and radioactivity was measured after magnetic separation. (c) Both Tz-driven removal of **12** based on bioorthogonal ligation to TCO-beads and biotin-driven removal by binding to streptavidin-beads was shown (in comparison to control experiments using compound **3** to correct for unspecific binding to the beads).

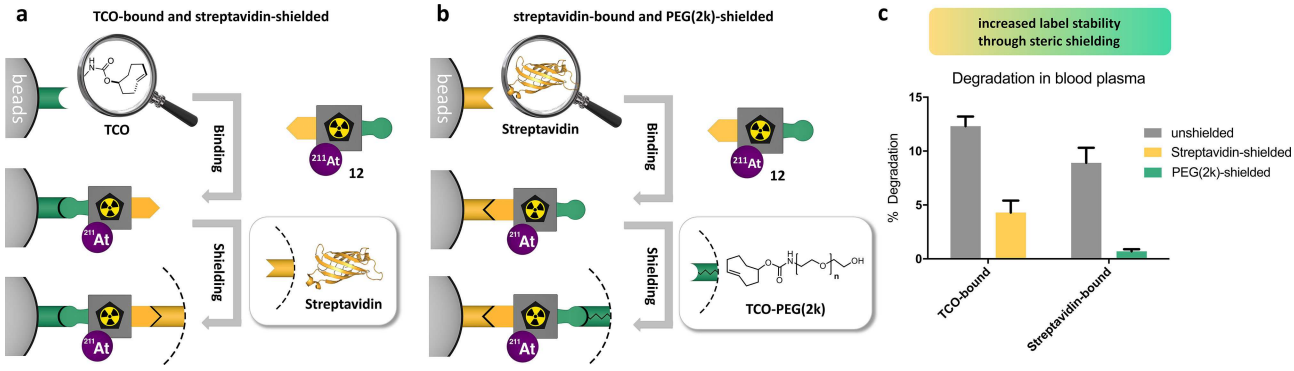

Figure 4. Radio-crosslinking using the biotinylated ^{211}At-tetrazine **12**. (a) TCO-beads were reacted with **12** by bioorthogonal Tz ligation followed by shielding with streptavidin. (b) In an analogous experiment, streptavidin-functionalized beads were radio-crosslinked with **12** to TCO-PEG(2k). (c) Steric shielding of **12** (on beads) with either streptavidin or TCO-PEG(2k) was shown to significantly reduce degradation/deastatination and thus increase label stability (up to > 10-fold) in human blood plasma (incubation time of 5 hours) in comparison to unshielded **12** on beads as a control.

show efficient Tz-driven and biotin-driven removal of **12** with remaining activities in solution of ~5% and ~7%, respectively, in comparison to ~72% and ~98%, respectively, for control **3** (Figure 3c). Hence, both moieties, Tz and biotin, were detected, strongly indicating successful three-component click-assembly. In addition, ^{211}At-labeled tetrazine **9** was compared to its iodine-labeled analog by HPLC showing similar retention times for both compounds (see Supporting Information).

Radio-crosslinking on beads was carried out following a similar approach by reacting functionalized beads with **12** followed by reaction with streptavidin or TCO-labeled poly(ethylene glycol) with an average molecular weight of 2 kDa (PEG(2k)), respectively (Figure 4a and Figure 4b). To investigate steric shielding of conjugated **12** (and the attached ^{211}At-label) by streptavidin or TCO-PEG(2k) the radio-crosslinked beads were incubated in human blood plasma at 37 °C for 5 hours. Radioactivity was measured in solution after magnetic separation of the beads revealing significantly reduced degradation compared to unshielded controls (Figure 4c). In case of PEG(2k)-shielding we observed a degradation (or deastatination) of less than 1% and thus a more than 10-fold increased stability in human blood plasma. Although these findings cannot directly be translated to *in vivo* conditions, we consider steric shielding through radio-crosslinking as a strategy to potentially further improve the stability of ^{211}At-labeled radiopharmaceuticals. Furthermore, multifunctional clickable ^{211}At-reagents such as **12** represent advantageous and broadly applicable tools for radiolabeling and subsequent surface modification of (nano)particles applying rapid radio-crosslinking. As click chemistry has become a commonly used method for engineering nanoparticle surfaces,[39,40] radio-crosslinking could be applied to simultaneously incorporate ^{211}At during surface modification to rapidly access nano-radiopharmaceuticals for theranostic applications[41–43] without the need for additional labeling procedures.

In summary, we have developed an efficient method for rapid assembly of astatinated compounds tolerating highly

reactive bioorthogonal functional groups such as 1,2,4,5-tetrazines. We thus present the synthesis of the first ^{211}At-labeled clickable tools that can be used for rapid radiolabeling applying tetrazine ligations. Moreover, multifunctional reagents can easily be prepared and used for radio-crosslinking. This approach was found to be a promising strategy for surface modification and simultaneous incorporation of ^{211}At. The observed increased stability in human blood plasma through steric shielding further illustrates the potential application of this methodology. Hence, we are convinced that our results will significantly contribute to further research in the field of ^{211}At-labeling and the development of new and improved radiopharmaceuticals.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: bioorthogonal chemistry · click chemistry · multicomponent reactions · radiochemistry · radiolabeling

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