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The inverse electron-demand Diels-Alder reaction as a new methodology for the synthesis of ^{225}Ac -labelled radioimmunoconjugates

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Abstract

The inverse electron-demand Diels-Alder reaction between tetrazine (Tz) and *trans*-cyclooctene (TCO) facilitates the efficient radiosynthesis of ^{225}Ac -radioimmunoconjugates in a two-step method, outperforming conventional approaches based on isothiocyanate couplings

Actinium-225 (^{225}Ac ; $t_{1/2} = 10.0$ d; 5.8 MeV α) is an α -particle emitting radionuclide that sequentially decays through six dominant daughters to stable ^{209}Bi , yielding 4 net α and 2 β -disintegrations. In light of this scheme, it has been dubbed a “nanogenerator” or “*in vivo* generator” of α -particles.¹ The 10.0 day physical half-life of ^{225}Ac advantageously matches the long biological half-life of immunoglobulins, making it particularly well suited for applications in radioimmunotherapy (RIT). An anti-CD33 monoclonal antibody (HuM195; Lintuzumab) radiolabelled with ^{225}Ac was recently translated to the clinic for Phase I and II trials for the treatment of relapsed or refractory acute myeloid leukemia.^{2,3} While the initial results have been promising, the clinical potential of ^{225}Ac -targeted alpha RIT remains limited by both a shortage of the radionuclide as well as a dearth of well-explored radiochemical protocols. High-energy proton spallation of ^{232}Th is currently evaluated for the production of gigabecquerel level of ^{225}Ac .⁴ However, with regard to the latter, the

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Conflicts of interest

There are no conflicts to declare.

current radiolabelling procedure for the clinical production of ^{225}Ac -labelled antibodies still relies on a two-step method that suffers from low radiochemical yields ($\sim 10\%$) (Figure 1A).⁵ Reported by McDevitt *et al.* in 2002, this method relies on the radiolabelling of an isothiocyanate-bearing, C-functionalized 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) derivative at $50\text{--}60^\circ\text{C}$ for $30\text{--}60$ minutes in a first step.⁵ Subsequently, this ^{225}Ac -DOTA-NCS conjugate is reacted with the IgG of interest at 37°C for $50\text{--}60$ min (pH 8.7).⁵ While this approach does indeed work, the hydrolysis of the isothiocyanate moiety reduces radiochemical yields to $8\text{--}11\%$ depending on the IgG.⁵ More recently, Maguire *et al.* have reported a one-step method that provides radiochemical yields up to 80% .⁶ However, this method, requires the functionalization of the IgG with >10 DOTA chelators per construct,⁶ a procedure which might not be feasible with most antibodies and could drastically reduce the immunoreactivity of the radioimmunoconjugate. Clearly the development of novel, modular, and efficient strategies for ^{225}Ac radiolabelling is an urgent unmet need.

In 2006, Marik and Sutcliffe pioneered the application of click chemistry for the synthesis of radiopharmaceuticals, with the use of a Cu^{I} -catalysed 1,3-dipolar cycloaddition for the synthesis of ^{18}F -radiolabelled peptides.⁷ Catalyst free click reactions such as the strain-promoted $[3+2]$ azide alkyne cycloaddition^{8,9,10} and Staudinger ligation^{11,12} were later used for the synthesis of radiotracers for use in small animal positron emission tomography (PET) or single photon emission computed tomography (SPECT). However, the low rate constant of these reactions, ($k \sim 10^{-2}\text{--}1\text{ M}^{-1}\text{s}^{-1}$ and $10^{-3}\text{ M}^{-1}\text{s}^{-1}$, respectively)^{13,14} limit their application *in vivo*. The remarkable selectivity and rapidity ($k \sim 1\text{--}10^6\text{ M}^{-1}\text{s}^{-1}$) of the bioorthogonal inverse electron-demand Diels-Alder (IEDDA) cycloaddition between tetrazine (Tz) and *trans*-cyclooctene (TCO) has most notably been leveraged for *in vivo* pretargeting.¹⁵ Pretargeting is a strategy for the administration of radioimmunoconjugates that relies on the separate injection of a TCO-antibody conjugate and a Tz-radioligand. This approach has been applied for both nuclear imaging and radiotherapy and has the major advantage of reducing the radiation dose to non-target tissues.¹⁵ The IEDDA reaction also holds promise for a wide variety of other applications in radiochemistry.^{15,16} The use of the IEDDA reaction was reported for the synthesis of ^{18}F -radiolabelled peptides by Fox *et al.*,¹⁷ but also for the synthesis of radioimmunoconjugates by Zeglis *et al.* (^{64}Cu and ^{89}Zr) and Rossin *et al.* (^{111}In).^{18,19,20} However, in these cases, this click-based radiolabelling method did not constitute a significant improvement compared to more traditional radiolabelling protocols. Here, we evaluate the application of inverse electron-demand Diels-Alder (IEDDA) chemistry to the *ex-vivo* synthesis of ^{225}Ac -labelled radioimmunoconjugates.

In the study at hand, two Tz-bearing chelators and two TCO-modified immunoconjugates were employed to evaluate the potential of the IEDDA reaction for the synthesis of ^{225}Ac -radioimmunoconjugates (Figure 1B). More specifically, a DOTA-PEG₇-Tz construct was synthesized according to previously reported protocols²¹, and a DO3A-PEG₇-Tz ligand was prepared via a similar synthetic route (see Supporting Information). With respect to the antibodies, 5B1 is a fully human antibody that targets carbohydrate antigen 19.9 (CA19.9), an antigen that is highly expressed by many cancers, most notably pancreatic ductal adenocarcinoma. huA33 is a humanized antibody that targets the A33 antigen, a transmembrane glycoprotein expressed by $>95\%$ of colorectal carcinomas. The conjugation

of TCO-NHS to 5B1 and huA33 as well as the subsequent determination of the average number of TCO moieties per antibody were performed according to previously published methods (see Supporting information).^{19,21}

²²⁵Ac is received as a nitrate salt and reconstituted using 0.2 M Optima grade HCl solution. DOTA-PEG₇-Tz (10 to 370 nmol) is radiolabelled with ²²⁵Ac (0.7 to 33.0 MBq) at 37°C in NH₄OAc (0.25 mM, pH 5.5) for 1 hour. Decay-corrected radiochemical yields range from 66 ± 2% to 96 ± 2% depending on the amount of activity added per μmol of Tz-chelator (Figure 2A). After purification on a C₁₈ light cartridge, instant thin layer chromatography (iTLC) indicates the presence of free non-equilibrium daughters at low level (<5%). At secular equilibrium, the radioligands are obtained with radiochemical purity >98% (see Supporting information) and molar activities up to 0.17 ± 0.03 MBq/μmol. The ligation of the Tz-bearing radioligands is performed by adding different molar ratios of 5B1-TCO (4:1 or 8:1 Tz-to-5B1) to the ²²⁵Ac-tetrazine complex. Not surprisingly given the speed of the click-reaction, the ligation is completed in less than 5 minutes. The ²²⁵Ac-labelled radioimmunoconjugates are purified using a PD-10 desalting column and obtained with excellent radiochemical purity (>98%) as confirmed by iTLC (see Supporting information). Specific activities for the ²²⁵Ac-DOTA-PEG₇-5B1 conjugates range from 2.8 ± 0.9 and 4.6 ± 0.2 MBq/mg depending on the molar ratio of Tz-to-5B1 (Figure 2B). These values are in the range of those produced using the currently clinically used two-step method (3.0–4.4 MBq/mg).⁵ Decay corrected radiochemical yields after purification vary from 35 ± 11 % to 45 ± 6 %. Taking into account the degree of labelling of TCO on the antibody (1.8 ± 0.4) and the molar ratio of Tz-to-5B1 used, the conjugation step is nearly quantitative. Clearly, our click-based two-step approach produces a significant improvement over the radiochemical yields obtained using the previously reported method (mAb = HuM195; radiochemical yield = 10.6 ± 3.6%; P<0.05). Similar methods are applied to the radiolabelling of 5B1-TCO with the Tz-PEG₇-DO3A derivative as well as for the labelling of the huA33-TCO conjugates. The results in these cases confirm the modularity of this approach and are provided in the supporting information.

The stability of the ²²⁵Ac-DOTA/DO3A-PEG₇-5B1 radioimmunoconjugates is determined in saline and human serum for up to 10 days. Radiochemical purity after 10 days was >70%, and the activity still bound to protein is >80%. The low activity concentrations observed in non-target organs during the *in vivo* evaluation experiments provide further evidence for the radiochemical stability of the radioimmunoconjugates. The *in vitro* immunoreactivity of the ²²⁵Ac-labelled 5B1 radioimmunoconjugates is determined to ensure that the conjugation and radiolabelling processes did not alter the affinity of the antibody for CA19.9. To this end, two human PDAC cell lines are used: BxPC3 (CA19.9 positive) and MIAPaCa-2 (CA19.9 negative). The immunoreactivity of ²²⁵Ac-DOTA-PEG₇-5B1 is determined to be 0.23 ± 0.01 % with MIAPaCa-2 cells and 61.3 ± 4.52 % with BxPC3 cells, confirming the selectivity of the radioimmunoconjugate for CA19.9. The immunoreactivity with BxPC3 cells is rather low but still acceptable for further *in vivo* evaluation. Similar results are obtained with ²²⁵Ac-DO3A-PEG₇-5B1 (see Supporting Information).

Acute biodistribution experiments are performed in healthy athymic nude mice as well as athymic nude mice bearing subcutaneous BxPC3 or MIAPaCa-2 xenografts. These mice are

administered either ^{225}Ac -DOTA-PEG₇-5B1 or ^{225}Ac -DO3A-PEG₇-5B1 (18.5 kBq, 8.6 μmol) in 150 μL of 0.9 % NaCl + 1.0 % BSA. Then, after 4 h, 1 d, 3 d, 7 d, or 10 d, tissues of interest are harvested, and each sample is weighed and counted for up to 10 min on a gamma counter using a 150 to 600 keV energy window. In healthy animals, the biodistribution data up to 3 days post-injection reveals good clearance of the radioimmunoconjugates with decreasing activity concentrations in the blood, heart, lung and kidneys (see Supporting Information). In mice bearing BxPC3 xenografts, ^{225}Ac -DOTA-PEG₇-5B1 produces high levels of prolonged tumor accumulation, with activity concentrations up to 32.4 ± 9.8 % ID/g, 10 days post-injection. The specificity of the radioimmunoconjugate for CA19.9 is confirmed by the drastic reduction in the tumoral activity concentrations in the mice bearing MIA PaCa-2 xenografts (5.3 ± 2.7 % ID/g, 3 days post-injection). Good clearance of ^{225}Ac -DOTA-PEG₇-5B1 is observed in this case as well, with decreasing concentrations of the radioimmunoconjugates in all other organs. This observation suggests that the radioimmunoconjugate is adequately stable *in vivo* since no persistent uptake is observed in the organs in which free ^{225}Ac and its daughters typically accumulate (*e.g.* liver, bone and kidneys). Similar biodistribution profiles — with high tumor uptake and efficient clearance — are observed with the ^{225}Ac -DO3A-PEG₇-5B1 radioligand as well as with ^{225}Ac -labelled variants of the huA33 antibody in mice bearing SW1222 human colorectal cancer xenografts (see Supporting Information).

In conclusion, this study positions the IEDDA ligation as an extremely promising radiosynthetic approach for the development and clinical production of ^{225}Ac -labelled radioimmunoconjugates. The Tz/TCO-based two step radiolabelling strategy described herein offers dramatically superior radiochemical yields compared to the two step method that is currently used in the clinic. This modular approach also yields radioimmunoconjugates with suitable specific activities and good *in vitro* stability. Finally, the excellent biodistribution profiles of the ^{225}Ac -labelled radioimmunoconjugates confirm the potential of this strategy. In the end, we contend that this Tz/TCO-based approach could be used in cases in which the targeting vector (*e.g.* antibody, protein, etc.) is unstable under the conditions necessary for the chelation of the radiometal. Two such scenarios that immediately come to mind are the creation of ^{227}Th -labelled antibodies for radioimmunotherapy^{22,23} and the synthesis of ^{89}Zr -DOTA-labelled biomolecules for PET imaging.²⁴

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Notes and references

‡ All animals were treated according to the guidelines approved by the Research Animal Resource Center and Institutional Animal Care and Use Committee at Memorial Sloan Kettering Cancer Center.

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Figure 1.

A) Schematic representation of the currently used two-step radiolabelling procedure for the clinical production of ^{225}Ac -radioimmunoconjugates; B) Schematic representation of the proposed click-reaction radiolabelling strategy between a ^{225}Ac -DOTA-PEG₇-Tz and TCO-antibody conjugate.

Figure 2. Two-step ^{225}Ac -radiolabelling method based on the IEDDA reaction (A) Dependence of the radiochemical yield and specific activity on the activity added (MBq) per nmol of DOTA-PEG₇-Tz; (B) Dependence of the radiochemical yield and specific activity on the Tz-to-5B1 ratio.

Figure 3.

In vivo biodistribution of in BxPC3 (CA19.9 positive) and MIAPaCa-2 (CA19.9 negative) tumor bearing athymic nude mice of ^{225}Ac -DOTA-PEG₇-Tz (18.5 kBq, 8.6 μmol , 0.06 nmol) up to 10 days post-injection. Only major organs are represented. Error bars represent the standard deviation (n = 5).