

ASPARAGINYL PEPTIDE LIGASE-MEDIATED RADIOLABELLING OF SINGLE-DOMAIN ANTIBODIES

FIELD OF THE INVENTION

The invention generally relates to the field of radiochemistry. In particular, the present invention provides an asparaginyl peptide ligase (PAL)-mediated method of producing site-specifically radiolabeled single-domain antibody (sdAb) tracers, which is improved in the presence of a glutaminyl cyclase (QC). The radiolabelled sdAb tracers prepared by the method of the present invention provide enhanced characteristics and are suitable for use in diagnosis, in surgery, in treatment, in therapy monitoring, and as an imaging agent.

BACKGROUND OF THE INVENTION

Molecular imaging techniques such as computed tomography (CT), positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), ultrasound imaging (US), and optical imaging offer non-invasive ways to characterize physiological processes and link molecular changes to clinical outcomes.

In recent times, significant advancements have been made in the field of molecular imaging, such as the development of new imaging equipment, the discovery of new molecular targets, and the creation of multifunctional contrast agents. In addition, the imaging moiety may be substituted with a therapeutic molecule such as a therapeutic radionuclide, or a fluorescent molecule that could be used in photodynamic therapy.

For *in vivo* diagnostic purposes in molecular imaging, the contrast agent or imaging tracer must preferably be composed of a targeting molecule that is able to specifically recognize the target of interest, and a signal-emitting molecule to allow for *in vivo* detection. The bioconjugation between the two molecules is also of importance, especially for small protein candidates such as single domain antibodies (sdAbs) in order to be able to achieve sufficient tissue penetration, selective binding to the target, a high signal/noise ratio at the target site, and low overall body retention or accumulation (as a consequence of elimination from the body; typically in liver or kidneys).

Single domain antibodies (sdAbs) are antibody-fragments that constitute interesting tools in nuclear medicine for imaging purposes (for example by PET imaging) and for therapeutic purpose with applications in various diseases (for example, site-specifically coupled sdAbs to a therapeutic radionuclide). A previously developed sdAb targeting the human Programmed Death Ligand 1 (hPD-L1) as described in patent application no. PCT/EP2019/055133 (WO2019/166622A1) displays promising properties as a PET tracer for stratification and

prediction of treatment outcome of patients potentially eligible for immune-therapy (Chigoho, D. M. et al., *Pharmaceuticals* 14, 550 (2021); Bridoux, J. et al., *Biomolecules* 10, 1–15 (2020)).

Fluorine-18 (^{18}F) is the most ideal PET radionuclide for clinical application. However, the harsh and complex conditions required for direct radiofluorination are hampering the development of ^{18}F -labelled sdAbs. With the aim of facilitating radiolabelling of sdAbs, enzymes are of interest, as they allow for radiolabelling in aqueous media under mild conditions, preserving the sdAbs' integrity.

The novel family of asparaginyl peptide ligases (PALs) (for example, as disclosed in patent publication nos. WO2020/226572A1, WO2018/056899A1, US2018/0274003A1, US2020/0172893A1 and WO2013/119184A1 herein incorporated by reference in their entirety) such as the VyPAL2 enzyme in its active form have kinetics unmatched by other ligases such as sortase A. Additionally, they can accept a wide range of substrates composed of a short polypeptide linker (stable or cleavable) or polyethylene glycol (PEG) and coupled to cytotoxic elements (e.g., monomethyl auristatin E (MMAE), monomethyl auristatin F (MMAF), DM1, PDB dimer, SN38) or metal chelators such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) or 1,4,7-triazacyclononanetriacetic acid (NOTA). The fast kinetics of the reaction and the substrate flexibility opens new possibilities for radiolabelling of sdAbs.

Accordingly, there is a need to provide improved methods of enzyme-mediated production of labelled antibodies, fragments or mimetics thereof that overcome or at least ameliorate, one or more of the drawbacks described above.

SUMMARY OF THE INVENTION

The present invention relates to a PAL-mediated method of preparing a site-specifically radiolabeled tracer with improved in vivo properties, wherein the yield is further improved to over 90% conversion in the presence of a glutaminyl cyclase (QC) and optimization of enzyme-to-substrate ratios.

In a first aspect, there is provided a method of preparing a site-specifically radiolabeled tracer, the method comprising providing

i) a peptidyl asparaginyl ligase (PAL);

ii) a first peptide comprising a P1-P1'-P2' tripeptide PAL motif at the C-terminus, wherein P1 is Asn or Asp, P1' is Gln or any non-proline amino acid and P2' is a hydrophobic amino acid such as Val or Leu or Ile, or a β -branched amino acid;

iii) a second peptide comprising a P1'-P2" motif at the N-terminus, wherein P1" is Gly and P2" is a hydrophobic amino acid such as Val or Leu or Ile, or a β -branched amino acid, wherein the second peptide is modified with a functional moiety to form a functionalized peptide;

- 5 iv) contacting the peptidyl asparaginyl ligase (PAL) with said first peptide and said functionalized peptide, to form a conjugate of said first peptide-functionalized peptide;

wherein the method further comprises

(a) radiolabeling the conjugate, or

(b) radiolabeling the functionalized peptide prior to contacting with the PAL.

- 10 In some embodiments, the contacting step may be carried out in the presence of a glutaminyl cyclase (QC).

In some embodiments, the first peptide is an antibody or its functional fragment thereof. In other embodiments, the antibody or its functional fragment thereof may be a single-domain antibody (sdAb). In some embodiments, the sdAb is a Programmed death-ligand 1 (hPD-L1)

- 15 sdAb or a Carcinoembryonic Antigen (CEA) sdAb. In some embodiments, the sdAb is selected from the group consisting of (hPD-L1)-NGL-His₆, (hPD-L1)-NQL-His₆, and (CEA)-NQL-His₆ sdAb.

In some embodiments, the P1-P1'-P2' tripeptide PAL motif may be Asn-Gln-Leu (NQL) or Asn-Gly-Leu (NGL).

- 20 In some embodiments, the functionalized peptide may comprise a -GIGK-, -GIGG-, or -GIGGGK- motif. In some embodiments, the functionalized peptide may be selected from the group consisting of GIGK-NOTA, GIGK-DOTA, GIGK-MeTz, GIGGGK-DOTA, GIGK-NOTA-[¹⁸F]AIF, GIGK-Py-DABCO, Ac-GIGK-Py-DABCO, GIGK-Py-NMe₃, Ac-GIGK-PyNMe₃, and GIGK-FPy.

- 25 In some embodiments, the PAL may be a VyPAL2, butelase-1, butelase-2, VyPAL3, OaAEP1b-C247A, HeAEP3, AtLEGy, VuPAL1, HaPAL1, OaAEP1b or a functional fragment or variant thereof.

In some embodiments, the PAL is a VyPAL2 or a functional fragment or variant thereof.

- 30 In other embodiments, the QC may be a Human glutaminyl cyclase, a Mouse glutaminyl cyclase, a Drosophila glutaminyl cyclase, an Arabidopsis glutaminyl cyclase, a Conus

glutaminy cyclase, a Sistrurus glutaminy cyclase, a Bacterial glutaminy cyclase and a functional fragment or variant thereof.

In some embodiments, the QC is a human QC or a functional fragment or variant thereof.

In some embodiments, the molar ratio of PAL: QC is 1:0.1 or 1:0.5.

- 5 In some embodiments, the functionalized peptide may comprise a chelator or a tetrazine group.

In some embodiments, the chelator is selected from the group consisting of 1,4,7-triazacyclononanetriacetic acid (NOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and 1,4,7-triazacyclononane-1-glutaric acid-4,7-diacetic acid (NODAGA).

- 10 In some embodiments, the conjugate or the functionalized peptide may be radiolabeled with a radionuclide selected from the group consisting of Gallium-68 (^{68}Ga), Gallium-67 (^{67}Ga), Lutetium-177 (^{177}Lu), and Fluorine-18 (^{18}F).

In some embodiments, the radionuclide is Fluorine-18 (^{18}F) in the form of [^{18}F]AIF.

In a second aspect, there is provided a site-specifically radiolabeled tracer prepared by the method of the first aspect.

- 15 In some embodiments, the site-specifically radiolabeled tracer comprises a human Programmed death-ligand 1 (hPD-L1) sdAb, or a Carcinoembryonic Antigen (CEA) sdAb.

- In some embodiments, the site-specifically radiolabeled tracer may be selected from the group consisting of [^{68}Ga]Ga-NOTA-KGIG-(hPD-L1) sdAb, [^{18}F]AIF-NOTA-KGIG-(hPD-L1) sdAb, [^{18}F]F-NODAGA-BCN-MeTz-KGIG-(hPD-L1) sdAb, and [^{177}Lu]Lu-DOTA-NHCS-KGIG-CEA
20 sdAb.

In a third aspect, there is provided a site-specifically radiolabeled tracer according to the second aspect for use as an imaging agent.

In a fourth aspect, there is provided a use of the site-specifically radiolabeled tracer according to the second aspect as an imaging agent.

- 25 In a fifth aspect, there is provided a use of the site-specifically radiolabeled tracer according to the second aspect in the manufacture of a diagnostic imaging agent for use in molecular imaging.

In a sixth aspect, there is provided a method of treating a disease in a subject, the method comprising administering the site-specifically radiolabeled tracer according to the second aspect to the subject.

5 In a seventh aspect, there is provided a use of the site-specifically radiolabeled tracer according to the second aspect in the manufacture of a medicament for the treatment of a disease in a subject.

In some embodiments, the disease is cancer.

10 Advantageously, the methods of the present disclosure provide for a fast and efficient generation of sdAb-based site-specifically radiolabelled tracers suitable for use in molecular imaging. More advantageously, the site-specifically radiolabeled antibody tracers of the present disclosure also display improved *in vivo* characteristics compared to ones obtained using chemical site-specific strategies. These and other advantages of the present disclosure will become readily apparent to those skilled in this art from the following detailed description.

BRIEF DESCRIPTION OF THE FIGURES

15 The accompanying drawings illustrate disclosed embodiments and serve to explain the principles of the disclosed embodiments. It is to be understood, however, that the drawings are designed for purposes of illustration only, and not as a definition of the limits of the invention.

20 **FIG. 1** shows the synthetic step of the solid phase peptide synthesis. (A) Boc-protected GIGK-peptidyl backbone (on resin). (B) p-SCN-Bn-NOTA used for the functionalization on the lysine's side chain free amine group. (C) GIGK- NOTA peptide after cleavable from the resin as a TFA salt, TFA = trifluoro acetic acid. (D) COOH-PEG4-MeTz used for the functionalization on the lysine's side chain free amine group. (E) GIGK-MeTz peptide after cleavage from the resin as a TFA salt.

25 **FIG. 2** shows the structure of BCN-NODAGA.

FIG. 3 depicts the Radio-RP-HPLC of the [^{68}Ga]Ga-NOTA-KGIGN-(hPD-L1) sdAb after purification and filtration. $\text{Rt}(^{68}\text{Ga}\text{-labelled sdAb}) = 7.42 \text{ min (99\%)}$, $\text{Rt}(\text{free } ^{68}\text{Ga}) = 3.30 \text{ min (1\%)}$.

30 **FIG. 4** depicts the Radio-SEC of the [^{67}Ga]Ga-DOTA-KGGGIGN-R3b23 sdAb (crude mixture). $\text{Rt}(^{68}\text{Ga}\text{-labelled sdAb}) = 4.85 \text{ min (30\%)}$, $\text{Rt}(\text{free } ^{68}\text{Ga}) = 5.70 \text{ min (70\%)}$.

FIG. 5 shows the Radio RP-HPLC on the XBridge C18 column of the [^{18}F]AIF-NODAGA-BCN after tC18 purification. $\text{Rt}([\text{F}^{18}]\text{AIF-NODAGA-BCN}) = \text{Rt} = 3.05 \text{ min (92\%)}$, $\text{Rt}([\text{F}^{18}]\text{AIF/NaF}) = 1.25 \text{ min (4\%)}$, $\text{Rt}(\text{radio-impurities due to degradation}) = 1.77 \text{ min (4\%)}$.

FIG. 6 depicts the Radio-SEC on the Superdex 75 Increase 3.2/300GL column of the chemically MeTz-functionalized hPD-L1 sdAb click to [^{18}F]AIF-NODAGA-BCN after purification and filtration. $\text{Rt}([\text{F}^{18}]\text{AIF-NODAGA-BCN-MeTz-mal-cys-(hPD-L1) sdAb}) = 11.4 \text{ min (>95\%)}$.

FIG. 7 shows the Radio-SEC and UV profile (280 nm) analysis on the TSKgel column of the crude mixture after 1 hour of reaction containing 1 eq. of NQL-His₆ hPD-L1 sdAb and 1 eq. of [^{18}F]AIF-NOTA-KGIG. $\text{Rt}([\text{F}^{18}]\text{AIF-NOTA-KGIG-(hPD-L1) sdAb}) = 9.87 \text{ min (41\%)}$, $\text{Rt}([\text{F}^{18}]\text{AIF-NOTA-KGIG}) = 11.97 \text{ min (59\%)}$. Sample diluted in PBS/Tween 20, which gives a signal in the UV at $\text{Rt}(\text{Tween 20}) = 6.8 \text{ min}$.

FIG. 8 shows the Radio-SEC and UV profile (280 nm,) of the purified and filtered [^{18}F]AIF-NOTA-KGIG-(hPD-L1) sdAb on a Superdex 75 Increase 3.2/300GL column. $\text{Rt}([\text{F}^{18}]\text{AIF-NOTA-KGIG-(hPD-L1) sdAb}) = 11.6 \text{ min (95\%)}$, $\text{Rt}([\text{F}^{18}]\text{AIF-NOTA-KGIG}) = 15.53 \text{ min (5\%)}$, sample diluted in PBS/Tween 20 0.1%. $\text{Rt}(\text{Tween 20}) = 6.8 \text{ min}$.

FIG. 9 depicts the Radio-SEC profile on a TSKgel column of the crude mixture after 30 min of incubation containing 1 eq. of [^{18}F]AIF-NOTA-KGIG, 1 eq. of NQL-His₆-tagged hPD-L1 sdAb and 0.01 eq. of enzymatic mix. $\text{Rt}([\text{F}^{18}]\text{AIF-NOTA-KGIG-(hPD-L1) sdAb}) = 10.02 \text{ min (79\%)}$. $\text{Rt}([\text{F}^{18}]\text{AIF-NOTA-KGIG}) = 11.25 \text{ min (9\%)}$. $\text{Rt}(\text{radio-impurities}) = 15.90 \text{ min (12\%)}$.

FIG. 10 shows the QCO (quality control) of radiolabelled sdAb after purification and filtration. (A) Radio-RP-HPLC analysis on the PLRP-S column. $\text{Rt}([\text{F}^{18}]\text{AIF-NOTA-KGIG-sdAb}) = 6.42 \text{ min (93\%)}$. $\text{Rt}(\text{smaller radio-impurities}) = 3.85 \text{ min (7\%)}$. (B) Same sample analysed in the UV spectrum at 280 nm. $\text{Rt}(\text{sdAb}) = 6.25 \text{ min (100\%)}$. A $\pm 20 \text{ sec}$ shift between the UV and the radio-signal is expected as the radio-detector is located after the UV detector of the HPLC system.

FIG. 11 shows the in vitro cell binding study at 3 nM of radiolabelled hPD-L1 sdAbs analogues on hPD-L1 positive (POS) cells. hPD-L1 Negative (NEG) cells and POS cells blocked with a 100-fold excess of non-radiolabelled sdAb were used as controls to assess specificity. CPM = counts per minutes. (***, $p < 0.001$; **, $p < 0.01$) (A) Results for the [^{68}Ga]Ga-NOTA-KGIG-(hPD-L1) sdAb. (B) [^{18}F]AIF-NOTA-KGIG-(hPD-L1) sdAb. (C) [^{18}F]F-NODAGA-BCN-MeTz-KGIG-(hPD-L1) sdAb. (D) [^{18}F]F-NODAGA-BCN-MeTz-mal-cys-(hPD-L1) sdAb.

FIG. 12 shows the affinity assay (K_D) of the ^{18}F -labelled sdAbs analogues by cell saturation on hPD-L1^{POS} 624-MEL cells. Cell bound activity in count per minutes (CPM) expressed as a function of the sdAb concentration (nM) for **(A)** [^{18}F]AIF-NOTA-KGIG-(hPD-L1) sdAb ($K_D = 2.5$ nM), **(B)** [^{18}F]F-NODAGA-BCN-MeTz-KGIG-(hPD-L1) sdAb ($K_D = 4.34$ nM) and **(C)** [^{18}F]F-NODAGA-BCN-MeTz-mal-cys-(hPD-L1) sdAb ($K_D = 3.18$ nM).

FIG. 13 depicts the biodistribution profiles in healthy mice (N = 3 mice per group) at 80 min post-injection (left bars with diagonal stripes) [^{18}F]F-NOTA-KGIG-(hPD-L1) sdAb and (right bars with horizontal stripes) [^{18}F]F-NOTA-KGIG-(R3b23) sdAb. Nb = sdAb = single-domain antibody. Results are expressed as mean $\pm$ standard deviation in %A/g = % of injected activity per gram of organ. (****, $p < 0.0001$).

FIG. 14 depicts the biodistribution profiles in healthy mice (N = 3 mice per group) at 80 min post-injection (left bars with checkerboard pattern) [^{68}Ga]Ga-NOTA-KGIG-(hPD-L1) sdAb and (right bars with diagonal stripes) [^{18}F]F-NOTA-KGIG-(hPD-L1) sdAb. Nb = sdAb = single-domain antibody. Results are expressed as mean $\pm$ standard deviation in %A/g = % of injected activity per gram of organ. (***, $p < 0.001$; **, $p < 0.01$).

FIG. 15 shows the biodistribution profiles in healthy mice (N = 3 mice per group) at 80 min post-injection (left bars with dotted pattern) [^{18}F]F-NODAGA-BCN-MeTz-KGIG-(hPD-L1) sdAb, (middle bars with diagonal stripes) [^{18}F]F-NOTA-KGIG-(hPD-L1) sdAb and (right bars with no pattern) [^{18}F]F-NODAGA-BCN-MeTz-mal-cys-(hPD-L1) sdAb. Nb = sdAb = single-domain antibody. Results are expressed as mean $\pm$ standard deviation in %A/g = % of injected activity per gram of organ. (**, $p < 0.01$; *, $p < 0.05$).

FIG. 16 shows the structure of peptidyl substrates for radiofluorination via nucleophilic substitution. The products are obtained as TFA salts (not represented on the figure). (Top) GIGK-Py-DABCO was obtained in 37% yield. The amine actyl-protected variant of the peptide (Ac-GIGK-Py-DABCO) was obtained in 47% yield. (Bottom) GIGK-Py-NMe₃ was obtained on 50% yield. The amine actyl-protected variant of the peptide (Ac-GIGK-Py-NMe₃) was obtained in 36% yield.

FIG. 17 depicts the Radio-RP-HPLC profile on a PLRP-S column of the crude mixture after 15 min of reaction at 40°C and pH 6.5, containing (hPD-L1)-NQL-His₆ sdAb (1 eq. at 1 mg/mL), 1 eq. (3.58 MBq) of [^{18}F]AIF-NOTA-NHCS-KGIG and 0.01 eq. of VyPAL2 (+GluC 0.5 eq. relative to VyPAL2). Rt([^{18}F]AIF-NOTA-KGIG-(hPD-L1) sdAb) = 7.13 min (92%), Rt([^{18}F]AIF-NOTA-KGIG) = 6.32 min (8%). (Internal reference SGD-2274-149).

FIG. 18 shows the Radio-RP-HPLC profile on a PLRP-S column of the crude mixture after 7 min of reaction at 40°C and pH 6.5, containing (hPD-L1)-NQL-His₆ sdAb (1 eq. at 1 mg/mL), 1 eq. (8.8 MBq) of [¹⁸F]AIF-NOTA-NHCS-KGIG and 0.1 eq. of VyPAL2 (+GluC 0.5 eq. relative to VyPAL2) which had been pre-irradiated with 900 MBq of [¹⁸F]NaF salts for 7 minutes.

5 Rt([¹⁸F]AIF-NOTA-KGIG-(hPD-L1) sdAb) = 6.83 min (85%), Rt([¹⁸F]AIF-NOTA-KGIG) = 6.03 min (15%). The injection peak at 2.5—4 min contains the excess of radioactive salt, therefore signal saturates in this region. Compared to FIG. 17, the retention times have a 0.3 min shift as a newer PLRP-S column is used with higher column pressure, leading to slightly earlier retention times. (Internal reference SGD-2686-017).

10 **FIG. 19** shows RP-HPLC profile in the UV at 215 nm on a PLRP-S column, of the non-radioactive reference peptide with a Rt(FPy-KGIG) = 6.28 min. (internal reference: JBR-2690-041). The Rt matches the expected Rt as compared with the radioactive peptide at 6.43 minutes with a typical shift of 0.15 min due to the delay between the UV and the radio-detector.

FIG. 20 depicts the UV profile at 280 nm on a C4 column of a crude mixture containing 1 eq. of R3b23-NQL-His₆ sdAb and 1 eq. of non-radioactive reference FPy-KGIG peptide, in presence of 0.1 eq. of VyPAL2 (+1 eq. of GluC relative to VyPAL2), for 5 minutes at 37°C.

15 Rt(R3b23-NQL-His₆), Rt(supposed R3b23-GIGK-PyF) = 7.53 min. (internal reference: SGD-2686-117).

FIG. 21 shows the Radio-RP-HPLC profile of the crude [¹⁷⁷Lu]Lu-DOTA-mal-CEA sdAb after 2 h at 60°C on a PLRP-S column. Rt([¹⁷⁷Lu]Lu-DOTA-mal-CEA) = 7.82 min, Rt(free [¹⁷⁷Lu]LuCl₃) = 3.20 min (internal reference: SGD-2686-097).

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FIG. 22 shows the Radio-RP-HPLC profile of the crude [¹⁷⁷Lu]Lu-DOTA-NHCS-KGIG-CEA sdAb after 45 min at 40°C on a PLRP-S column. Rt([¹⁷⁷Lu]Lu-DOTA-NHCS-KGIG-CEA) = 8.00 min, Rt(free [¹⁷⁷Lu]LuCl₃) = 3.40 min (internal reference: JBR-2690-044).

25 **FIG. 23** shows a table depicting the optimization of parameters influencing the radiofluorination of the NOTA-GIGK peptide. % of complexed AIF was measured on the XBridge C18 analytical column.

DETAILED DESCRIPTION OF THE INVENTION

The following detailed description refers to, by way of illustration, specific details and

30 embodiments in which the invention may be practiced. These embodiments are described in sufficient detail to enable those skilled in the art to practice the invention. Other embodiments may be utilized and structural, and logical changes may be made without departing from the scope of the invention. The various embodiments are not necessarily mutually exclusive, as

some embodiments can be combined with one or more other embodiments to form new embodiments.

Bibliographic references mentioned in the present specification are for convenience listed in the form of a list of references and added at the end of the examples. The whole content of
5 such bibliographic references is herein incorporated by reference but their mention in the specification does not imply that they form part of the common general knowledge.

Definitions

For convenience, certain terms employed in the specification, examples and appended claims are collected here.

10 In general, technical, scientific and medical terminologies used herein has the same meaning as understood by those skilled in the art to which this invention belongs. Further, the following technical comments and definitions are provided. These definitions should in no way limit the scope of the present invention to those terms alone, but are put forth for a better understanding of the following description.

15 As used herein, "a" or "an" may mean one or more than one unless indicated to the contrary or otherwise evident from the context.

As used herein, the term "amino acid" may refer to natural and/or unnatural or synthetic amino acids, including both the D and L optical isomers, amino acid analogs (for example norleucine is an analog of leucine) and peptidomimetics. As used in the context of the present application,
20 the term "amino acid" typically refers to the 20 naturally occurring L-amino acids, namely Gly, Ala, Val, Leu, He, Phe, Cys, Met, Pro, Thr, Ser, Glu, Gln, Asp, Asn, His, Lys, Arg, Tyr, and Trp.

As used herein, the term "comprising" or "including" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not
25 preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. However, in context with the present disclosure, the term "comprising" or "including" also includes "consisting of". The variations of the word "comprising", such as "comprise" and "comprises", and "including", such as "include" and "includes", have correspondingly varied meanings.

30 As used herein, the terms "peptide", "polypeptide" and "protein" are used interchangeably to denote a polymer of at least two amino acids covalently linked by an amide bond. Whereas

peptides are considered to be short amino acid chains, polypeptides are long amino acid chains and proteins tend to have a stable structure and may comprise modifications (e.g., glycosylation or phosphorylation). The term "protein" may encompass a naturally-occurring as well as artificial (e.g., engineered or variant) full-length protein as well as a functional fragment of the protein.

As used herein, the term "functional fragment" refers to a portion of a protein that retains some or all of the activity or function (e.g., biological activity or function, such as enzymatic activity, or antigen-binding properties) of the full-length protein, such as, e.g., the ability to catalyse a ligation reaction between two peptides. The functional fragment can be any size, provided that the fragment retains the activity/functionality of the full-length protein /enzyme/antibody. For example, an antibody fragment may retain the essential specificity and affinity of the full-length antibody. Examples of an antibody fragment include but not limited to antigen-binding fragment (Fab), F(ab')₂, single chain variable fragment (scFv), single-domain antibody (sdAb) also known as a nanobody, variable domain of heavy-chain only antibody (VhH), minibody, diabody, and the like.

As used herein, the term "functionalized peptide" refers to a peptide sequence that has been modified and/or complexed with other functional group(s), moieties, compound(s), and or agent(s) to enhance and/or broaden the peptide functionality to achieve its intended purpose, for example, for application in medicine. For example, a cancer-targeting peptide may be functionalized to comprise a linker carrying a therapeutic agent as part of a drug delivery system. As used in the present invention, a peptide may be functionalized to preferably comprise an agent that is suitable for use as a diagnostic agent and/or for therapeutic application. For example, the functionalized peptide of the present disclosure may be modified to comprise a radiolabelled chelator. Other functional group(s), moieties, compound(s), and or agent(s) suitable for use in the present invention include but not limited to chelators such as 1,4,7-triazacyclononanetriacetic acid (NOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and 1,4,7-triazacyclononane-1-glutaric acid-4,7-diacetic acid (NODAGA), tetrazine moieties such as MeTz, 1,2,3,4-tetrazines, 1,2,3,5-tetrazines, and 1,2,4,5-tetrazines, a probe/linker such as bicyclononyne, functional group such as DABCO and NMe₃, and other detectable and/or optical labels such as horseradish peroxidase (HRP) or green fluorescent protein (GFP).

As used herein, "single-domain antibody" (sdAb), or a nanobody, refers to an antibody fragment that comprises a single monomeric variable antibody domain or one engineered constant domain that solely facilitates target binding. It is able to bind selectively to a specific

antigen, like a full-length antibody. Methods of generating sdAbs are known in the art. It would be understood that, for the purpose of the invention, any antibody or a functional fragment thereof may be suitable for use in the present invention, provided that it is suitably adapted to comprise a P1-P1'-P2' tripeptide PAL recognition motif to facilitate a ligation reaction using
5 PAL, optionally in the presence of a glutaminyl cyclase (QC).

As used herein, the term "radiolabelling" refers to a method of labelling peptides/proteins using radioactive isotopes. Various radiolabelling strategies to incorporate a radionuclide into a peptide/protein are known in the art. For example, via direct labelling, via indirect labelling via a prosthetic group and, through indirect labelling via complexation. It would be appreciated
10 that radioactive isotopes may be directly integrated into a peptide molecule by electrophilic substitution or indirectly via conjugation. In other instances, radioactive metals may be labelled via complexation with a chelator/chelating agent. Examples of radionuclides/radioisotopes include but not limited to gallium-68 (⁶⁸Ga), gallium-67 (⁶⁷Ga), indium-111 (¹¹¹In), lutetium-177 (¹⁷⁷Lu), fluorine-18 (¹⁸F), iodine-124 (¹²⁴I), iodine-131 (¹³¹I), iodine-123 (¹²³I), iodine-125 (¹²⁵I),
15 phosphorus-32 (³²P), strontium-90 (⁹⁰Sr), strontium-89 (⁸⁹Sr), yttrium-90 (⁹⁰Y), radium-223 (²²³Ra), radium-226 (²²⁶Ra), caesium-137 (¹³⁷Cs), technetium-99m (^{99m}Tc), tin-117 (^{117m}Sn), samarium-153 (¹⁵³Sm), erbium-169 (¹⁶⁹Er), rhenium-188 (¹⁸⁸Re), rhenium-186 (¹⁸⁶Re), thallium-201 (²⁰¹Tl), carbon-11 (¹¹C), oxygen-15 (¹⁵O-), copper-64 (⁶⁴Cu), lead-212 (²¹²Pb) astatine-211 (²¹¹At), actinium-225 (²²⁵Ac), Terbium-149/161/155/152 (^{149/161/155/152}Tb), and
20 iridium-192 (¹⁹²Ir).

As used herein, the term "peptide asparaginyl ligases (PALs)" refers to a special group of enzymes that primarily catalyse peptide bond formation. In this regard, PALs typically perform site-specific ligation reactions and recognize a P1-P1'-P2' tripeptide motif, for ligation after P1, wherein P1 is typically Asn or Asp, and P1' and P2' may be any of the naturally occurring
25 amino acids Gly, Ala, Val, Leu, Ile, Phe, Cys, Met, Thr, Ser, Glu, Gln, Asp, Asn, His, Lys, Arg, Tyr, and Trp. PALs cleave the first peptide/protein after P1 in the tripeptide PAL motif and ligates said first peptide/protein to the P1"-P2" motif of said second peptide/protein.

As used herein, the term "QC" refers to glutaminyl cyclase (QC) enzyme and QC-like enzymes. QC and QC-like enzymes have identical or similar enzymatic activity, i.e., catalysing
30 the intramolecular cyclization of N-Terminal glutaminyl and glutamyl residues of peptides and proteins to form pyroglutamyl residue (pGlu). In this regard, QC-like enzymes can fundamentally differ in their molecular structure from QC.

As used herein, the term "variant", refers to an amino acid sequence that is altered by one or more amino acids of the non-variant reference sequence, but retains the ability to recognize

its target and affect its function. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties (e.g., replacement of leucine with isoleucine). More rarely, a variant may have "non-conservative" changes (e.g., replacement of glycine with tryptophan). Analogous minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological activity may be found using computer programs well known in the art, for example, DNASTAR® software (DNASTAR, Inc. Madison, Wisconsin, USA).

A description of exemplary, non-limiting embodiments of the invention follows.

10 The present invention provides a new and improved method for the rapid and reagent-effective generation of radiolabelled single-domain antibodies (sdAbs) with improved *in vivo* properties.

To this end, provided in one aspect of the present disclosure is of preparing a site-specifically radiolabeled tracer, the method comprising providing

- i) a peptidyl asparaginyl ligase (PAL);
 - 15 ii) a first peptide comprising a P1-P1'-P2' tripeptide PAL motif at the C-terminus, wherein P1 is Asn or Asp, P1' is Gln or any non-proline amino acid, and P2' is a hydrophobic amino acid such as Val, Leu or Ile, or a β -branched amino acid;
 - iii) a second peptide comprising a P1"-P2" motif at the N-terminus, wherein P1" is Gly and P2" is a hydrophobic amino acid such as Val or Leu or Ile, or a β -branched amino acid, wherein the second peptide is modified with a functional moiety to form a functionalized peptide;
 - 20 iv) contacting the peptidyl asparaginyl ligase (PAL) with said first peptide and said functionalized peptide to form a conjugate of said first peptide-functionalized peptide;
- wherein the method further comprises
- 25 (a) radiolabeling the conjugate, or
 - (b) radiolabeling the functionalized peptide prior to contacting with the PAL.

It would be appreciated that PALs perform site-specific ligation reactions and require a minimal tripeptide recognition motif, P1-P1'-P2', for ligation after P1, wherein P1 is typically Asn or

Asp, and P1' and P2' may be any of the naturally occurring amino acids Gly, Ala, Val, Leu, Ile, Phe, Cys, Met, Thr, Ser, Glu, Gln, Asp, Asn, His, Lys, Arg, Tyr, and Trp.

For the purposes of the present invention, P1 is preferably Asn or Asp, P1' is preferably Gln or any non-proline amino acid and P2' is preferably a hydrophobic amino acid such as Val or Leu or Ile, or a β -branched amino acid. In some embodiments, P1 is preferably Asn and P1' is preferably Gln, Gly or Ser. In some embodiments, the P1-P1'-P2' tripeptide PAL motif is a Asn-X-Leu motif, wherein X is Gln (Q), Gly (G) or Glu (E). In some preferred embodiments, the first peptide may comprise a Asn-Gln-Leu (NQL) or Asn-Gly-Leu (NGL) tripeptide PAL motif.

It would be appreciated that any peptide may be suitable for use in the present invention as the first peptide, provided that it is suitably adapted to comprise a P1-P1'-P2' tripeptide PAL recognition motif as described herein to facilitate a ligation reaction.

It would also be appreciated that the first peptide may be further modified to comprise an affinity tag such as a His tag to facilitate its purification process. Accordingly in some embodiments, the first peptide may comprise a His₆ tag at its C-terminus. In further embodiments, the first peptide may comprise a -NGL-His₆ or a -NQL-His₆ tag at its C-terminus.

In some embodiments, the first peptide may be an antibody, a scaffold protein, an antibody mimetic, or its functional fragment thereof.

In some embodiments, the first peptide may be an antibody or a functional fragment thereof. In particular, the antibody or a functional fragment thereof may be selected from the group consisting of Fab, F(ab')₂, scFv, and sdAb.

In some embodiments, the antibody or a functional fragment thereof is an sdAb. In some embodiments, the sdAb may be selected from the group consisting of hPD-L1 sdAb, R3b23 sdAb, and CEA-targeting sdAb. In some embodiments, the sdAb is selected from the group consisting of (hPD-L1)-NGL-His₆, (hPD-L1)-NQL-His₆, R3b23-NGL-His₆, R3b23-NQL-His₆, and (CEA)-NQL-His₆ sdAb.

In some embodiments, the first peptide may also be a scaffold protein and/or an antibody mimetic, or a functional fragment thereof. Examples of antibody mimetics may include, but not limited, to affibody molecules, DARPins, affimers, anticalins, alphabodies, anticalins, avimers, monobodies, Kunitz domain peptides, and nanoCLAMPs etc. In relation to the present invention, it would be appreciated that the PAL cleaves the first peptide after P1 in the

tripeptide PAL motif, and ligates said first peptide to the P1"-P2" motif of the said functionalized peptide.

In some embodiments, P2" may be a hydrophobic amino acid or a β -branched amino acid. Examples of a hydrophobic amino acid may include Gly, Ala, Val, Leu, Ile, Pro, Phe, Met, Tyr
5 and Trp. Examples of a β -branched amino acid include Thr, Val, and Ile. In some embodiments, P2" is a hydrophobic amino acid selected from Val, Leu or Ile.

In some embodiments, the P1"-P2" motif of the functionalized peptide may be a -G-I motif. In various other embodiments, the functionalized peptide may comprise a -GIGK-, -GIGG-, or -GIGGGK- motif at its N-terminus.

10 In some embodiments, the functionalized peptide is selected from the group consisting of GIGK-NOTA, GIGK-DOTA, GIGK-MeTz, GIGGGK-DOTA, GIGK-NOTA-[¹⁸F]AIF, GIGK-Py-DABCO, Ac-GIGK-Py-DABCO, GIGK-Py-Nme₃, Ac-GIGK-PyNMe₃, and GIGK-FPy.

It would be appreciated by a person skilled in the art that different PALs and variants thereof having the desired protein ligase activity may be suitable for the practice of the present
15 invention. Accordingly in some embodiments, the PAL may be a VyPAL2, butelase-1, butelase-2, VyPAL3, OaAEP1b-C247A, HeAEP3, AtLEGy, VuPAL1, HaPAL1, OaAEP1b or a functional fragment or variant thereof. In other embodiments, the PAL is a VyPAL2 or a functional fragment or variant thereof.

In certain embodiments, the PAL may be selected from the group comprising VyPAL2
20 comprising the amino acid sequence set forth in SEQ ID NO: 32, butelase-1 comprising the amino acid sequence set forth in SEQ ID NO: 33, butelase-2 comprising the amino acid sequence set forth in SEQ ID NO: 34 or SEQ ID NO: 35, , VyPAL3 comprising the amino acid sequence set forth in SEQ ID NO: 36, OaAEP1b-C247A comprising the amino acid sequence set forth in SEQ ID NO: 37, HeAEP3 comprising the amino acid sequence set forth in SEQ ID
25 NO: 38, AtLEGy comprising the amino acid sequence set forth in SEQ ID NO: 39, VuPAL1 comprising the amino acid sequence set forth in SEQ ID NO: 440, HaPAL1 comprising the amino acid sequence set forth in SEQ ID NO: 41, OaAEP1b comprising the amino acid sequence set forth in SEQ ID NO: 42 and a functional fragment or a variant thereof.

In other preferred embodiments, the PAL may be VyPAL2 comprising the amino acid
30 sequence set forth in SEQ ID NO: 32 or a functional fragment or a variant thereof.

It would be appreciated that the presence of a glutaminyl cyclase (QC) improves the ligation reaction between the substrates catalysed by the PAL. Accordingly in some embodiments, the contacting step is carried out in the presence of a glutaminyl cyclase (QC).

5 It is also envisaged that various QCs having the desired QC enzymatic activity may be suitable for use in the practice of the present invention. Accordingly in some embodiments, the QC may be a Human glutaminyl cyclase, a Mouse glutaminyl cyclase, a Drosophila glutaminyl cyclase, an Arabidopsis glutaminyl cyclase, a Conus glutaminyl cyclase, a Sistrurus glutaminyl cyclase, a Bacterial glutaminyl cyclase or a functional fragment or variant thereof.

10 In some embodiments, the QC is a human glutaminyl cyclase or a functional fragment or variant thereof.

In some embodiments, the QC may be selected from the group comprising Human glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 43, Mouse glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 44, Drosophila glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 45, 15 Arabidopsis glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 46, Conus glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 47, Sistrurus glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 48, Bacterial glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 49 and a functional fragment or a variant thereof.

20 In some preferred embodiments, the QC is Human glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 43 or a functional fragment or a variant thereof.

As those skilled in the art would appreciate, a protein/enzyme's function is directly related to its structure and sequence, and that there is a positive relationship between sequence identity and function similarity. In this regard, methods of determining a protein sequence identity are 25 known in the art. Accordingly, the sequences of the enzymes of the present disclosure may be sufficiently varied so long as the enzymes maintain their functionality and can exhibit the required activity.

In some embodiments, the PAL may be a VyPAL2 comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 30 99% identity to the amino acid sequence set forth in SEQ ID NO: 33, a butelase-1 comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth in SEQ ID

NO: 33, a butelase-2 comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth in SEQ ID NO: 34 or 35, a VyPAL3 comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth in SEQ ID NO: 36, a OaAEP1b-C247A comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth in SEQ ID NO: 37, a HeAEP3 comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth in SEQ ID NO: 38, a AtLEGy comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth in SEQ ID NO: 39, a VuPAL1 comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth in SEQ ID NO: 40, a HaPAL1 comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth in SEQ ID NO: 41 or a OaAEP1b comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth in SEQ ID NO: 42.

In some embodiments, the QC may be a Human glutaminyl cyclase comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth SEQ ID NO: 43, a Mouse glutaminyl cyclase comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth SEQ ID NO: 44, a Drosophila glutaminyl cyclase comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth SEQ ID NO: 45, an Arabidopsis glutaminyl cyclase comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth SEQ ID NO: 46, a Conus glutaminyl cyclase comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth SEQ ID NO: 47, a Sistrurus glutaminyl cyclase comprising the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth SEQ ID NO: 48, or a Bacterial glutaminyl cyclase comprising

the amino acid sequence with at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence set forth SEQ ID NO: 49.

Table 1. Single-domain antibodies (sdAbs), functionalized peptides, conjugates, radiolabelled sdAb tracer, peptide asparaginyl ligases (PALs), and glutaminyl cyclases (QC) of the present invention and their amino acid sequences.

Name /SEQ ID NO.	Amino Acid Sequence
(hPD-L1)-NGL-His₆ sdAb SEQ ID NO: 1 Length: 129 amino acids	DVQLVESGGG LVQPGGSLRL SCAASGFTFS SFAMSWVRQA PGKGLEWVSD IDTTGRTTDY ADSVKGRFTI SRDNAENTLY LQMNDLKPED TAVYYCANVP KELVLSFGSW GPGTQVTVSS NGLHHHHHHH
(hPD-L1)-NQL-His₆ sdAb SEQ ID NO: 2 Length: 129 amino acids	DVQLVESGGG LVQPGGSLRL SCAASGFTFS SFAMSWVRQA PGKGLEWVSD IDTTGRTTDY ADSVKGRFTI SRDNAENTLY LQMNDLKPED TAVYYCANVP KELVLSFGSW GPGTQVTVSS NQLHHHHHHH
R3b23-NGL-His₆ sdAb SEQ ID NO: 3 Length: 137 amino acids	DVQLVESGGG SVQAGGSLRL SCAASGDTGY MGWFRQAPGK EREGVAVINS DSGVGSTYYA DSVKGRFTIS RDNAKNTVYL QMNSLKPEDT AIYYCAAGHF SDYVSPWTWR EIYRYNVWGQ GTQVTVSSNG LHHHHHHH
R3b23-NQL-His₆ sdAb SEQ ID NO: 4 Length: 137 amino acids	DVQLVESGGG SVQAGGSLRL SCAASGDTGY MGWFRQAPGK EREGVAVINS DSGVGSTYYA DSVKGRFTIS RDNAKNTVYL QMNSLKPEDT AIYYCAAGHF SDYVSPWTWR EIYRYNVWGQ GTQVTVSSNQ LHHHHHHH
(hPD-L1)-linker-his₆-cys sdAb SEQ ID NO: 5 Length: 141 amino acids	QVQLQESGGG LVQPGGSLRL SCAASGFTFS SFAMSWVRQA PGKGLEWVSD IDTTGRTTDY ADSVKGRFTI SRDNAENTLY LQMNDLKPED TAVYYCANVP KELVLSFGSW GPGTQVTVSS HHHHHHSPST PPTPSPSTPP C
carcinoembryonic antigen (CEA)-NQL-His₆ sdAb SEQ ID NO: 6 Length: 132 amino acids	DVQLVESGGG SVQAGGSLRL SCAASGDTYG SYWMGWFRQA PGKEREGVAA INRGGGYTVY ADSVKGRFTI SRDTAKNTVY LQMNSLRPDD TADYYCAASG VLGGLHEDWF NYWGQGTQVT VSSNQLHHHHH HH

CEA-linker-His₆-cys sdAb SEQ ID NO: 7 Length: 150 amino acids	QVQLVESGGG SVQAGGSLRL SCAASGDTYG SYWMGWFRQA PGKEREGVAA INRGGGYTVY ADSVKGRFTI SRDTAKNTVY LQMNSLRPDD TADYYCAASG VLGGLHEDWF NYWGQGTQVT VSSLPETGGH HHHHHSPSTP PTPSPSTPPC
CEA-mal-DOTA sdAb SEQ ID NO: 8 Length: 150 amino acids	QVQLVESGGG SVQAGGSLRL SCAASGDTYG SYWMGWFRQA PGKEREGVAA INRGGGYTVY ADSVKGRFTI SRDTAKNTVY LQMNSLRPDD TADYYCAASG VLGGLHEDWF NYWGQGTQVT VSSLPETGGH HHHHHSPSTP PTPSPSTPPC -mal-DOTA
[¹⁷⁷Lu]Lu-DOTA-mal-CEA sdAb SEQ ID NO: 9 Length: 150 amino acids	QVQLVESGGG SVQAGGSLRL SCAASGDTYG SYWMGWFRQA PGKEREGVAA INRGGGYTVY ADSVKGRFTI SRDTAKNTVY LQMNSLRPDD TADYYCAASG VLGGLHEDWF NYWGQGTQVT VSSLPETGGH HHHHHSPSTP PTPSPSTPPC -mal-DOTA- [¹⁷⁷ Lu] Lu
GIGK-NOTA peptide SEQ ID NO: 10 Length: 4 amino acids	GIGK - (NHCS-Bn-NOTA)
GIGK-DOTA peptide SEQ ID NO: 11 Length: 4 amino acids	GIGK - (NHCS - DOTA)
GIGK-MeTz peptide SEQ ID NO: 12 Length: 4 amino acids	GIGK - (NH-PEG4-Bn-MeTz)
GIGGGK-DOTA peptide SEQ ID NO: 13 Length: 6 amino acids	GIGGGK - (DOTA)

GIGK-NOTA-¹⁸F]AIF peptide SEQ ID NO: 14 Length: 4 amino acids	GIGK - (NHCS-Bn-NOTA) - [¹⁸ F]AlF
GIGK-Py-DABCO peptide SEQ ID NO: 15 Length: 4 amino acids	GIGK - Py - DABCO
Ac-GIGK-Py-DABCO peptide SEQ ID NO: 16 Length: 4 amino acids	Ac - GIGK - Py - DABCO
GIGK-Py-NMe₃ peptide SEQ ID NO: 17 Length: 4 amino acids	GIGK - Py - NMe ₃
Ac-GIGK-Py-NMe₃ peptide SEQ ID NO: 18 Length: 4 amino acids	Ac - GIGK - Py - NMe ₃
GIGK-FPy peptide SEQ ID NO: 19 Length: 4 amino acids	GIGK - PyF
(hPD-L1)-GIGK-MeTz conjugate SEQ ID NO: 20 Length: 125 amino acids	DVQLVESGGG LVQPGGSLRL SCAASGFTFS SFAMSWVRQA PGKGLEWVSD IDTTGRITDY ADSVKGRFTI SRDNAENTLY LQMNDLKPED TAVYYCANVP KELVLSFGSW GPGTQVTVSS NGIGK-PEG ₄ -MeTz

(hPD-L1)-GIGK- NOTA conjugate SEQ ID NO: 21 Length: 125 amino acids	DVQLVESGGG LVQPGGSLRL SCAASGFTFS SFAMSWVRQA PGKGLEWVSD IDTTGRTTDY ADSVKGRFTI SRDNAENTLY LQMNDLKPED TAVYYCANVP KELVLSFGSW GPGTQVTVSS NGIGK-NHCS-NOTA
R3b23-GIGK- MeTz conjugate SEQ ID NO: 22 Length: 133 amino acids	DVQLVESGGG SVQAGGSLRL SCAASGDTGY MGWFRQAPGK EREGVAVINS DSGVGSTYYA DSVKGRFTIS RDNAKNTVYL QMNSLKPEDT AIYYCAAGHF SDYVSPWTWR EIYRYNVWGQ GTQVTVSSNG IGK-PEG ₄ -MeTz
R3b23-GIGGGK- DOTA conjugate SEQ ID NO: 23 Length: 135 amino acids	DVQLVESGGG SVQAGGSLRL SCAASGDTGY MGWFRQAPGK EREGVAVINS DSGVGSTYYA DSVKGRFTIS RDNAKNTVYL QMNSLKPEDT AIYYCAAGHF SDYVSPWTWR EIYRYNVWGQ GTQVTVSSNG IGGGK-DOTA
R3b23-GIGK-PyF conjugate SEQ ID NO: 24 Length: 133 amino acids	DVQLVESGGG SVQAGGSLRL SCAASGDTGY MGWFRQAPGK EREGVAVINS DSGVGSTYYA DSVKGRFTIS RDNAKNTVYL QMNSLKPEDT AIYYCAAGHF SDYVSPWTWR EIYRYNVWGQ GTQVTVSSNG IGK-PyF
CEA-GIGK-DOTA conjugate SEQ ID NO: 25 Length: 128 amino acids	DVQLVESGGG SVQAGGSLRL SCAASGDTYG SYWMGWFRQA PGKEREVAA INRGGGYTVY ADSVKGRFTI SRDTAKNTVY LQMNSLRPDD TADYYCAASG VLGGLHEDWF NYWGQGTQVT VSSNGIGK-NHCS-DOTA
[⁶⁸Ga]Ga-NOTA- KGIG-(hPD-L1) sdAb SEQ ID NO: 26 Length: 125 amino acids	DVQLVESGGG LVQPGGSLRL SCAASGFTFS SFAMSWVRQA PGKGLEWVSD IDTTGRTTDY ADSVKGRFTI SRDNAENTLY LQMNDLKPED TAVYYCANVP KELVLSFGSW GPGTQVTVSS NGIGK-NHCS-NOTA- [⁶⁸ Ga] Ga
[¹⁸F]AIF-NOTA- KGIG-(hPD-L1) sdAb SEQ ID NO: 27 Length: 125 amino acids	DVQLVESGGG LVQPGGSLRL SCAASGFTFS SFAMSWVRQA PGKGLEWVSD IDTTGRTTDY ADSVKGRFTI SRDNAENTLY LQMNDLKPED TAVYYCANVP KELVLSFGSW GPGTQVTVSS NGIGK-NHCS-NOTA-Al [¹⁸ F] F

[¹⁸F]F-NODAGA-BCN-MeTz-KGIG-(hPD-L1) sdAb SEQ ID NO: 28 Length: 125 amino acids	DVQLVESGGG LVQPGGSLRL SCAASGFTFS SFAMSWVRQA PGKGLEWVSD IDTTGRTTDY ADSVKGRFTI SRDNAENTLY LQMNDLKPED TAVYYCANVP KELVLSFGSW GPGTQVTVSS NGIGK-PEG ₄ -MeTz-BCN-NODAGA-Al [¹⁸ F] F
[¹⁸F]F-NODAGA-BCN-MeTz-mal-cys-(hPD-L1) sdAb SEQ ID NO: 29 Length: 141 amino acids	QVQLQESGGG LVQPGGSLRL SCAASGFTFS SFAMSWVRQA PGKGLEWVSD IDTTGRTTDY ADSVKGRFTI SRDNAENTLY LQMNDLKPED TAVYYCANVP KELVLSFGSW GPGTQVTVSS HHHHHHSPST PPTPSPSTPP C-mal-PEG ₄ -MeTz-BCN-NODAGA-Al [¹⁸ F] F
[¹⁸F]F-NOTA-KGIG-(R3b23) sdAb SEQ ID NO: 30 Length: 135 amino acids	DVQLVESGGG SVQAGGSLRL SCAASGDTGY MGWFRQAPGK EREGVAVINS DSGVGSTYYA DSVKGRFTIS RDNAKNTVYL QMNSLKPEDT AIYYCAAGHF SDYVSPWTWR EIYRYNVWGQ GTQVTVSSNG IGGGK-NHCS-NOTA-Al [¹⁸ F] F
[¹⁷⁷Lu]Lu-DOTA-NHCS-KGIG-CEA sdAb SEQ ID NO: 31 Length: 128 amino acids	DVQLVESGGG SVQAGGSLRL SCAASGDTYG SYWMGWFRQA PGKEREVAA INRGGGYTVY ADSVKGRFTI SRDTAKNTVY LQMNSLRPDD TADYYCAASG VLGGLEDHDF NYWGGGTQVT VSSNGIGK-NHCS-DOTA- [¹⁷⁷ Lu] Lu
VyPAL2 (Viola yedoensis) SEQ ID NO: 32 Length: 483 amino acids	MQLFAAGVIL FFLALSGTI AGGLDVDSLQ LPSEAAKFFH NDNSTNDDDS IGTRWAVLIA GSKGYHNYRH QADVCHMYQI LRKGGVKDEN IIVFMYDDIA YNESNPFFGI IINKPGGENV YKGVPKDYTG EDINNVNFLA AILGNKSAII GGSGKVLDT PNDHIFIYYA DHGAPGKIGM PSKPPLYADD LVDTLKQKAA TGTYSKSMVFY VEACNAGSMF EGLLPEGTNI YAMAASNSTE GSWITYCPGT PDFPPEFDVC LGDLWSITFL EDCDAHLNRT ETVHQGFELV KKKIAYASTV SQYGDIPISK DSLSVYMGTD PANDNRTFVD ENSLRPPLKV IHQHDADLYH IWCKYNMAPE GSSKKIEAQK QLELMASHRA HVDNSITLIG KLLFGVNKAS KVLNTPRPVG QPLVDDWQCL KAMIRTFETH CGSLSEYGMK HTLSFANMCN AGIQKEQLAE AAAQACVTFP SNPYSSLAE G FSA
Butelase-1 (Clitoria ternatea) SEQ ID NO: 33 Length: 482 amino acids	MKNPLAILFL IATVVAVVSG IRDDFLRLPS QASKFFQADD NVEGTRWAVL VAGSKGYVNY RHQADVCHAY QILKKGGLKD ENIIIVFMYDD IAYNESNPHP GVIINHPYGS DVIKGVPKDY VGEDINPPNF YAVLLANKSA LTGTGSGKVL DSGPNDHVF YYTDHGGAGV LGMPSPKPIA ASDLNDVLKK KHASGTYKSI VFYVESCESG SMFDGLLPED HNIYVMGASD TGESSWVTYC PLQHPSPPPE YDVCVGDLSF VAWLED CDVH NLQTETFOQQ YEVVKNKTIV ALIEDGTHV QYGDVGLSKQ TLFVYMGTD ANDNNTFTDK NSLGTPRKAV SQRDADLIHY WEKYRRAPEG SSRKAEAKKQ LREVMHRMH IDNSVKHIGK LLFGIEKGHK MLNNVRPAGL PVVDDWDCFK TLIRTFETHC GSLSEYGMKH

	MRSFANLCNA GIRKEQMAEA SAQACVSIPD NPWSSLHAGF SV
Butelase-2 (Clitoria ternatea) G252V G182A SEQ ID NO: 34 Length: 480 amino acids	MGHHHHHHSS GVDLG TENLY FQSMARLNPQ KEWDSVIRLP TEPVDADTDE VGTRWAVLVA GSNGYENYRH QADVCHAYQL LIKGGLKEEN IIVFMYDDIA WHELNPRPGV IINNPRGEDV YAGVPKDYTG EDVTAENLFA VILGDRSKVK GGSGKVINSK PEDRIFIFYS DHGAPGVLGM PNEQILYAMD FIDVLKKKHA SGGYREMYIY VEACESGSLF EGIMPKDLNV FVTTASNAQE NSWVTYCPGT EPSPPPEYTT CLGDLYSVAV MEDSESHNLR RETVNQYRS VKERTSNFKD YAMGSHVMQY GDTNITAEKL YLFQGFDPAT VNLPPHNGRI EAKMEVVHQR DAELLFMWQM YQRSNHLGK KTHILKQIAE TVKHRNHLDG SVELIGVLLY GPGKGSPLVQ SVRDPGLPLV DNWACLKSMV RVFESHCGSL TQYGMKHMRA FANICNSGVS ESSMEEACMV ACGGHDAGHL
Butelase-2 (Clitoria ternatea) G252V P183A SEQ ID NO: 35 Length: 480amino acids	MGHHHHHHSS GVDLG TENLY FQSMARLNPQ KEWDSVIRLP TEPVDADTDE VGTRWAVLVA GSNGYENYRH QADVCHAYQL LIKGGLKEEN IIVFMYDDIA WHELNPRPGV IINNPRGEDV YAGVPKDYTG EDVTAENLFA VILGDRSKVK GGSGKVINSK PEDRIFIFYS DHGGAGVLGM PNEQILYAMD FIDVLKKKHA SGGYREMYIY VEACESGSLF EGIMPKDLNV FVTTASNAQE NSWVTYCPGT EPSPPPEYTT CLGDLYSVAV MEDSESHNLR RETVNQYRS VKERTSNFKD YAMGSHVMQY GDTNITAEKL YLFQGFDPAT VNLPPHNGRI EAKMEVVHQR DAELLFMWQM YQRSNHLGK KTHILKQIAE TVKHRNHLDG SVELIGVLLY GPGKGSPLVQ SVRDPGLPLV DNWACLKSMV RVFESHCGSL TQYGMKHMRA FANICNSGVS ESSMEEACMV ACGGHDAGHL
VyPAL3 (Viola yedoensis) SEQ ID NO: 36 Length: 449 amino acids	MQLFAAGVIL FFLALSGTI AGGLDVDSLQ LPSEAAKFFH NDNSTNDSS AGTKWAVLIA GSKGYQNYRH QADVCHAYQI LRRGGVKDEN IIVFMYDDIA YDIRNPYPGT ITNSPDKKDV YKGVPKDYTG EDVNVQNFLA VILGNKTALT GGSGKVLDT PNDHIFIYYT DHGYAGVLGM PTQPPLYAND LIDTLKKKHA SGTYESLVFY VEACESASIF EGLLPDGLNI YVSTAAKAGE GSWVVYCPTQ QPPVPAEYGT CVGDLYSVTW MEDCDLYNLR TQTLHQYEM VKKKIAYAST VSQFGDLTIT KDSLFEYMG DPANEKHHYE DQENSLRPHV DAVHQREADL YHFWDKYQKA SEGSRNKVAA RKQLVEVMLH RMHVDDSI ES IAKLLFGSDA KASEMMNTIR PPGQPLVSDW DCLKTMVRTF ETHCGSLSEY GMKYTRFLA

OaAEP1b-C247A (oldenlandia affinis) SEQ ID NO: 37 Length: 537 amino acids	MGMAHHHHHH MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL EDGRTLSDYN IQKESTLHLV LRLRGGARDG DYHLHPSEVS RFFRPQETND DHGEDSVGTR WAVLIAGSKG YANYRHQAGV CHAYQILKRG GLKDENIVVF MYDDIAYNES NPRPGVIINS PHGSDVYAGV PKDYTGEEVN AKNFLAAILG NKSATITGSG KVVDSGPN DH IFIYYTDHGA AGVIGMPSKP YLYADELND LKKKHASGTY KSLVFYLEAC ESGSMFEGIL PEDLNIYALT STNTTESSWA YYCPAQENPP PPEYNVCLGD LFSVAWLED DVQNSWYETL NQQYHHVDKR ISHASHATQY GNLKLGEGL FVYMGSNPAN DNYTSLDGNA LTPSSIVVNQ RDADLLHLWE KFRKAPEGSA RKEEAQTQIF KAMSHRVHID SSIKLGKLL FGIEKCTEIL NAVRPAGQPL VDDWACLRLS VGTFFETHCGS LSEYGMRHTR TIANICNAGI SEEQMAEAS QACASIP
HeAEP3 (Afrohybanthus enneaspermus) SEQ ID NO: 38 Length: 481 amino acids	MKLLVPGVLL LFLALSGIA AGRPDDFLRL PSEAAKSFLLH NDDDSVGTRW AVLIAGSKGW QNYRHQADVC HAYQILKKGG LKDENIIVFM YDDIAYNESN PRPGIVINKP KGEDVYKGVF KDYTGENVNA VNFLAVLLAN RSALTGGSGK VLDSGPNDR I FIYYTDHGAP VTIGMPSPY LVAKDLVDTL KKKHAAGTYK SMVFYIESCE SGSMFDGLLP EDANIYGMTA TNSTEGSWVT YCPGQTDDYP EDDEYDVCFG DLWSVAWLED CDAHNLRTET LDQQYEVVKK KIEYAHIP AQ YGNVSLAKDS LFFVYMGTDPA NDNKT FVEEN TLRRPLKAVH SRDADLLHFW HKYHKAPEGT SRKIDAQKQL VEVLSHRTHV DNSIKLVGEL LFGVGKASEV LNTIRPAGQP LVDDWDCLKT MVRTFETHCG SLSEYGMKHM RSFANMCNAG VQKEQMAVAA GQACVTFPSN PWSSLDGEGFS V
AtLEGy (Arabidopsis thaliana) SEQ ID NO: 39 Length: 455 amino acids	SLEHHHHHHE NLYFQGVGTR WAVLVAGSSG YGNYRHQADV CHAYQILKRG GLKEENIVVL MYDDIANHPL NPRPGTLINH PDGDDVYAGV PKDYTGSSVT AANFYAVLLG DQKAVKGGSG KVIASKPN DH IFVYYAXHGG PGVLGMPNTP HIYAADF IET LKKKHASGTY KEMVIYVEAA ESGSIFEGIM PKDLNIYVT ASNAQESSYG TYCPGMNPSP PSEYITCLGD LYSVAWMEDS ETHNLKKETI KQYHTVKMR TSNYNTYSGG SHVMEYGNNS IKSEKLYLYQ GFDPATVNLP LNELPVKSKI GVVNQRDADL LFLWHMYRTS EDGSRKKDDT LKELTETTRH RKHL DASVEL IATILFGPTM NVLNLVREPG LPLVDDWECL KSMVRVFEEH CGSLTQYGMK HMRAFANVCN NGVSKELMEE ASTAACGGYS EARYTVHPSI LGYSA
VuPAL1 (Viola uliginosa) SEQ ID NO: 40 Length: 484 amino acids	MKLLAAGVIL VSLALSGTV AGGLDVDPLR LPSEAAKFFH NDNSTNDDDS IGTRWAVLIA GSKDYHNYRH QADVCHMYQI LRKGGVKDEN IIVFMYDDIA YNESNPHPGI IINKPGGEDV YKGVPKDYTG EDVNNINFLA AILGNKSAII GGSGKVLDTS PNDHIFIYYT DHGAPGKIGM PSKPPLYADD LVDTLKQKAA TGTYKSMVFY VEACNAGSMF EGLLPEGTNI YAMAASNSTE

	GSWITYCPGA TPDFPPEYDI CLGDLWSITF LEDCDAHNLR TETVHQQFEL VKKNIAYAST VSQYGDIPIS KDSLSVYMG DPANDNRFTV DENSLKPPLK VIHQRDADLY HLWYKYNKAP EGSSKKIEAQ KQLLELMshr AHVDNSITLI GKLLFGVDKA SKVLNtvrpv GQPLVDDWQC LKAMIRTFET HCGSLSEYGM KHTLSFANMC NAGIQKEQLA EAAAQACVTF PSNSYSSSLAE GFSA
HaPAL1 (<i>Helianthus annuus</i>) SEQ ID NO: 41 Length: 487 amino acids	MACFSYRLIC LLLVLMVMMA LPNGAAAARR GSDYWDPFIR SPVDLEDDEL GNGTRWALLV AGSKGYQSYR HQANVCHAYQ ILKRGGLKDE NIVVEMYDDI ATCDENPRPG TIIHHPEGGD VYAGVPKDYT GDAVTADNFF AVILGDKSSV KGGSGKVIDS KPDDRIFLYY TDHGAAGLLG MPEKPYVVAN DFVEVLKKKH AMGTYKEMVI YLEACESGSI FEGLLPEDLN IYAITSTKPE EPSYIIYCPD MNPPPPPEYT TCLGDTFSVA WMEDSETHNL KKESLAQQIN KVKERTSMFG TYANGSHVME YGTVIKPEK VYLYQGYNPE TANLPANRIH FDKKMESVNQ RDGDLIYLWQ KYKRSSVSNR AEALKQMTET LRYMAHLDSS VDMIGVLLFG PQNGGSILRS SRGRGLPLVD DWDCLKSMTR LFEKHCGLLT EYGMKHMRAF ANICNNLVEE TEVEEAIAT CSGKNIGPYA SLGAYSV
OaAEP1b (<i>oldenlandia affinis</i> wild-type) SEQ ID NO: 42 Length: 537 amino acids	MGMAHHHHHH MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL EDGRTLSDYN IQKESTLHLV LRLRGGARDG DYHLHPSEVS RFFRPQETND DHGEDSVGTR WAVLIAGSKG YANYRHQAGV CHAYQILKRG GLKDENIVVF MYDDIAYNES NPRPGVIINS PHGSDVYAGV PKDYTGEEVN AKNFLAAILG NKSATIGGSG KVVDSGPN DH IFIYYTDHGA AGVIGMPSKP YLYADELND LKKKHASGTY KSLVFYLEAC ESGSMFEGIL PEDLN IYALT STNTTESSWC YYCPAQENPP PPEYNVCLGD LFSVAWLED S DVQNSWYETL NQQYHHVDKR ISHASHATQY GNLKLGE E GL FVYMGSNPAN DNYTSLDGNA LTPSSIVVNQ RDADLLHLWE KFRKAPEGSA RKEEAQTQIF KAMSHRVHID SSIKLI GKLL FGIEKCTEIL NAVRPAGQPL VDDWACLRLS VGTFFETHCGS LSEYGM RHTR TIANICNAGI SEEQMAEAS QACASIP
Human glutaminyl cyclase (QC) SEQ ID NO: 43 Length: 339 amino acids	VSPSASAWPE EKNYHQPAI L NSSALRQIAE GTSISEMWQN DLQPLLIERY PGSPGSYAAR QHIMQRIQRL QADWVLEIDT FLSQTPYGYR SFSNIISTLN PTAKRHLVLA CHYDSKYFSH WNNRVFVGAT DSAVPCAMML ELARALDKKL LSLKTVSDSK PDLSQLIFF DGEEAFLHWS PQDSLYGSRH LAAKMASTPH PPGARGTSQL HGMDLLVLLD LIGAPNPTFP NFFPNSARWF ERLQAIEHEL HELGLLKDHS LEGRYFQNY S YGGVIQDDHI PFLRRGVPVL HLIPSPFPEV WHTMDDNEEN LDESTIDNLN KILQVFVLEY LHLHHHHHH

	(the underlined C-ter sequence is a His ₆ tag added to facilitate purification)			
Mouse glutaminyl cyclase (QC) SEQ ID NO: 44 Length: 327 amino acids	AWTQEKNNHQ IERYPGSPGS YGYRSFSNII VGATDSAVPC LIFFDGEEAF TNQLDGMDDL EKELYELGLL VPVLHLIASP VLEYLHL	PAHLNSSLQ YSARQHIMQR STLNPEAKRH AMMLELARAL HHWSPQDSLY VLLDLIGAN KDHSLERKYF FPEVWHTMDD NEENLHASTI	QVAEGTISE IQRLQAEWV LVLACHYDSK DKKLHSLKDV GSRHLAQKMA PTFPNFFPKT QNFYGNIIQ DDHIPFLRKG DNLNKIIQVF	MWQNDLRPLL EVDTFLSRTP YFPRWDSRVF SGSKPDLSLR SSPHPPGSRG TRWFNRLQAI DDHIPFLRKG DNLNKIIQVF
Glutaminyl cyclase (Drosophila melanogaster) SEQ ID NO: 45 Length: 340 amino acids	MAIGSVVFAA FNRTLDSILV EFKQRPVFG PNDPGFVGAT GLMLIFFDGE AQLAPRNIDR LVQIEKSLRT NVPVLHLVAT FVYQYLKRHT	AGLLLLLLPP PRVVGSRGHQ ELTFANVVG DSAVPCAILL EAFKEWTDAD IEVLVLLDLI AGQLEGNNNM PFPDVWHTPR SPVNLRFYRT	SHQQATAGNI QVREYLVQSL INPQAQNFIA NTAKTLGAYL SVYGSKHLAA GARNPKFSSF FLSRVSGGLV DNAANLHWPS IRNFNVRFRN	GSQWRDDEVH NGLGFQTEVD LACHYDSKYF QKEFRNRSDV KLASKRSGSQ YENTDGLHSS DDDHRPFLDE IRNFNVRFRN
Glutaminyl cyclase (Arabidopsis thaliana) SEQ ID NO: 46 Length: 300 amino acids	MATRSPYKRQ IPLALLSGAV AEFPHDPDAF TGKVEILEKM LRNLSKVKPF PQTMKVTDKH LSRGLLKSGH IKLKQASAKS	TKRSMIQSLP FLFFMPFNSW TOGLLYAGND DNTYFGEGLT KHHMKDGWGL IVRYNGRES RGIDVLNGIA GNYIEQQCLV	ASSSASSRRR GQSSGSSLDL TLFESTGLYG LLGERLFQVA ATDGKALFGS CIARISPKDG WDSKQQLFV TGKLWPKLYQ	FISRKRFAMM SHRINEIEVV KSSVRKVDLR WLTNTGFTYD DGTSTLYRMD SLLGWILLSK TGKLWPKLYQ
Glutaminyl cyclase (Conus frigidus) SEQ ID NO: 47 Length: 345 amino acids	MMEKVTTAAT VDNLSNLTVG SIIKWMRREG ARRVVLACHY KLRQVFQEKV YGARHLAQKW TRFANLFNQT TSEQKQGTIE NLHALDFQRT	YVRLLLLCSA MSDDGLRKA WSVQEDPFIA DSKLILFHGL ADASFQELTL ASTPDPTAAG AGVYAKLQSI DDHLPFLRRG ENLARILRLF	VASNRALQNL LPPLLKPRVS KTPYGVWRFS SFVGATDSAV QFIFFDGEEA LNYLQTIGVF EMCLTENGYL VPVHLLISTP LVDLL	GCGSLTSQYT GTRGNFNVN NVIATLNPRA PCALLMDSAK YVQWSRSDSL ILLDLIGSAD DATANPLPLF FPSVWHKLS LVDLL
Glutaminyl cyclase (Sistrurus tergeminus) SEQ ID NO: 48	MARERRDSKA TQEKYSHRPT RYPGSPGSYA YRTFSNIIIST GATDSAVPCA	ATFFCLAWTL ILNATCILQV VRQHIKHLRQ LNPLAKRHLV MMLELARSLD	CLALPGFPQH TSQTNVNRMW GLQAGWLVEE IACHYDSKYF RQLSFLKQSS	VSGREDRADW QNDLHPILIE DTFQSHTPYG PPQLDGKVFV LPPKADLSLK

Length: 368 amino acids	LIFFDGEEAF VRWSPSDSLY GSRSLAQKMA STPHPPGARN TYQIQGIDLF VLLDLIGARN PVFPVYFLNT ARWFGRLAI ERNLYDLGLL NNYSSERQYF RSNLRRHPVE DDHIPFLRRG VPILHLIPSP FPRVWHTMED NEENLDKPTI DNLSKILQVF VLEYLNLG
Bacterial glutaminyl cyclase (Xanthomonas campestris) SEQ ID NO: 49 Length: 267 amino acids	MPRLVPALLL ILALLPAMAV ARDPVPTQGY RVVKRYPHDT TAFTEGLFYL RGHLYESTGE TGRSSVRKVD LETGRILQRA EVPPPYFGEG IVAWRDLIQ LTWRNHEGFV YDLATLTPRA RFRYPGEGWA LTSDDSHLYM SDGTAVIRKL DPDTLQQVGS IKVTAGGRPL DNLNELEWVN GELLANVWLT SRIARIDPAS GKVVAWIDLQ ALVPDADALT DSTNDVLNGI AFDAEHDRLF VTGKRWPMLY EIRLTPLPHA AAGKHAQ

The rate of reaction of the method of the present disclosure may be controlled by varying the ratio of the enzyme(s) to the substrate in question. In some embodiments, a small amount of enzyme (for example 0.01 eq of PAL + QC mix in relation to the substrate) may be sufficient to carry out the invention. The ratio of enzyme to substrate to use is largely dependent on the substrate and the specific application.

In some embodiments, the ratio of the first peptide: the functionalized peptide: PAL is in the range of 1-5: 1-25 : 0.1 to 0.5 respectively. In some embodiments, the ratio of the first peptide: the functionalized peptide: PAL is 1:1:0.25. In some embodiments, the ratio of the first peptide: the functionalized peptide: PAL is selected from the group consisting 1:5:0.25; 1:25:0.25; 2:1:0.25, 1:5:0.25, and 1:1:0.25.

In some embodiments, the ratio of the first peptide: the functionalized peptide: PAL+QC mix is in the range of 1:1: 0.01 to 1:1:0.1. In other embodiments, the ratio of the first peptide: the functionalized peptide: PAL+QC mix is 1:1: 0.01.

It would be appreciated that varying the amounts of QC may also affect the rate of reaction. Accordingly in some embodiments, the molar ratio of PAL: QC is in the range of 1:0.1 to 1:1. In some embodiments, the molar ratio of PAL: QC is 1:0.1. In other embodiments, the molar ratio of PAL: QC is 1: 0.5.

For the purposes of the present invention, the functionalized peptides as described herein may be suitably functionalized to comprise a functional group(s), moiety(ies), compound(s), and or agent(s) that may facilitate radiolabelling, its use as a diagnostic agent and/or its use as a therapeutic agent. In this regard, it would be appreciated that functionalized peptides for

use in the present invention may be prepared using standard techniques known to those skilled in the art of synthetic organic chemistry, or may be deduced by reference to the pertinent literature.

In some embodiments, the functionalized peptide is functionalized with a chelator or a
5 tetrazine moiety. In some embodiments, the tetrazine moiety is – MeTz.

In some embodiments, the chelator is selected from the group consisting of 1,4,7-triazacyclononanetriacetic acid (NOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and 1,4,7-triazacyclononane-1-glutaric acid-4,7-diacetic acid (NODAGA).

In some embodiments, the functionalized peptide may further comprise a bicyclononyne
10 (BCN) or a trans-cyclooctene (TCO).

In some embodiments, the tetrazine-functionalized peptide may form a conjugate of the first peptide-(tetrazine-functionalized peptide), and the conjugate is further complexed with a bicyclononyne/TCO-functionalized chelator/reactive moiety prior to radiolabeling. In some
15 embodiments, the bicyclononyne-functionalized chelator comprises a chelator selected from the group consisting of 1,4,7-triazacyclononanetriacetic acid (NOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and 1,4,7-triazacyclononane-1-glutaric acid-4,7-diacetic acid (NODAGA).

In various embodiments, the conjugate or the functionalized peptide may be radiolabeled with a radionuclide selected from the group consisting of gallium-68 (⁶⁸Ga), gallium-67 (⁶⁷Ga),
20 indium-111 (¹¹¹In), lutetium-177 (¹⁷⁷Lu), fluorine-18 (¹⁸F), iodine-131 (¹³¹I), iodine-123 (¹²³I), iodine-125 (¹²⁵I), phosphorus-32 (³²P), strontium-90 (⁹⁰Sr), strontium-89 (⁸⁹Sr), yttrium-90 (⁹⁰Y), radium-223 (²²³Ra), radium-226 (²²⁶Ra), caesium-137 (¹³⁷Cs), technetium-99m (^{99m}Tc), tin-117 (^{117m}Sn), samarium-153 (¹⁵³Sm), erbium-169 (¹⁶⁹Er), rhenium-188 (¹⁸⁸Re), rhenium-186 (¹⁸⁶Re), thallium-201 (²⁰¹Tl), carbon-11 (¹¹C), oxygen-15 (¹⁵O-), copper-64 (⁶⁴Cu), lead-212 (²¹²Pb) astatine-211 (²¹¹At), actinium-225 (²²⁵Ac), Terbium-149/161/155/152 (^{149/161/155/152}Tb),
25 and iridium-192 (¹⁹²Ir).

In some embodiments, the conjugate or the functionalized peptide is radiolabeled with a radionuclide selected from the group consisting of Gallium-68 (⁶⁸Ga), Gallium-67 (⁶⁷Ga), Lutetium-177 (¹⁷⁷Lu), and Fluorine-18 (¹⁸F).

30 In some embodiments, the radionuclide is Gallium-68 (⁶⁸Ga) or Fluorine-18 (¹⁸F). In other embodiments, the Fluorine-18 (¹⁸F) is in the form of [¹⁸F]AIF.

In another aspect, there is provided a site-specifically radiolabeled tracer prepared by the method described herein.

In some embodiments, the site-specifically radiolabeled tracer as provided herein is selected from the group consisting of [⁶⁸Ga]Ga-NOTA-KGIG-(hPD-L1) sdAb, [¹⁸F]AIF-NOTA-KGIG-(hPD-L1) sdAb, [¹⁸F]F-NODAGA-BCN-MeTz-KGIG-(hPD-L1), and [¹⁷⁷Lu]Lu-DOTA-NHCS-KGIG-CEA sdAb.

In another aspect, there is provided a site-specifically radiolabeled tracer as described herein for use as a nuclear imaging agent.

In a further aspect, there is provided a use of the site-specifically radiolabeled tracer of the present disclosure as a nuclear imaging agent.

In another further aspect, there is provided a use of the site-specifically radiolabeled tracer of the present disclosure in the manufacture of a diagnostic imaging agent for use in nuclear imaging.

In another aspect, there is provided a method of treating a disease in a subject, the method comprising administering the site-specifically radiolabeled tracer according to the second aspect to the subject.

In a further aspect, there is provided a use of the site-specifically radiolabeled tracer according to the second aspect in the manufacture of a medicament for the treatment of a disease.

In some embodiments, the sdAb targets the diseased cell such as cancer cell, and the site-specifically radiolabeled antibody tracer described herein is radiolabeled with a therapeutic radionuclide such as ¹⁷⁷Lu, ²¹¹At or ²²⁵Ac.

The overexpression of PD-L1 in cancers such as gastric carcinoma, hepatocellular carcinoma, renal-cell carcinoma, oesophageal carcinoma, pancreatic cancer, ovarian cancer, melanoma, breast cancer, non-small cell lung cancer, papillary thyroid cancer, testicular cancer, and bladder cancers is associated with poor clinical outcomes, and the detection of its levels has important clinical significance. Carcinoembryonic antigen (CEA) overexpression is observed in patients with a variety of carcinomas, including in the gallbladder, urinary bladder, endometrium, colon, thyroid, lung, uterus, pancreas, gastric, breast and ovary. CEA can be used as a cancer marker in clinical testing; it can also be used as a prognostic marker for cancer after radiotherapy and chemotherapy, a predictive factor for cancer treatment, and as a therapeutic target.

In some embodiments, the disease is a tumour or a cancer. In further embodiments, the cancer may be a pancreatic cancer, prostate cancer, ovarian cancer, melanoma, breast cancer, non-small cell lung cancer, papillary thyroid cancer, testicular cancer, bladder cancer, gastric carcinoma, hepatocellular carcinoma, renal-cell carcinoma, oesophageal carcinoma, and/or carcinomas of the gallbladder, urinary bladder, endometrium, colon, thyroid, lung, uterus, pancreas, gastric, breast and ovary. In some embodiments, the tumour is a neuroendocrine or a carcinoid tumour.

Unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in various embodiments, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. "About" in reference to a numerical value generally refers to a range of values that fall within $\pm 10\%$, in some embodiments $\pm 5\%$, in some embodiments $\pm 1\%$, in some embodiments $\pm 0.5\%$ of the value unless otherwise stated or otherwise evident from the context. In any embodiment in which a numerical value is prefaced by "about", an embodiment in which the exact value is recited is provided. Where an embodiment in which a numerical value is not prefaced by "about" is provided, an embodiment in which the value is prefaced by "about" is also provided. Where a range is preceded by "about", embodiments are provided in which "about" applies to the lower limit and to the upper limit of the range or to either the lower or the upper limit, unless the context clearly dictates otherwise. Where a phrase such as "at least", "up to", "no more than", or similar phrases, precedes a series of numbers, it is to be understood that the phrase applies to each number in the list in various embodiments (it being understood that, depending on the context, 100% of a value, e.g., a value expressed as a percentage, may be an upper limit), unless the context clearly dictates otherwise. For example, "at least 1, 2, or 3" should be understood to mean "at least 1, at least 2, or at least 3" in various embodiments. It will also be understood that any and all reasonable lower limits and upper limits are expressly contemplated.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLES

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Green and Sambrook, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (2012).

EXAMPLE 1: Materials and General Methods**1.1 Reagents**

All the reagents and solvents were purchased from Sigma–Aldrich (Overijse, Belgium) or VWR (Oud-Heverlee, Belgium). Buffers used for coupling reactions and for radiolabeling were prepared with metal free water (Honeywell, Fluka, Brussels, Belgium) and purified from metal contamination using Chelex 100 resin (Sigma Aldrich, Belgium). p-SCN-Bn-NOTA was purchased from Macrocyclics (Plano, United States of America). BCN-NODAGA was purchased from Macrocyclics (Dijon, France). VyPAL2 preparation (proenzyme expression, low-pH proenzyme activation and purification of the activated form) was as described by Zhang et al. and Hemu et al.. Human Glutaminyl Cyclase (QC) was purchased from Abcam (ab206806), aliquoted and stored at -80 °C until mixed with VyPAL2 with a QC: VyPAL2 stoichiometry of 0.1:1.

1.2 Analytical

Quality control (QCO) of the peptidyl substrates and intermediates during their synthesis was performed on a Chromolith HR RP18 50-4.8 mm column, at a flow rate of 3 mL/min with the following gradient: 3-97% ACN/H₂O in 4 min with 0,1% TFA. 300, 280, 254 & 214 nm detection. Preparative HPLC of peptidyl substrates and intermediates during their synthesis was performed on a Vydac C18 22 ×150 mm 10 µm column, at a 20 mL/min flow rate with the following gradient: 10-60% ACN/H₂O in 20 min with 0,1% TFA.

Analytical and preparative HPLC SEC columns were purchased from Cytiva (Hoegaarden, Belgium). SEC purification of the site-specifically functionalized (non-radioactive) sdAbs was performed on a Superdex 75 Increase 10/300 GL column using PBS pH 7, at a flow rate of 0.8 mL/min accommodated on an NGC medium-pressure chromatography system (BioRad, Temse, Belgium).

QCO of modified sdAbs was performed by SEC on a Superdex 75 Increase 3.2/300 GL using 2× PBS (5.36 mM KCl, 273.8 mM NaCl, 2.94 mM KH₂PO₄, 16.2 mM Na₂HPO₄) at a flow rate of 0.150 mL/min, monitoring at 280 and 215 nm, accommodated on a Hitachi HPLC system equipped with a 5110 Hitachi pump, a 5210 Hitachi autosampler, and a 5430 Hitachi Diode Array Detector.

Radiochemical purity (RCP) of [¹⁸F]AIF-NODAGA-BCN was assayed by radio-RP-HPLC on the Hitachi system connected to a radio-detector (GABI STAR, Elysia Raytest, Tilman, Belgium) and equipped with an analytical XBridge C18 5 µm 4.6×150 mm (Waters, Antwerp,

Belgium) using the following gradient; A = H₂O + 0.1% TFA and B = ACN + 0.1% TFA (HPLC grade); 0—2 min 20—37% B; 2—6 min 37—50% B; 6—7 min 90% B at 2.5 mL/min.

RCP of the [¹⁸F]AIF-NOTA-KGIg was assayed by radio-RP-HPLC on the Hitachi system equipped with the analytical XBridge column using the following gradient: A = H₂O + 0.1% TFA and B = ACN + 0.1% TFA (HPLC grade); 0—5 min 5—50% B; 5—6.5 min 90% B at 2.5 mL/min, or on a PLRP-S 300 A 5 μM 250×4.6MM column (Agilent) using the following gradient: A = H₂O + 0.1% TFA and B = ACN + 0.1% TFA (HPLC grade); 0—6 min 7—90% B; 6—10 min 90% B at 1 mL/min.

RCP of ¹⁸F-labelled sdAbs was assessed on the Hitachi system by radio-SEC on the Superdex 75 Increase 3.2/300 GL in 2x PBS at 0.15 mL/min, and/or on a TSKgel SUPER SW2000 (Tosoh Biosciences, Amsterdam, The Netherlands) in 2x PBS at a flow rate of 0.35 mL/min, by diluting the samples were diluted with 2xPBS + 0.1% Tween 80, and were analysed by radio-RP-HPLC on the PLRP-S column using the same method as described above.

RCP of the ⁶⁸Ga-labelled sdAb was assessed on the RLRP-S column using the same method as described above by diluting the sample 2x with 0.1 M NaCitrate pH 4.5 + 0.1% Tween 80.

RCP of the ⁶⁷Ga-labelled sdAb was assessed by radio-SEC on the Superdex 75 Increase 5/150 GL in 2x PBS at a flow rate of 0.45 mL/min for 12 min. Samples were diluted with 0.1M NaCitrate pH 4.5 + 0.1% Tween 80.

RCP of ^{68/67}Ga-labelled compounds was assayed with binderless glass microfiber paper that was impregnated with silica gel (instant thin layer chromatography, iTLC-SG) (Agilent Technologies, Diegem, Belgium) using 0.1 M sodium citrate buffer pH 4.5—5 as eluent.

1.3 Production of the sdAbs and extraction for the periplasmic extract

The nanobodies were produced as described before (Vincke, C. et al., *Methods mol. biol.* 907, 145—176 (2012)). Briefly, the hPD-L1 sdAb and control sdAb R3b23 were produced and purified including cloning of the sdAbs encoding cDNAs into the vector pHEN6 (containing an N-terminal pelB signal for periplasmic translocation), incorporating a C-terminal NXL-His6 tag (X = G, Q) (2 L production), or a C-terminal linker-His₆-cys-tag (5 L production). Expression plasmids were transformed in WK6 E. coli cells, grown in baffled shaker flasks at 37°C under antibiotic selection, and expression was induced with IPTG at 28°C overnight. Overnight induction cultures were harvested by centrifugation and periplasmic extracts were harvested by osmotic shock. To the falcon tubes containing the sdAb in periplasmic extract (typically 8 x 50 mL) was added Ni-NTA beads (Thermo Fisher, 250 μL per tube) and the mixtures were

shaken for 1 h. The tubes were centrifuged, and the supernatant was discarded. The tubes were topped up to 50 mL with metal free PBS, shaken for 30 min, centrifuged and the supernatant was discarded. The beads were added to a PD-10 column, the tubes were rinsed twice with PBS (10 mL) which was added to the column. The sdAbs were eluted from the column with 5x1 mL of imidazole buffer (34 g/L in PBS; pH = 7,4). The recovered sdAb was purified by SEC on a HiLoad 16/600 Superdex 75 pg in PBS at a flow rate of 1 mL/min. The fractions of interest were pooled, allowing to recover the purified sdAbs: 20 mg of (hPD-L1)-NGL-His₆ (production yield = 16.8 mg/mL), 25.7 mg of (hPD-L1)-NQL-His₆ (production yield = 5.14 mg/mL), 35.1 mg of R3b23-NGL-His₆ (production yield = 17.6 mg/mL), 22.2 mg of R3b23-NQL-His₆ (production yield = 11.1 mg/mL) and 50 mg of (hPD-L1)-linker-his₆-cys sdAbs (production yield = 10 mg/L).

1.4 Surface Plasmon Resonance

All measurements were performed on a Biacore T200 device (GE Healthcare) at 25°C and using Hepes-buffered saline (0.01 M Na HEPES, pH 7.4; 0.15M NaCl, 3 mM EDTA, 0.005 % Tween 20) as running buffer. All recombinant proteins were dissolved to 10 µg/mL in 10 mM Na-acetate (pH 5.0) for immobilization on a CM5 sensor chip using linkage chemistry with 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide (EDC) and N-hydroxy-succinimide (NHS). Unreacted EDC-NHS linkers were blocked with 1 M ethanolamine-HCl. For all measurements, SPR signals in the flow cell with immobilized protein were subtracted with those in a flow cell that underwent the same manipulations but where recombinant protein was omitted, to obtain specific binding signals (response units, RU). Affinity for human PD-L1 of the purified sdAbs was evaluated on immobilized PD-L1 protein.

1.5 Thermostability of the sdAbs (Melting point)

The melting temperature of the starting sdAbs and modified sdAbs was determined using the Protein Melting program of a RealTime PCR machine. Samples were prepared by mixing 12.5 µg of sdAb with 7.5 µL of Cypro Orange dye (Thermo fisher, 300 × dilution) in PBS to a 25 µL final volume. Blank samples contained NH₄OAc. Samples were prepared in triplicates.

1.6 SDS-PAGE and western blot

Samples were prepared by diluting the sdAbs in Leammli reducing buffer (Bio-Rad) and heating at 95°C for 4 min. The 40 µL protein sample was added to a Novex™ WedgeWell™ 8-16% Tris-Glycine gel (Live Technologies Europe BV). A sample containing unmodified His₆-tagged sdAb was prepared as a positive control. The PageRuler™ Prestained Protein Ladder

was added to the gel (10 to 180 kDa, ThermoFisher). The gel placed in a XCell Sure Lock™ Mini-Cell Electrophoresis System connected to a ZOOM® Dual Power Supply (ThermoFisher) and run was performed in the following conditions: 80 V for 5 — 10 min to allow protein to penetrate the gel, then 180 V for 45 min – 1 h, in TGS buffer (BioRad). The SDS-PAGE gel was stained with Instant Blue Coomassie protein stain (Abcam) for 20 min. The gel was scanned on an Amersham Imager (GE Healthcare) using the colorimetric trans-illumination (automatic) mode. For WB, transfer on the membrane (Bio-Rad) was performed at 70 - 100 V for 45 min – 1 h in TG transfer buffer (BioRad) pH 8.3 containing 20% MeOH. The membrane was blocked with 1 g of Milk powder (Nestlé) in 50 mL PBS, incubated at RT with the primary antibody (Mouse anti-His₆, 4E3D10H2/E3, dilution ThermoFisher Scientific) for 1 h under gentle shake, then with the secondary antibody (Goat anti-mouse IgG HRP, Sigma Aldrich) following manufacturer recommended dilutions. Between each step, the membrane was washed twice with 10 mL of PBS + 0.1% Tween 80 and 30 mL of PBS. The membrane was incubated for 20 min in the dark at RT with a freshly made HRP-revelation solution (18 mg chloronaphtol (Sigma Aldrich), in MeOH and 20 µL H₂O₂ (Sigma Aldrich) in TPA buffer pH 7.5 (0.5 M NaCl, 23 mM Tris-maleate)). The membrane was scanned on the Amersham Imager using the following parameters Chemiluminescence, Colorimetric marker, Binning = default (U) 8x, Exposure = 6 sec (manual).

1.7 Mice and cell lines

Dr. S.L. Topalian (National Cancer Institute, USA) provided HLA-A*0201+ 624- MEL cells. The 624-MEL cells were stably transduced to express hPD-L1 and they have been characterized, as previously described (Broos, K. et al., *Cancers (Basel)* 11, (2019)). The 624-MEL cells were cultured in RPMI1640 medium supplemented with 10% Fetal clone I serum (Thermo Scientific, Belgium), 2 mM L-Glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, and nonessential amino acids. Female, five to six weeks old C57BL/6 mice were purchased from Charles River. All of the experiments were performed in accordance with the European guidelines for animal experimentation under the license LA1230272. Experiments were approved by the Ethical Committee for the use of laboratory animals of the Vrije Universiteit Brussel (18-272-14 and 22-272-3). Intravenous injections were performed in the tail vein. The animals were anesthetized with 2.5% isoflurane in oxygen (Abbott) for injections and euthanasia.

1.8 Peptide synthesis

The GIGK sequence was synthesized using Solid-Phase Peptide Synthesis. In a 5 mL fritted syringe reactor, rink amide resin (244 mg, 0.41 mmol/g loading, 0.1 mmol scale) was swelled

in DMF (3 mL) for 10 min, before being vacuum filtered out. Secondly, the resin was standardly washed with DMF (3x3 mL) and DCM (3x3 mL), then the resin was Fmoc deprotected using a basic solution 4-methylpiperidine (4MP) in DMF (20 % vol., 2 mL) and shaken, after 5 min, the solution was filtered away and the deprotection step was repeated with a shaking time of 5 15 min. The solution was filtered away and the resin was washed. In a glass vial, HBTU (3 eq) and the first amino acid (Fmoc-L-Lys(Alloc)-OH) (3 eq) were weighed and dissolved in DMF (3 mL). After complete dissolution, DIEA (4 eq) was added to the solution which was then stirred for 5 min. The mixture was added to the resin, after which it was shaken for 60 min. Afterwards, the reagents were filtered away and the resin was washed with DMF and DCM. A 10 Kaiser test was performed to ensure that all free primary amines have reacted. The steps from the deprotection to the Kaiser test were repeated for each amino acid desired on the peptide, respectively Fmoc-Gly-OH, Fmoc-L-Ile-OH and lastly Boc-Gly-OH, affording the GIGK(Alloc) sequence.

For the acetylation, see paragraph below "Ac-GIGK-Py-DABCO, GIGK-Py-DABCO, Ac-GIGK-Py-NME₃ and GIGK-Py-NME₃". 15

The Alloc protecting group on the lysine was removed using a solution of PhSiH₃ (24 eq) and Pd(PPh₃)₄ (0.2 eq) in DCM (3 mL) which was added to the syringe and shaken for 30 min, this deprotection step was repeated a second time with a fresh solution, affording the GIGK sequence. The resin was then standard washed, and the residual palladium was removed by 20 6 washes with a mixture of 2 mg/mL diethyldithiocarbamate solution (1 mL) and of a 0.5 % (v) DIEA solution (1 mL). The resin was standard washed again. The desired reactive moiety was then coupled (see paragraphs below).

GIGK-NOTA synthesis

The coupling was done by adding a solution of p-NCS-Bn-NOTA (1.4 eq) and DIEA (9 eq) in 25 DCM/DMF (2:1) (3 mL) to the resin and letting it shake a room temperature for 16 h. Then the solution was filtered off and the resin was washed with DMF and DCM. The product was cleaved from the resin using an acidic TFA/TIS/H₂O (9:0.5:0.5 (v)) solution for 1 h. The filtrate was collected, and the product was cleaved a second time from the resin using a fresh acidic solution. The collected filtrates were concentrated under reduced pressure, the peptide was 30 precipitated cold in diethylether and centrifuged. The precipitate was isolated, and dried. The resulting crude peptide was dissolved in ACN/H₂O (1:1) and lyophilized.

The product was then purified with a preparative HPLC on a Vydac C18 22x150mm 10µm column, at a 20 mL/min flow rate with the following gradient: 10-60% ACN/H₂O + 0,1% TFA

in 20 min. The collected fractions were combined depending on their purity and lyophilized. GIGK(NHCS-Bn-NOTA) was obtained as a white powder (12.9 mg, 14.7 μ mol, 14 % yield). Purity (HPLC): >97 %, t_r = 1.44 min.

GIGK-MeTz synthesis

- 5 From this point forward, the product was protected from light. The coupling was performed by adding a solution of commercially available MeTz-PEG₄-COOH (1.4 eq.), HBTU (1.5 eq.) and DIEA (9 eq.) in DCM/DMF (2:1) (3 mL). The reaction was shaken for 16 h. The solution was filtered off and the resin was washed with DMF and DCM. The cleavage of the peptide was done using an acidic TFA/DCM (1:1) solution over 1 h. The filtrate was collected, and the
- 10 product was cleaved a second time using a fresh acidic solution. The collected filtrates were concentrated under reduced pressure, the peptide was precipitated cold in diethylether and centrifuged. The precipitate was isolated, and dried. The resulting crude peptide was dissolved in ACN/H₂O (1:1) and lyophilized.

- The product was then purified with a preparative HPLC on a Vydac C18 22x150mm 10 μ m column, at a 20 mL/min flow rate with the following gradient: 10-60% ACN/H₂O + 0,1% TFA in 20 min. The collected fractions were combined depending on their purity and lyophilized.
- 15 GIGK(NH-PEG₄-Bn-MeTz) was obtained as a bright pink powder (20.2 mg, 22.4 μ mol, 22 % yield). Purity (HPLC): >97 %, t_r = 2.60 min.

Ac-GIGK-Py-DABCO, GIGK-Py-DABCO, Ac-GIGK-Py-NME₃ and GIGK-Py-NME₃

- 20 For Ac-GIGK-Py-DABCO and Ac-GIGK-Py-NME₃ the acetylation reaction was done with acetic anhydride (10 eq.), DIPEA (5 eq.) in DCM (3 mL). The mixture was added to the resin and shaken for 1 h. The solution was filtered out under vacuum, the resin was standard washed and a Kaiser test was performed. The Alloc deprotection was then performed as described above.
- 25 The precursor DABCO-Py-TFP or NMe₃-Py-TFP (2 eq.) was coupled to the side chain of the lysine in presence of DIEA (5 eq.) and in DMF (3 mL). The mixture was shaken for 2 h. A standard wash was performed. The resin cleavage was performed using a solution of TFA/Triisopropylsilane (TIS)/H₂O (90:5:5 (v/v/v)) which was added to the resin and shaken for 2 h. The filtrate and the 2x3mL DCM washes were collected in a falcon tube. The solvent
- 30 was mostly evaporated under reduced pressure. Diethyl ether (cold) was added and the falcon tubes were left in the freezer overnight. Diethyl ether was removed under reduced pressure and the product was lyophilized. The crude product was purified by preparative HPLC. All the

fractions were analyzed by analytical HPLC, combined according to their purity and lyophilized. The obtained product was analyzed by HPLC and MS.

GIGK-Py-DABCO: LC-MS (m/z): C₂₈H₄₆N₉O₅⁺ calculated 588.73, found 588.39. Purity (HPLC): 98%, Rt=2.09 min, 37% yield. **GIGK-Py-NME₃**: LC-MS (m/z): C₂₅H₄₃N₈O₅⁺ calculated 535.67, found 535.38. Purity (HPLC): ≥99%, Rt=2.12 min, 50% yield. **Ac-GIGK-Py-DABCO**: LC-MS (m/z): C₃₀H₄₈N₉O₆⁺ calculated 630.77, found 630.37. Purity (HPLC): 90%, Rt=2.26 min, 36% yield. **Ac-GIGK-Py-NME₃**: LC-MS (m/z): C₂₇H₄₅N₈O₆⁺ calculated 577.71, found 577.39. Purity (HPLC): ≥99%, Rt=2.29 min, 36% yield

1.9 Site-specific VyPAL2-mediated sdAb functionalization

10 The GIGK-MeTz peptide (25 eq., 4.85 mg, 5.19 μmol) was dissolved in the reaction buffer (PBS pH = 6.5; 600 μL). To the mixture was added the (hPD-L1)-NGL-His₆ sdAb (1 eq., 3 mg, 0.215 μmol) and the VyPAL2 enzyme (0.025 eq, 166 μg, 5.37 nmol). The reaction mixture volume was completed to 3 mL with Fluka water (calculated for a sdAb concentration of 71.5 μM in the reaction mixture) and was then incubated in a warm bath at 37 °C. After 2 hours,

15 the reaction was stopped with a 10x PBS stop buffer (pH = 7.4, 300 μL). Ni-NTA beads (100 μL/mg of protein, Thermo Scientific) were added to an Eppendorf, centrifuged and the supernatant was discarded. The stop buffer (315 μL) was added to the beads, centrifuged and the supernatant was discarded, this was repeated two times more. The beads were transferred in the reaction mixture with 200 μL of stop buffer and the reaction mixture was shaken for 1

20 hour. The reaction mixture was centrifuged, and the supernatant was filtered (Millex®- GV Low Protein Binding Durapore® 13 mm, 0.22 μm membrane). The beads were washed with 200 μL of stop buffer, vortexed, and centrifuged, the supernatant was recovered and used to rinse the filter. The filtrate was collected and transferred to a vivaspin tube (5MWCO, Sartorius, Schaerbeek, Belgium). The reaction mixture was concentrated by centrifugation at 3900 RPM

25 to a volume of 300 μL. The solution was recovered by centrifuging the tube upside-down at 2100 RPM. The membrane was rinsed with metal free PBS (200 μL) and the two solutions were combined. The functionalized sdAb was purified by SEC. The collected fractions with highest concentration were combined allowing to recover the purified (hPD-L1)-GIGK-MeTz sdAb (51±12% recovery yield, N = 2). QCO was performed by SEC, SDS PAGE, WB and MS.

30 The procedure was repeated on the control R3b23-NGL-His₆ sdAb to afford R3b23-GIGK-MeTz in 59% recovery yield (N = 1). The procedure was repeated on a 1.2 mg sdAb-scale (keeping the ratio constant) for the functionalization with the GIGK-NOTA peptide to obtain (hPD-L1)-GIGK-NOTA (47% recovery yield, N = 1), and with GIGGGK-DOTA to obtain R3b23-GIGGGK-DOTA (39% recovery yield, N = 1).

1.10 Site-specific sdAb functionalization via the Michael addition

The site-specific chemical Michael addition was performed following an existing procedure (Chigoho, D. M. et al., *Pharmaceuticals* 14, 550 (2021)). First, the cys-tagged sdAb was reduced. To this aim, a solution of 2.5 mL (1.2 mg/mL, 37 μ M, 3 mg) of dimerized sdAb in PBS (pH 7.4) was reduced with 25 μ L of a 0.5 M stock solution of ethylenediaminetetraacetic acid (EDTA) pH 7 and a 90-fold molar excess of 2-MEA (Acros Organics, Fisher Scientific, Merelbeke, Belgium). The reduction was completed after incubating the mixture at 37 °C for 90 min. The solution obtained after reduction was buffer-exchanged and purified on a PD-10 column pre-equilibrated with 0.2 M NH₄OAc pH 6. Secondly the mal-PEG₄-MeTz (Conju-
5 probes, San Diego, USA) was coupled to the sdAb. To this aim, the purified reduced sdAb (1 mg/mL) was incubated in presence of a 5-fold molar excess of mal-PEG₄-MeTz and 30 μ L of a 0.5 M stock solution EDTA pH 7 at 37 °C for 2 h. The reaction mixture was then concentrated and purified by SEC. QCO of the product was performed through SEC, SDS-PAGE and ESI-Q-ToF-MS. (hPD-L1)-linker-cys-mal-PEG₄-MeTz was obtained in 52%
10 recovery yield (N = 1).

1.11 Direct ⁶⁸Ga-labelling of the NOTA-KGIG-(hPD-L1) sdAb

1 mL (850 MBq) of [⁶⁸Ga]GaCl₃ was eluted from a germanium-68/gallium-68 generator (Galli Eo™, IRE ELiT, Fleuru, Belgium) and was added to the NOTA-KGIG-(hPD-L1) sdAb (7.97 nmol, 3.47 μ M in final volume) diluted in 1 mL of 1 M NaOAc pH 5. After incubating for 10 min
20 at RT, the reaction mixture was applied to a PD10 column (Cytiva) preequilibrated with 25 mL of PBS. The sample was allowed to penetrate the column and the application sample was topped up to 2.5 mL. The product was eluted fractions (total elution volume of 3.5 mL) of PBS. The first fraction of 0.5 mL was discarded, and the following 2 mL were combined and filtered on a 0.22 μ m PVDF low protein binder filter (Merck Millipore). RCP was assessed by radio-
25 RP-HPLC on the PLRPS column and radio-iTLC. Decay-corrected radiochemical yield (DC-RCY) was calculated based on the starting activity of [⁶⁸Ga]GaCl₃ and the sample after filtration, both values decay-corrected for the same time point.

1.12 Direct ⁶⁷Ga-test-labelling of the DOTA-KGGGIG-R3b23 sdAb

[⁶⁷Ga]Ga-citrate solution was purchased from Curium Pharmaceuticals (Amsterdam, The Netherlands) was performed. [⁶⁷Ga]GaCl₃ was obtained as previously described (Bridoux, J. et al., *Biomolecules* 10, 1–15 (2020); Scasnar, V. & van Lier, J. E., *Eur J Nucl Med* 20, 273–
30 273 (1993)). The DOTA-KGGGIG-R3b23 sdAb solution (0.67 nmol, 14.2 μ M in total reaction mixture) was brought to pH 5 with 5 M NH₄OAc pH 5 (volume calculated to reach 0.5 M in

total reaction mixture), to which was added 10 μL (12.3 MBq) of $[^{68}\text{Ga}]\text{GaCl}_3$. The reaction mixture was incubated for 10 min at 65°C. RCP was assessed on the Superdex 75 Increase 5/150GL column and by radio-(iTLC).

1.13 Production of $[^{18}\text{F}]\text{AIF}$

- 5 ^{18}F was produced on site using a cyclotron (Cyclone KIUBE, IBA, Ottignies-Louvain-la-Neuve, Belgium) by irradiation of 2.55 mL of $[^{18}\text{O}]\text{H}_2\text{O}$ with 18-MeV protons for about 3 min (50 μA , 4.8 bars). Enriched water was transferred to an AllInOne (Trasis) automated system and passed through a preconditioned Sep-Pak Light QMA cartridge (WAT023525, Waters, Belgium). The QMA was rinsed twice with 3 mL of water and $[^{18}\text{F}]\text{NaF}$ was eluted with 300 μL of 0.9% NaCl solution. 100 μL (1 — 2.5 GBq) of $[^{18}\text{F}]\text{NaF}$ solution was added to 10 μL (20 nmol) of AlCl_3 solution (2 mM AlCl_3 trace-metal in 0.1 M NaOAc buffer pH 4.5) and left at RT for 5 min.

1.14 Enzyme-mediated radiofluorination of the (hPD-L1)-NXL sdAb

Synthesis of $[^{18}\text{F}]\text{AIF-NOTA-GIGK}$ (Reaction volume = 220 μL)

- 15 In an Eppendorf, a solution of 3 mM GIGK-NOTA in 0.2 M NaOAc pH 4.5 buffer (0.2 mM in the total reaction volume) was diluted in 0.2 M NaOAc pH 4.5 buffer (0.05 M in total volume) and EtOH (25% of total volume). To the solution was added $[^{18}\text{F}]\text{AIF}$ (50% of total volume, 0.300 — 1 GBq), and the Eppendorf was heated at 100 °C for 15 min. The reaction mixture was diluted with ice-cold Fluka water (900 μL). The reaction mixture was applied to a tC18 SEP-PAK Vac 1cc (50 mg) cartridge (Waters, pre-activated with 2 mL of metal free EtOH and 2 mL of Fluka water). The cartridge was dried, rinsed with Fluka water (2x 2.5 mL) and eluted with ACN (200 μL). The eluted fraction was evaporated at 43°C under an N_2 flow in a 1-conical vial of 1 mL.

Enzyme-mediated coupling between the NXL-tagged sdAbs and the $[^{18}\text{F}]\text{AIF-NOTA-GIGK}$ (Reaction volume = 611 μL)

- 25 The reaction was carried in a sdAb:peptide:enzyme(s) ratio of 1:1:0.25 when using the NGL-tagged sdAb in combination with the VyPAL2 enzyme or in a ratio of 1:1:0.01 when using the NQL-tagged sdAb in combination with the VyPAL2 + QC enzyme cocktail. The sdAb concentration was kept constant at 71.5 μM in the reaction mixture.
- 30 To the dry $[^{18}\text{F}]\text{AIF-NOTA-KGIG}$ (50 — 150 MBq) was added PBS buffer pH 6.5 (20% of total volume), the NXL-tagged sdAb and the enzyme(s). Water was added to reach the desired

reaction volume. After 30 min, the solution was purified by SEC using either a 5 mL Pierce cartridge (Thermo Scientific) or a PD10 desalting column following manufacturer's recommendations. Elution the pierce cartridge was performed in fractions of PBS (0.5 mL), the first fraction was discarded, and the following 1.5 mL were kept and filtered on 0.22 µm PVDF low protein binder filter (Merck Millipore). Elution from the PD10 column was performed fractions of PBS (0.5 mL). The first fraction of 0.5 mL was discarded, and the following 1.5 mL were combined and filtered on a 0.22 µm PVDF low protein binder filter (Merck Millipore). RCP was assessed by radio-RP-HPLC on the PLRPS column and/or by radio-SEC on the TSKgel or Superdex 75 Increase 3.2/300GL column. DC-RCY was calculated based on the starting activity of [¹⁸F]NaF used and the activity after filtration.

1.15 Click-radiofluorination of the (hPD-L1) MeTz-functionalized sdAbs analogues

Synthesis of [¹⁸F]AIF-NODAGA-BCN

A solution of 4 mM BCN-NODAGA in 0.2 M NaOAc buffer pH 4.5 (10 µL) was diluted in 0.2 M NaOAc (40 µL) and EtOH (50 µL). To this solution was added the 110 µL of [¹⁸F]AIF (1 GBq), and the Eppendorf was heated at 95 °C for 7 min. Right after the end of the reaction, the solution was quickly diluted with ice-cold Fluka water (900 µL) and transferred to a new Eppendorf. The reaction mixture was applied to a tC18 SEP-PAK Vac 1cc (50 mg) cartridge (Waters, pre-activated with 2 mL of metal free EtOH and 2 mL of Fluka water). The cartridge was dried, rinsed with Fluka water (2x 2.5 mL) and eluted with EtOH (200 µL). RCP was assessed before and after tC18 purification by RP-HPLC.

Click-coupling between the MeTz-functionalized sdAbs analogues and [¹⁸F]AIF-NODAGA-BCN

To the enzymatically or chemically MeTz-functionalized sdAbs (200 µg, 232.5 µL), was added 223.6 µL of Fluka water (calculated to have a sdAb concentration of 0.35 mg/mL in the reaction mixture) and 114.4 mL of EtOH containing ±90 MBq of [¹⁸F]AIF-NODAGA-BCN (calculated to have 20% volume of EtOH in the reaction mixture). After 30 min, the reaction mixture was applied to a PD10 column (Cytiva) preequilibrated with 25 mL of PBS. The sample was allowed to penetrate the column and the application sample was topped up to 2.5 mL. The product was eluted in 3.5 mL of PBS fractionated in 0.5 mL. The first fraction was discarded, and the following 1.5 mL were combined and filtered on a 0.22 µm PVDF low protein binder filter (Merck Millipore). RCP was assessed by radio-SEC on the TSKgel column before purification. After purification, the RCP was assessed on the Superdex 75 3.2/300 column.

1.16 Test-radiofluorination of the GIGK-NME₃ peptide

The GIGK-NME₃ peptide in a lyophilized form was solubilized in anhydrous DMSO (Sigma Aldrich). Enriched water containing [¹⁸F]F⁻ was collected in a vial using the automated AllInOne Trasis module. It was applied to a QMA carbonate Plus Light cartridge (Waters, P#186004540). When NEt₃•HCO₃ solution (6 mg/mL in 80% ACN) was used as phase transfer catalyst (PTC) to elute the ¹⁸F, the QMA was pre-conditioned with 0.5 M NaHCO₃. When K₂CO₃/K₂CO₃ solution (ABX, P#K-2620SYN) was used for elution, 8.4% NaHCO₃ was used to pre-condition the QMA. For the test, 1.78 μmol of peptide or 0.178 μmoles of peptide were used, calculated to be at 10 mg/mL or 1 mg/mL during the radiolabelling. For the NEt₃•HCO₃ conditions, 64 μL of eluted solution was used (2 μmoles of PTC). For K₂CO₃/K₂CO₃ 100 μL per test was used (corresponding to 1.44 μmoles of base and 9.98 μmoles of PTC). Peptide concentration, amount of solvent and temperature were varied during the radiolabelling. RCP was assessed after 15 min of reaction by radio-RP-HPLC on the PLRP-S column.

1.17 Cell binding studies

The radiolabelled sdAbs binding capacity was tested on hPD-L1 positive (hPD-L1^{POS}) 624-MEL cells. 5x10⁴ cells in 1 mL of RPMI (Thermo Fisher) medium per well were allowed to attach in a 24 well plate at 37°C two days prior to experiment. The plate was cooled to 4°C 1 h prior to experiment. Supernatant was removed and cells were incubated for 1 h at 4°C with 500 μL of a 3 nM or a 6 nM radiolabelled sdAb solution in unsupplemented medium (N = 3 wells per conditions). Unbound fractions were collected, wells were washed 2x with ice-cold PBS. Lysis of the cells was performed 2x with 0.75 mL of 1 M NaOH at RT for 5 min. All fractions were collected and counted in the γ-counter (PerckinElmer). Specificity was assayed on hPD-L1^{NEG} 624-MEL cells, and on hPD-L1^{POS} cells in presence of a 100-molar excess of unlabelled competitor (unmodified sdAb) following the same procedures. Percentage of bound activity was calculated as followed: measured activity in bound fractions divided by the total activity of the well.

1.18 Cell saturation assays

The affinity of the radiolabelled hPD-L1 sdAbs was tested on hPD-L1^{POS} 624-MEL cells. 5x10⁴ cells in 1 mL of medium per well were allowed to attach in a 24 well plate at 37 °C two days prior to the experiment. The plate was cooled to 4 °C 1 h prior to the experiment. The supernatant was removed, and the cells were incubated for 1 h at 4 °C with 500 μL of a radiolabelled sdAb solution at different concentrations (300 nM, 100 nM, 33.3 nM, 11.1 nM, 3.7 nM, 1.2 nM, 0.4 nM, and 0.1 nM) in unsupplemented medium (N = 3 wells per condition).

The wells were processed the same way as the cell binding study. To correct for a specific binding, the same procedure was simultaneously applied to a second plate containing 100-molar excess of unlabelled competitor (unmodified sdAb) in each well. The KD was calculated while using a “One site — total and nonspecific binding” analysis in Prism software.

5 1.19 In vivo biodistribution studies

Healthy female C57BL/6 mice (five to six weeks old, N = 3 per group) were injected with either 22 µg of [¹⁸F]AIF-NOTA-KGIG-(hPD-L1) sdAb (2.75 GBq/µmol at injection time), 22 µg of [¹⁸F]AIF-NOTA-KGIG-R3b23 sdAb (0.98 GBq/µmol at injection time), 5 µg of [⁶⁸Ga]Ga-NOTA-KGIG-(hPD-L1) sdAb (30.87 GBq/µmol at injection time), 22 µg of [¹⁸F]AIF-NODAGA-BCN-MeTz-KGIG-(hPD-L1) sdAb (1.73 GBq/µmol at injection time) or 24.7 µg of [¹⁸F]AIF-NODAGA-BCN-MeTz-mal-cys-(hPD-L1) sdAb (1.78 GBq/µmol at injection time). Animal were allowed to wake up after injection and were euthanized 80 min post-injection by neck dislocation under isoflurane anaesthesia. After euthanasia, main organs and tissues were isolated, weighed, and counted against a standard of known activity using a γ-counter. The amount of radioactivity in organs and tissues was expressed as percentage of injected activity per gram (%IA/g).

1.20 Statistical analysis

Results are expressed as mean ± standard error of the mean. A non-parametric Mann-Whitney U test was carried out to compare data sets. Sample sizes and number of times experiments were repeated are indicated in the figure legends. The number of asterisks in the figures indicates the statistical significance as follows: *P < 0.05; ** P < 0.01; ***P<0.001; ****P<0.0001.

EXAMPLE 2: SdAb generation, quality controls and characterization

The sdAbs were generated in usual yields following standard quality controls procedures as well as characterization which were as expected (Table 2).

Table 2. Summary of the production yield, quality controls and characterization performed on the starting sdAbs. SEC = Size-exclusion chromatography. MS = mass spectrometry. SPR = surface plasmon resonance. SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis. MP = melting point. NM = not measured. NA = not applicable.

SdAb	Production yield	SEC (280 nm)	MS (expected kDa, obtained kDa)	SDS-PAGE	SPR (nM)	MP (°C)
(hPD-L1)-NGL-His ₆	16.8 mg/mL	99%	13.961, 13.961	>99%	3.25	68°C
(hPD-L1)-NQL-His ₆	5.14 mg/mL	NM	14.032, 14.032	>99%	NM	68°C
R3b23-NGL-His ₆	17.6 mg/mL	94%	15.009, 15.009	>99%	NA	NM
R3b23-NQL-His ₆	11.1 mg/mL	>99%	15.080, 15.080	>99%	NA	NM
(hPD-L1)-linker-his ₆ -cys	10.0 mg/L	NM	NM	Dimer and monomers visible	4.3	70°C

EXAMPLE 3: Peptide synthesis

The first peptidyl substrate, GIGK-(NHCS-Bn-NOTA) peptide (FIG. 1C) (referred to as GIGK-NOTA), is a chelator-functionalized peptide that can be coupled to a sdAb engineered with the asparagine-glycine-leucine-hexahistidine (NGL-His₆) recognition tag. The VyPAL2 enzyme specifically recognizes and cleaves the NGL-tag to form a sdAb-acyl-enzyme covalent intermediate. Upon nucleophilic substitution of the intermediate with the peptidyl substrate, the reaction results in a sdAb coupled site-specifically at its C-terminal end. The NOTA chelator allows complexation of (radio) metals, such as ⁶⁸Ga or [¹⁸F]AIF. The commercially available chelator, p-NCS-Bn-NOTA (FIG. 1B), was coupled to the GIGK tetrapeptide sequence (FIG. 1A) in presence of a base. The peptide was then simultaneously cleaved from the resin and Boc deprotected with an acidic solution of TFA in presence of scavengers. After purification with preparative HPLC, the GIGK-NOTA peptide (FIG. 1C) was recovered as a TFA salt with a 14 % yield and a purity of ≥97 %, determined by HPLC (214 nm).

Similarly, the second peptidyl substrate, GIGK(NH-PEG₄-Bn-MeTz peptide (referred to as GIGK-MeTz), a tetrazine-functionalized peptide, can be coupled to an NGL-His₆ tagged sdAb via catalysis with the VyPAL2 enzyme. This tetrazine functionalization strategy allows for subsequent click-radiolabeling with a commercially available bicyclononyne functionalized chelator (BCN-NODAGA) (FIG. 2) offering the advantage that the functionalized sdAb can be properly purified and isolated and more intermediate QCOs can be performed. Moreover, this functionalized nanobody could be click coupled to a prosthetic group (PG) with other functions.

The commercially available MeTz-PEG₄-COOH (FIG. 1D) was coupled to the GIGK tetrapeptide sequence by a condensation between the carboxylic acid of the MeTz-PEG₄-COOH and the free amine on the peptide, leading to an amide bond formation. The condensation was done in presence of a base and the HBTU activator. As the tetrazine moiety shows a sensibility to light, the handling of tetrazine functionalized products was done with minimal light exposure. The peptide was then simultaneously cleaved from the resin and Boc deprotected. However, due to a TFA sensibility of the tetrazine moiety, a 1:1 TFA/DCM solution was used, affording the GIGK-MeTz peptide (FIG. 1E) as a TFA salt with a 22 % yield and a purity of ≥97 %, determined by HPLC (214 nm).

10 **EXAMPLE 4: VyPAL2-mediated site-specific functionalization of sdAbs, quality control and characterization**

The NGL-His₆ tagged hPD-L1 sdAb had first to be functionalized site specifically with the MeTz peptide to allow click-radiolabelling with the [¹⁸F]AlF-labelled BCN-NODAGA PG in a subsequent step. A functionalization with the NOTA peptide was also performed as a reference for non-radioactive analysis and ⁶⁸Ga-test labelling. The R3b23-NGL-His₆ sdAb was also coupled to the MeTz-GIGK peptide as control conditions. The synthetic GIGGK-DOTA peptide was coupled to the R3b23 sdAb in order to perform test-labelling with gallium-67 (⁶⁷Ga, SPECT radionuclide), in view of future test labelling with therapeutic radiometals. The peptides were allowed to react with the sdAb in presence of the VyPAL2 enzyme at 37 °C in Phosphate Buffer Saline (PBS) at pH = 6.5 for 2 h.

The enzyme, the unreacted sdAb and the free His₆-tag were removed from the mixture by Immobilized Metal Affinity Chromatography (IMAC) with Ni-NTA resin. Using Size Exclusion Chromatography (SEC), the functionalized sdAb was separated from the impurities that were not removed with IMAC, such as the unreacted peptide. Table 3 displays the recovery yields of the bioconjugation process as well as the quality control and characterization assays.

Table 3. Summary of the VyPAL2-mediated bioconjugations to NGL-His₆-tagged sdAbs and their quality control and characterization. SdAb = single-domain antibody. QCO = quality control. SEC = Size-exclusion chromatography. MS = mass spectrometry. SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis. NM = not measured. NA = not applicable. N = number of repetitions in case N > 1.

SdAb	Recovery yield	QCO by SEC (280 nm)	MS (expected kDa, obtained kDa)	SDS-PAGE
(hPD-L1)-GIGK-	51±12%	99%	13.741, 13.741	> 99%

MeTz	(N=2)			
(hPD-L1)-GIGK- NOTA	47%	98%	13.773, 13.773	> 99%
R3b23-GIGK-MeTz	48%	98%	14.788, 14.789	> 99%
R3b23-GIGGGK- DOTA	39%	NM	NM	NM

The western blotting (WB) gel (data not shown) indicated that the functionalized sdAbs contained the His₆-tagged, which indicates that the IMAC purification requires further optimization. The enzyme was not detected even though it is His₆-tagged. The samples were analysed by mass spectrometry, which showed expected molecular weights of the functionalized sdAbs. Yet the unreacted sdAbs could be seen on the spectrum, confirming the results of the SDS-PAGE/WB analysis: a small amount of unreacted sdAb remains in solution, meaning that the process may be optimized.

EXAMPLE 5: Site-specific Michael addition between the cys-tagged sdAb and the mal-PEG₄-MeTz. QCO and characterization

The (hPD-L1)-linker-His₆-cys-tag (dimeric form) was first reduced in presence of 2-mercaptoethylamine (2MEA). The reduction step was followed by site-specific coupling of mal-PEG₄-MeTz on the free thiol of the C-terminal cysteine. SEC purification of the conjugated sdAb resulted in a recovery yield of 52%. QCO by SDS-PAGE revealed presence of monomer, and SEC revealed a purity >98%. No dimeric sdAb was observed. MS characterization showed a major peak at the expected Mw (15.752 kDa). Starting dimeric sdAb or monomeric sdAb were not observed.

EXAMPLE 6: Direct ⁶⁸Ga-labelling of the NOTA-KGIG-(hPD-L1) sdAb

Before going further with the ¹⁸F strategy, the NOTA-KGIG-(hPD-L1) sdAb was labelled with ⁶⁸Ga as the radiochemistry is more straightforward. The sdAb was successfully radiolabelled in 45% decay-corrected radiochemical yield (DC-RCY) and radiochemical purity (RCP) was > 99% (FIG. 3) as assessed by radio-RP-HPLC on the PLRP-S column. Apparent molar activity at end of production was 44.79 GBq/μmol.

EXAMPLE 7: Direct ⁶⁷Ga-test-labelling of the DOTA-KGGGIG-R3b23 control sdAb

Before going further with the therapeutic strategy involving therapeutic radio metals, the DOTA-KGGGIG-R3b23 control sdAb was labelled with ⁶⁷Ga as the radiochemistry is more

straightforward. The test-labelling was performed at 65°C. Radio-SEC at 10 and 30 min indicated 30% of radiolabelled sdAb (FIG. 4). The yield of the reaction can be improved, however further optimization steps will be performed with therapeutic radionuclide lutetium-177 (^{177}Lu) as the radiochemistry conditions differ from ^{67}Ga .

5 **EXAMPLE 8: Indirect click-radiofluorination of MeTz-sdAbs analogues**

The BCN-NODAGA chelator was radiolabelled for further click-reaction with the site-specifically MeTz-functionalized sdAbs. The radiofluorination was performed by complexation with $^{18}\text{F}[\text{AlF}]$ which is produced from the reaction between $^{18}\text{F}[\text{NaF}]$ obtained from an automated sequence of the AllInOne Trasis and AlCl_3 at room temperature. The complexation
10 reaction between BCN-NODAGA and $^{18}\text{F}[\text{AlF}]$ was done at 95°C in 7 min and the obtained product was immediately cooled down to avoid degradation, as the BCN-NODAGA was unstable at 100°C for prolonged times. QCO of the mixture before purification showed that it contains 20% of the radiofluorinated BCN-NODAGA and 80% of free ^{18}F (in the form of $^{18}\text{F}[\text{NaF}]$ and/or $^{18}\text{F}[\text{AlF}]$). The $^{18}\text{F}[\text{AlF}]$ -NODAGA-BCN intermediate was then purified using a
15 tC18 cartridge and the elution was done in EtOH. After purification, the RCP of the intermediate was 92% (FIG. 5). Even if the product was not completely pure it was used for the next step because radio-impurities such as free $^{18}\text{F}[\text{NaF}]/\text{AlF}$ do not influence the click reaction.

The click coupling between the electron-rich dienophile BCN moiety and the electron-deficient
20 diene MeTz of the enzymatically or chemically functionalized sdAbs, is known as an inverse electron-demand Diels-Alder (IEDDA) reaction. The reaction is fast and performed in mild conditions with only nitrogen as the by-product. It was performed at RT for 30 min in aqueous solution (PBS, pH 7) and 20% of EtOH. The mixture was then purified using a PD-10 column pre-equilibrated with a buffer compatible with in vivo injection (0.9% NaCl + 5 mg/mL vitamin
25 C, or PBS). The estimated coupling yield was 25% based on the activity measured in the collected fractions. The Radio-SEC analysis of the product after filtration showed a RCP of >95% (FIG. 6). The decay-corrected radiochemical yield (DC-RCY) was 2%, calculated from starting activity (measured in the $^{18}\text{F}[\text{AlF}]/\text{NaF}$ solution) and the activity after filtration, both values decay-corrected for the same time point. The apparent molar activity was 3.87
30 GBq/ μmol ($N = 2$).

EXAMPLE 9: Enzyme-mediated radiofluorination of the NXL-His₆-tagged sdAbs

First, the conditions to produce GIGK-NOTA- $^{18}\text{F}[\text{AlF}]$ were optimized, and suitable analytical methods were developed. The NOTA-functionalized peptide is diluted in a mixture of 0.2 M

NaOAc buffer pH 4.5 and solvent supplemented with [^{18}F]AIF/NaF. The reaction mixture is then heated at 100 °C for 15 min.

To determine the best conditions for the chelation of [^{18}F]AIF with GIGK-NOTA, first, EtOH or ACN were tried as solvents, then heating for either 10 min or 15 min. For these tests the following parameters were kept constant: 0.5 eq. AlCl_3 to 1 eq. of GIGK-NOTA, [GIGK-NOTA]_{in reaction vial} = 0.2 mM, [AlCl_3]_{in reaction vial} = 0,020 mM, 25% solvent in final volume, pH = 4.5. Various parameters such as the concentrations of AlCl_3 and GIGK-NOTA as well as the numbers of [^{18}F]AIF equivalents were evaluated. After the reaction, each sample was diluted in cold water to stop the reaction. Samples were analyzed by radio RP-HPLC on the XBridge C18 analytical column, [^{18}F]AIF was observed at R_t = 0.83 min and [^{18}F]AIF-GIGK-NOTA at R_t = 3 min. Labeling conditions and [^{18}F]AIF complexation yields are reported in FIG. 23.

Having 0.1 eq. of AlCl_3 (line 1 of FIG. 23) led to a 16% complexation while a better complexation of 35% could be obtained with a 0.5 eq. reaction in the same conditions (line 2 of FIG. 23). Having a higher number of equivalents leads to a lower yield; 1.5 eq. of AlCl_3 (line 5 of FIG. 23) led to a 9% complexation against a 15% complexation with a 0.5 eq. reaction in the same conditions (line 4 of FIG. 23). Thus, it is hypothesized that the non-radioactive Al-species are competing with [^{18}F]AIF for NOTA chelation. Decreasing the concentration of GIGK-NOTA by two (line 3 & 2 of FIG. 23) leads to similar complexation yields (30 % against 35 %). However, a decrease in concentration by a factor 3 (line 4 of FIG. 23) has a negative impact on the yield, as it was only 15 %, against 35 % for a higher concentration (line 2 of FIG. 23).

Before proceeding with the enzyme-mediated labelling of the sdAb, the [^{18}F]AIF-NOTA-KGIG species was isolated. The reaction mixture was first diluted in cold water to reach a total volume of 1.1 mL. The solution was then applied to a tC18 cartridge which was rinsed twice with water to remove salts and [^{18}F]AIF/NaF. Next, the product was eluted with ACN. The analytical method to perform the QCO of [^{18}F]AIF-NOTA-KGIG was transferred to a PLRP-S method allowing the use of a single gradient to separate the free ^{18}F , the peptide and the radiolabelled sdAb final product.

Having successfully formed the [^{18}F]AIF-NOTA-KGIG, the best conditions for coupling with the (hPD-L1)-NGL-His₆ sdAb were determined. First, the impact of the peptide/sdAb ratio and the reaction time were assessed. The ACN eluent containing the [^{18}F]AIF-NOTA-KGIG was evaporated under N_2 flow at 43°C. A solution of sdAb (at various eq.) and VyPAL2 (0.025 eq.) diluted in 20% (v) PBS pH = 6.5 and 30% (v) water (to keep the concentration of sdAb constant) was added to the dried peptide and the mixture was heated at 40°C for the duration

of the reaction. The samples were diluted in 2 ×PBS + Tween 80 0.1% and analysed by radio-SEC on the TSKgel column to determine the percentage of radioactivity coupled to the sdAb.

Table 4. Optimization of the coupling reaction between ¹⁸F-labelled NOTA-peptide and the NGL-His₆-tagged sdAb using the VyPAL2 enzyme. % of coupled activity was measured by radio-SEC.

N°	Peptide eq.	sdAb eq.	Reaction time	% coupled activity
1	25	1	1 h	4%
2	5	1	1 h	18%
3	1	1	1 h	41%
4	1	2	1 h	60%
5	1	5	1 h	72%
6	1	1	30 min	50%
			45 min	48%
			1 h	51%

The results listed in table 4 show that, with a 0.04 and 0.20 sdAb/peptide ratio (respectively reactions 1 & 2), almost all the expected radioactivity was coupled to the sdAb in one hour (4% and 18% respectively). With a ratio of 1:1 between the sdAb and peptide (reaction 6), the percentage of activity coupled to the sdAb seemed to be stable (around 50%) after 30, 45 or 60 min of heating, which shows that the reaction is complete after only 30 min. This further demonstrates the high efficacy of the VyPAL2 enzyme, because coupling with other enzymes such as sortase A have to be performed over 16 h. A higher ratio was also tried by using 2 times and 5 times more sdAb as compared with PG (lines 4 and 5 respectively) which allowed to increase the % of coupling to 60 and 72% respectively. However, a lot of sdAb protein must be used for these reactions which may not be cost-effective for future translation application. The reaction mixture from line 3 (FIG. 7) was further purified on a PD10 desalting column. The elution from the PD10 was fractionated to investigate whether peptide is coming through in the eluent.

After PD10 purification the initial eluted volume of 0.5 mL was discarded, and the following 1.5 mL were pooled and analysed by radio-SEC analysis. The analysis (FIG. 8) showed that the product was recovered with an RCP of 95.2%. The other 4.8% were peptide that was probably eluted with fraction 4. Hence, adding more fractions to the pool would certainly elute more free-peptide too and reduced the final RCP.

The purified radioactive product was allowed to decay and was then analysed by SDS-PAGE / WB. The SDS-PAGE analysis showed a band around the expected Mw of the sdAb. However, the WB analysis shows that some unlabelled sdAb were still present in solution, which was

expected since the labelling was not total and minority of unreacted sdAb still carrying the His₆-tag were present. On the WB another band corresponding the His-tagged enzyme was visible, suggesting that some of the enzyme was not eliminated by the PD10 column desalting and are still present in the purified solution.

- 5 Overall, since the product was recovered with an RCP>95%, it is suitable for *in vivo* studies. The product was obtained with a DC-RCY of 7% (measured in the diluted reaction mixture before purification on the tC18) and the activity after filtration. Both values were decay-corrected at the same time point. The apparent molar activity after production was 2.94 GBq/μmol.

10 9.2 Use of the VyPAL2 + QC mix for the cascade labelling reaction

- In order to improve the radiochemical yield, the enzymatic mix (VyPAL2 + QC enzyme) was used to mediate the fluorination between the hPD- L1 sdAb bearing an NQL-tag and the radiofluorinated GIGK-NOTA. First, the GIGK-(NHCS-Bn-NOTA) peptide was radiolabelled and isolated as described above. The enzyme-mediated radiofluorination was done using 1 eq. of the hPD-L1-NQL-His₆, 1 eq of the labelled peptide [¹⁸F]AIF-GIGK-NOTA) and 0.01 eq. of the enzymatic mix, in PBS buffer (pH 6.5). The reaction was performed at 43°C for 30 min. Radio-SEC after 30 min of incubation showed 11 % of peptide remaining (FIG. 9). Small radio-impurities were detected at 15.90 min which could represent free ¹⁸F or degraded peptide. They are more likely due to the presence of impurities attached to this particular column as no unexpected radio-impurities were visible by radio-RP-HPLC (data not shown). An estimated ratio of 72% of the radioactivity was associated with the sdAb.

- The mixture was then purified using a PD-10 column. The QCO by radio RP-HPLC after purification and filtration showed a RCP of 93% (FIG. 10A). Smaller radio- impurities were detected (7%). The UV spectrum at 280 nm showed a chemical purity of 100% (FIG. 10B). The product is obtained with good purity suitable for *in vivo* studies.

The DC-RCP was 11% which is higher than the value obtained in previous coupling with VyPAL2 enzyme (DC-RCP=7%).

Example 10: In vitro binding studies

- To assess the binding specificity of the radiolabelled sdAbs to its hPD-L1 target expressed on cells, radiolabeled sdAbs were added to either hPD-L1^{POS} or hPD-L1^{NEG} 624-MEL cells at two different concentrations (3 nM and 6 nM). The blocked MEL624 hPD-L1^{POS} cells with a 100-fold molar excess of non-radioactive sdAb were used as control to assess binding specificity.

After incubation, the unbound fractions were removed, and the cell-associated activity was measured. FIGs. 11A, B, C and D show the results for the following tested compounds respectively; [^{68}Ga]Ga-NOTA-KGIG-(hPD-L1) sdAb, [^{18}F]AIF-NOTA-KGIG-(hPD-L1) sdAb, [^{18}F]F-NODAGA-BCN-MeTz-KGIG-(hPD-L1) sdAb and [^{18}F]F-NODAGA-BCN-MeTz-mal-cys-(hPD-L1) sdAb. For all sdAbs analogues, the cell-associated activity of both radiolabelled showed specific binding on hPD-L1^{POS} cells, which was confirmed by absence of cell-associated activity in control conditions (hPD-L1^{NEG} cells and excess of unlabelled sdAb). This assay confirms the specificity of the hPD-L1 sdAbs for their target but does not allow to assess the affinity.

10 **EXAMPLE 11: In vitro cell saturation assay**

The affinity (dissociation constant K_D) of the radiolabelled sdAbs analogues was tested on hPD-L1^{POS} 624-MEL cells. The cells were incubated in the plate with the radiolabelled sdAbs at concentrations ranging from 300 nM to 0.1 nM and the wells were processed the same way as in the cell binding study. FIG. 12A, B and C show the results for the following tested compounds respectively; [^{18}F]AIF-NOTA-KGIG-(hPD-L1) sdAb, [^{18}F]F-NODAGA-BCN-MeTz-KGIG-(hPD-L1) sdAb and [^{18}F]F-NODAGA-BCN-MeTz-mal-cys-(hPD-L1) sdAb. All obtained values were in the expected range for the hPD-L1 sdAb, indicating that there was no negative impact on the affinity of the hPD-L1 sdAb after their functionalization and radiolabelling.

EXAMPLE 12: In vivo biodistribution studies in healthy mice

20 To study their in vivo biodistribution, the radiolabelled sdAbs were injected in three healthy mice. The mice were euthanized at 80 minutes post injection (p.i.). The organs were collected, and the amount of activity was measured in each organ.

The hPD-L1 and control R3b23 sdAbs radiofluorinated with [^{18}F]AIF-NOTA-KGIG were first injected via the enzymatic mix (FIG. 13). No background is observed as expected except for retention in the kidneys due to the excretion pathway of sdAbs. As expected, the hPD-L1 sdAbs displays significant lower kidney retention compared to the control sdAbs.

The biodistribution of the [^{68}Ga]Ga-NOTA-KGIG-(hPD-L1) sdAb which had been pre-functionalized with VyPAL2 was then investigated, isolated and then directly labelled with ^{68}Ga . This biodistribution was compared with previously obtained data from the [^{18}F]AIF-labelled analogues (FIG. 14). As the two end products are the same, except for the radionuclide, it was interesting to see the impact on using an enzyme-mediated strategy to perform the

radiofluorination of the sdAb instead of the usual pre-functionalization strategy followed by direct ^{68}Ga -labelling.

Finally the biodistribution obtained [^{18}F]AIF-NOTA-KGIG-(hPD-L1) sdAb was compared with the biodistribution profile of the enzymatically or chemically MeTz-pre-functionalized sdAbs.

- 5 This comparison is interesting as the chemical strategy is one of the usual site-specific strategies performed to radiolabel sdAbs.

In conclusion the enzymatically pre-functionalization of the sdAb lowers by two times its kidney retention as compared with the chemical pre-functionalization. When using the enzyme to mediate the radiofluorination strategy to yield [^{18}F]F-NOTA-KGIG-(hPD-L1), the kidney
10 retention further decreased with a 3.2x reduction as compared with the chemical strategy.

Taken together, these data indicate that using the VyPAL2 enzyme (in combination with the QC enzyme) for the radiofluorination of the hPD-L1 sdAb brings an advantage for in vivo properties in addition to facilitating and improving the overall efficiency of the radiochemical process.

15 **EXAMPLE 13: Novel peptides for radiofluorination via nucleophilic substitution**

To obtain the data shown previously, [^{18}F]AIF was used as a metal-like radionuclide because the complexation chemistry is more straightforward compared to performing nucleophilic substitutions with the radiohalogen [^{18}F]F $^-$. However, using [^{18}F]F $^-$ would increase the yield of radiofluorination of the peptide and increase specific activity by separating non-radiolabelled
20 peptidyl precursor from the ^{18}F -labelled peptide. Moreover, this would benefit the overall enzymatic coupling yield. Furthermore, radiometals are known to increase kidney retention as compared with radiohalogens. For these reasons, one aim is to develop peptides with leaving groups such as DABCO and NMe_3 for radiofluorination by nucleophilic substitution.

The precursors DABCO-Py-TFP and NMe_3 -Py-TFP were obtained in good yields from 6-chloronicotinic acid in two steps. The esterification reaction of the starting material using N,N'-dicyclohexylcarbodiimide (DCC) and 2,3,5,6-tetrafluorophenol (TFP-OH) produced the ester Cl-Py-TFP. The side-product dicyclohexylurea (DCU) was removed by filtration while the unreacted reagents were easily removed by liquid extraction. The nucleophilic aromatic substitution of the chlorine using DABCO and NMe_3 was favored as Cl-Py-TFP was highly
25 activated by the electron withdrawing TFP ester moiety. The DABCO-Py-TFP precursor was obtained by reacting Cl-Py-TFP with DABCO using the same condition described by Zlatopolskiy et al. (2019). The product was obtained after a filtration step with 72% yield. The
30

NMe₃-Py-TFP precursor was also obtained with 72% yield and a purity ≥99% by reacting the NMe₃ solution in THF with Cl-Py-TFP using the conditions described by Bouvet et al. (2016).

The coupling between the precursors and the peptidyl backbone (consisting of the GIGK sequence) was performed similarly as for the NOTA and MeTz peptides described in Example 3. The coupling step was performed using 2 eq. of the precursor in presence of 5 eq. of the base in 2 h. As the precursor was already activated by the presence of the TFP group, no activator was needed. To prevent nucleophilic substitution with the amine-group of the glycine residue on the leaving group of the peptide during radiofluorination, acetyl-protected variants of the peptides were generated. The products were purified by RP-HPLC and obtained with good purities and with various yields (FIG. 16).

Next, test-radiofluorination was performed on the GIGK-NMe₃ peptide by varying several parameters as described in table 5.

Table 5. Test-radiofluorination of the GIGK-NME3 peptide (pep) varying the base, the phase transfer catalyst (PTC), the solvent, the temperature, and the activity. The radiochemical purity (RCP) was assessed by radio-RP-HPLC after 15 min of reaction.

[pep] (1 eq.)	Base (eq.)	PTC (eq.)	Solvent	T°	Ratio ¹⁸ F/peptide	RCP by radio-RP-HPLC @ ±15 min
10 mg/mL	NaHCO ₃	NEt ₃ •HCO ₃ (1.1 eq.)	20% DMSO in ACN	40°C	NA	0%
			20% DMSO in ACN	80°C	NA	0%
	NaHCO ₃ K ₂ CO ₃ (0.8 eq.)	K ₂₂₂ (5.6 eq.)	DMSO	40°C	1497	85%
			DMSO	95°C	2144	95%
1 mg/mL	NaHCO ₃ K ₂ CO ₃ (8 eq.)	K ₂₂₂ (56 eq.)	DMSO	95°C	54	65%

In the next steps we will confirm that the measured peak is the expected product, isolate the ¹⁸F-labelled peptide by testing various cartridges or by preparative RP-HPLC. We will also attempt to radiofluorinate the DABCO-functionalized peptide.

EXAMPLE 14: Radiofluorination of the hPD-L1 sdAb - optimization of the coupling with the GIGK-NHCS-NOTA peptide.

The synthesis and radiolabeling of the GIGK-NHCS-NOTA peptide with [¹⁸F]AIF was described earlier. The enzyme-mediated radiofluorination was reported with 1 eq. of the hPD-L1-NQL-His₆ single domain antibody (sdAb), 1 eq. of the labelled peptide [¹⁸F]AIF-GIGK-

NOTA and 0.01 eq. of the enzymatic mix (VyPAL2 + 0.1 eq. of GluC enzyme relative to VyPAL2), in PBS buffer (pH 6.5). The reaction was performed at 43°C for 30 min. This condition yielded about 75% of coupled radioactive peptide to the sdAb.

Optimization of the time and efficiency of the reaction was investigated.

- 5 In this regard, non-radioactive optimization on the hPD-L1 sdAb was carried with the 0.1 eq. of VyPAL2 (+ 0.5 eq. of GluC relative to VyPAL2) and yielded >90% conversion in 5 minutes with the NOTA peptide.

For the coupling with the radiolabelled peptide, increasing the equivalents of GluC enzyme to 0.5 eq. (relative to VyPAL2) yielded 92% of coupled peptide within 15 minutes of reaction as
10 displayed on FIG. 17.

With the [¹⁸F]AIF peptide we currently cannot increase the amount of ¹⁸F due to the presence of metal contaminants coming from the cyclotron, (unrelated of the enzymatic coupling). However, we are interested to study the resistance of the enzyme when exposed to higher amounts of activity.

- 15 In this experiment, 900 MBq of [¹⁸F]NaF salts were dried and resolubilized using the enzyme mix. The mix was heated at 43°C for 7 minutes to allow irradiation, after which was added the (hPD-L1)-NQL sdAb (1 eq. at 1 mg/mL) and the ¹⁸F-labelled peptide substrate (1 eq.), and incubated for another 7 minutes in presence of 0.1 eq. of VyPAL2 (+ 0.5 eq. of GluC relative to VyPAL2). 85% of radiolabeled peptide was coupled to the sdAb in these conditions as
20 displayed on FIG. 18.

This data seems to indicate that irradiation with higher activity (suitable for preclinical research) did not impact the enzyme's efficiency. The next steps will involve increasing the amount of activity on the peptide itself, investigate reproducibility of the process and develop a robust purification method in order to repeat the preclinical studies in tumor-bearing mice.

25 **EXAMPLE 15: Radiolabeling via nucleophilic substitution using the FPy-KGIG peptide.**

To allow to work with higher amount of radioactivity on the peptide, we are investigating in parallel the use of a novel type of peptides including leaving groups to allow for radiofluorination by nucleophilic substitution, as described in Example 3.

- The synthesis of two novel peptides: GIGK-Py-DABCO and GIGK-Py-NMe₃ was described
30 earlier. The test labelling with the NMe₃ peptide resulted in 65% of the radioactivity at 6.43 minutes, representing the incorporation of ¹⁸F onto the peptide at 1 mg/mL in DMSO, using

kryptofix as a phase transfer catalyst and K_2CO_3 as a base, at 95°C within 15 minutes. A non-radioactive reference has now been synthesized which allowed to confirm the presence of the radiolabeled peptide at the expected R_t (see FIG. 19).

5 Using the non-radioactive reference, non-radioactive couplings onto the (hPD-L1)-NQL-His₆ and the control R3b23-NQL-His₆ sdAbs were attempted. Coupling of the peptide was observed by following the UV signal on a C4 column as displayed in FIG. 20, however this needs to be confirmed by mass spectrometry.

1:1 sdAb (tested with hPD-L1 and control R3b23) :peptide ratio, 5 min reaction time with 0.1 eq. Of VyPAL2 (+1 eq. Of GluC relative to VyPAL2)

10 This test displayed in FIG. 20 shows results performed on the control sdAb R3b23 R_t = 7.15 min = unmodified sdAb, R_t = 7.53 = supposed sdAb coupled to the FPy-KGIG peptide.

EXAMPLE 16: Enzymatic coupling to produce sdAbs for labelling with therapeutic radionuclides.

15 The sdAb targeting the carcinoembryonic antigen (CEA) was synthesized with a NQL-His₆ or a cysteine tag at its C-terminal.

The aim was to couple the DOTA moiety to the sdAb to allow radiolabeling with ^{177}Lu , a radionuclide for therapeutic applications. To this aim, a GIGK-NHCS-DOTA peptide was synthesized following protocol described in Example 3 above.

20 The GIGK-NHCS-DOTA peptide was coupled to the CEA sdAb following the protocol described in Example 4 above.

In parallel, maleimide-DOTA (mal-DOTA) was purchased from CheMatech (Dijon, France) and coupled to the cysteine-tagged CEA sdAb analogue following procedure described in Example 5 above.

25 Test-labelling with ^{177}Lu were performed by adding ^{177}Lu to the DOTA-sdAbs (19 μM in the mix) at pH 4.5 for 2 hours. In these conditions, the CEA chemically coupled to mal-DOTA resulted in only 10% incorporation of ^{177}Lu at 50°C and temperature required to be increased to 60°C to reach 94% incorporation as seen in FIG. 21.

The enzymatically coupled CEA-GIGK-NHCS-DOTA was labeled with ^{177}Lu at 16.6 μM for 45 min at 40°C, yielding satisfactory 80% of incorporation of ^{177}Lu as showed in FIG. 22.

Summary

The present disclosure describes the use of asparaginyl peptide ligase enzymes (PALs) either alone or in tandem with a glutaminyl cyclase (QC) enzyme, as a new method for the rapid and reagent-effective generation of radiolabelled single-domain antibodies (sdAbs) with improved in vivo properties. In particular, the cascade reaction (PAL + QC) applied to sdAbs introduces a tool that is unmatched: it allows streamlined generation (eg < 1 hour) in a time compatible with short (<12 hours) half-life radioelements of pure homogeneous radiolabeled sdAbs with close to 100% yield and minimal radioactive waste generation.

Various strategies were employed to generate radiolabelled sdAbs tracers using either the VyPAL2 enzyme alone or VyPAL2 in combination with the QC enzyme typically at a 1: 0.1 or a 1: 0.5 molar ratio. These bioconjugation strategies were compared with another site-specific strategy relying on a chemical reaction instead of the enzymatic reaction. Remarkably, using the enzymatic cocktail allowed almost total bioconjugation (>70%) between the sdAb carrying a NQL-His₆ tag at its C-terminal end with the radiofluorinated peptide [¹⁸F]AIF-NOTA-KGIG. For the latter reaction, an equimolar ratio of sdAb:peptide was used. The generated product [¹⁸F]AIF-NOTA-KGIG-(hPD-L1) sdAb displayed better in vivo behaviour as compared with the one obtained using chemical site-specific strategy. The characteristics of the sdAb conjugate obtained using the enzymatic cocktail indicate that this method is extremely well-suited for the fast and efficient generation of sdAb-based site-specifically radiofluorinated tracers with improved in vivo characteristics for molecular imaging.

Furthermore, the peptidyl substrate may comprise other radiolabels, and/or reactive moieties for the purpose of radiolabelling. This can be combined with specific amino acid sequences that facilitate the radiochemistry processes and further improve the in vivo behaviour, based on the desired application such as for use in diagnosis, in surgery, in treatment, or for use in therapy monitoring, and, more specifically, for use as an imaging agent. This will be of great interest in hospitals and medical centres routinely using radio-medicine.

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Claims

1. A method of preparing a site-specifically radiolabeled tracer, the method comprising providing
- 5 i) a peptidyl asparaginyl ligase (PAL);
- ii) a first peptide comprising a P1-P1'-P2' tripeptide PAL motif at the C-terminus, wherein P1 is Asn or Asp, P1' is Gln or any non-proline amino acid, and P2' is a hydrophobic amino acid such as Val or Leu or Ile or a β -branched amino acid;
- 10 iii) a second peptide comprising a P1''-P2'' motif at the N-terminus, wherein P1'' is Gly and P2'' is a hydrophobic amino acid such as Val or Leu or Ile, or a β -branched amino acid, wherein the second peptide is modified with a functional moiety to form a functionalized peptide;
- 15 iv) contacting the peptidyl asparaginyl ligase (PAL) with said first peptide and said functionalized peptide to form a conjugate of said first peptide-functionalized peptide;
- wherein the method further comprises
- (a) radiolabeling the conjugate, or
- (b) radiolabeling the functionalized peptide prior to contacting with the PAL.
- 20 2. The method of claim 1, wherein the first peptide is an antibody, a scaffold protein, an antibody mimetic, or a functional fragment thereof.
3. The method of claim 2, wherein the first peptide is an antibody or a functional fragment thereof.
4. The method of claim 3, wherein the antibody or a functional fragment thereof is a single-domain antibody (sdAb).
- 25 5. The method of claim 4, wherein the sdAb is hPD-L1-targeting sdAb or a CEA-targeting sdAb.
6. The method of any one of the preceding claims, wherein P2' and/or P2'' is a hydrophobic amino acid selected from the group consisting of Val, Leu and Ile.
- 30 7. The method of any one of the preceding claims, wherein the P1-P1'-P2' tripeptide PAL motif is a Asn-X-Leu motif, wherein X is Gln (Q), Gly (G) or Glu (E).

8. The method of any one of the preceding claims, wherein the P1-P1'-P2' tripeptide PAL motif is Asn-Gln-Leu (NQL) or Asn-Gly-Leu (NGL).
9. The method of any one of the preceding claims, wherein the first peptide comprises a His₆ tag at its C-terminus.
- 5 10. The method of any one of the preceding claims, wherein the first peptide comprises a -NGL-His₆ or a -NQL-His₆ tag at its C-terminus.
11. The method of claim 10, wherein the first peptide is selected from the group consisting of (hPD-L1)-NGL-His₆, (hPD-L1)-NQL-His₆, and (CEA)-NQL-His₆ sdAb.
12. The method of any one of the preceding claims, wherein the P1"-P2" motif is a -G-I motif.
10
13. The method of any one of the preceding claims, wherein the functionalized peptide comprises a -GIGK-, -GIGG-, or -GIGGGK- motif at its N-terminus.
14. The method of claim 13, wherein the functionalized peptide is selected from the group consisting of GIGK-NOTA, GIGK-DOTA, GIGK-MeTz, GIGGGK-DOTA, GIGK-NOTA-
15 [18F]AIF, GIGK-Py-DABCO, Ac-GIGK-Py-DABCO, GIGK-Py-NMe₃, Ac-GIGK-PyNMe₃, and GIGK-FPy.
15. The method of any one of the preceding claims, wherein the PAL is selected from the group comprising VyPAL2 comprising the amino acid sequence set forth in SEQ ID NO:32, butelase1 comprising the amino acid sequence set forth in SEQ ID NO: 33,
20 butelase-2 comprising the amino acid sequence set forth in SEQ ID NO: 34 or 35, VyPAL3 comprising the amino acid sequence set forth in SEQ ID NO: 36, OaAEP1b-C247A comprising the amino acid sequence set forth in SEQ ID NO: 37, HeAEP3 comprising the amino acid sequence set forth in SEQ ID NO: 38, AtLEGy comprising the amino acid sequence set forth in SEQ ID NO: 39, VuPAL1 comprising the amino
25 acid sequence set forth in SEQ ID NO: 40, HaPAL1 comprising the amino acid sequence set forth in SEQ ID NO: 41, OaAEP1b comprising the amino acid sequence set forth in SEQ ID NO: 42 and a functional fragment or a variant thereof.
16. The method of any one of the preceding claims, wherein the PAL is VyPAL2 comprising the amino acid sequence set forth in SEQ ID NO: 32, or a functional
30 fragment or variant thereof.
17. The method of any one of the preceding claims, wherein the contacting step is carried out in the presence of a glutaminyl cyclase (QC).

18. The method of claim 17, wherein the PAL:QC stoichiometry is from 1:0.1 to 1:1.
19. The method of claim 18, wherein the PAL:QC stoichiometry is 1:0.1 or 1:0.5.
20. The method of any one of claims 17 to 19, wherein the QC is selected from the group comprising Human glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 43, Mouse glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 44, Drosophila glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 45, Arabidopsis glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 46, Conus glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 47, Sistrurus glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 48, Bacterial glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 49 and a functional fragment or a variant thereof.
21. The method of any one of claims 17 to 20, wherein the QC is human glutaminyl cyclase comprising the amino acid sequence set forth in SEQ ID NO: 43, or a functional fragment or variant thereof.
22. The method of any one of the preceding claims, wherein the functionalized peptide is functionalized with a chelator or a tetrazine moiety.
23. The method of claim 22, wherein the chelator is selected from the group consisting of 1,4,7-triazacyclononanetriacetic acid (NOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and 1,4,7-triazacyclononane-1-glutaric acid-4,7-diacetic acid (NODAGA).
24. The method of claim 22 or 23, wherein the chelator is further functionalized with a bicyclononyne.
25. The method of claim 22, wherein the tetrazine moiety is – MeTz.
26. The method of claim 22 or 25, wherein the tetrazine-functionalized peptide forms a conjugate of said first peptide-(tetrazine-functionalized peptide), and the conjugate is further complexed with a bicyclononyne-functionalized chelator prior to radiolabeling.
27. The method of claim 26, wherein the bicyclononyne-functionalized chelator comprises a chelator selected from the group consisting of 1,4,7-triazacyclononanetriacetic acid (NOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and 1,4,7-triazacyclononane-1-glutaric acid-4,7-diacetic acid (NODAGA).

28. The method of any one of the preceding claims, wherein the conjugate or the functionalized peptide is radiolabeled with a radionuclide selected from the group consisting of Gallium-68 (^{68}Ga), Gallium-67 (^{67}Ga), Lutetium-177 (^{177}Lu), and Fluorine-18 (^{18}F).
- 5 29. The method of claim 28, wherein the radionuclide is Gallium-68 (^{68}Ga), or Fluorine-18 (^{18}F) in the form of [^{18}F]AIF.
30. A site-specifically radiolabeled tracer prepared by the method of any one of claims 1 to 29.
- 10 31. A site-specifically radiolabeled tracer of claim 30, selected from the group consisting of [^{68}Ga]Ga-NOTA-KGIG-(hPD-L1) sdAb, [^{18}F]AIF-NOTA-KGIG-(hPD-L1) sdAb, [^{18}F]F-NODAGA-BCN-MeTz-KGIG-(hPD-L1), and [^{177}Lu]Lu-DOTA-NHCS-KGIG-CEA sdAb.
32. A site-specifically radiolabeled tracer according to claim 30 or 31 for use as a nuclear imaging agent.
- 15 33. Use of the site-specifically radiolabeled tracer according to claim 30 or 31 as a nuclear imaging agent.
34. Use of the site-specifically radiolabeled tracer according to claim 30 or 31 in the manufacture of a diagnostic imaging agent for use in nuclear imaging.
35. A method of treating a disease in a subject, the method comprising administering the site-specifically radiolabeled tracer according to claim 30 or 31 to the subject.
- 20 36. Use of the site-specifically radiolabeled tracer according to the claim 30 or 31 in the manufacture of a medicament for the treatment of a disease in a subject.
37. The method of claim 35, or the use of claim 36, wherein the disease is a tumour or a cancer.
- 25 38. The method of claim 35, or the use of claim 36 wherein the sdAb targets the diseased cell, and the radiolabeled tracer is labeled with a therapeutic radionuclide such as ^{177}Lu , ^{211}At or ^{225}Ac .