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(54) Title: BIOMOLECULAR COMPLEXES

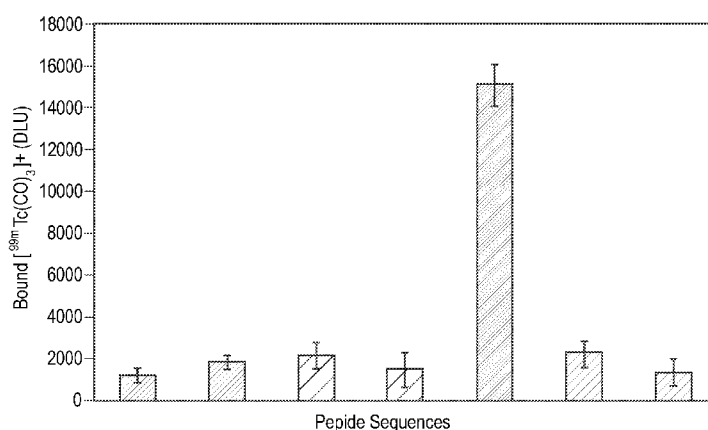


Fig. 20

(57) **Abstract:** Disclosed are biomolecular complexes that are useful in the labelling of proteins with metal tricarbonyl groups. The biomolecular complex include a protein of interest and a labelling sequence capable of conjugation to a metal tricarbonyl ([M(CO)₃]⁺). The labelling sequence consists of between 6 and 15 amino acid residues, of which between 4 and 6 are histidine residues and of which at least 2 residues are selected from the group consisting of lysine and/or arginine residues. The labelling sequence has an isoelectric point (pI) of at least 9. The biomolecular complexes of the invention are useful in molecular imaging, diagnostic and therapeutic applications. Also disclosed are nucleic acid molecules encoding such biomolecular complexes, methods of producing such biomolecular complexes; biomolecular complexes for use as medicaments in radiotherapy; and methods of treatment, diagnosis and molecular imaging utilising such molecular complexes.



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BIOMOLECULAR COMPLEXES

Field of the invention

The present invention relates to biomolecular complexes. The biomolecular complexes of the invention are useful in molecular imaging, diagnostic and therapeutic applications. The invention also relates to nucleic acid molecules encoding such biomolecular complexes, methods of producing such biomolecular complexes; biomolecular complexes for use as medicaments in radiotherapy; and methods of treatment, diagnosis and molecular imaging utilising such molecular complexes.

Background of the invention

Radiopharmaceuticals are agents that may be used in a wide range of applications, including diagnostic, therapeutic or research uses. Examples of radiopharmaceuticals include molecular imaging agents in which the detectable signal used in imaging is provided by a radionuclide that emits a gamma photon or a positron and which is attached or incorporated into a molecular or particulate entity which endows it with an affinity for a specific molecular target which is present *in vivo*. Typically the target molecule or pathway or physiological state may be one that acts as a biomarker for a specific physiological, biochemical, metabolic or gene expression phenotype such as may be present in disease sites (such as tumours or inflamed tissues).

The same molecular targeting principle can be used therapeutically by use of a particle-emitting radioisotope in place of the gamma or positron emitter. Here the aim is to target the radioisotope to a location where emitted particles (e.g. alpha particles, beta particles, Auger electrons) can kill cells associated with the disease site.

The targeting vehicle is often a protein (e.g. a monoclonal antibody, antibody fragment, signalling protein etc.) or peptide with specific affinity for a receptor, transporter or other molecular target. To be used as molecular imaging agents, these protein or peptide vehicles must be modified in order to incorporate a suitable radionuclide. The modification may consist of direct attachment of a radionuclide, for example the substitution of a hydrogen in the phenolic group of a tyrosine residue with a radioactive iodine. More often the radioisotope is a metal (e.g. Tc-99m, In-111, Cu-64, Ga-68 etc. for imaging and Re-188, Y-90, Lu-177 etc. for therapy). Tc-99m is a preferred radioisotope for imaging because of its

favourable gamma energy and ready availability from the Mo-99/Tc-99m generator. Re-188 is an attractive therapeutic radioisotope because it too is available from a generator (W-188/Re-188) and it is chemically analogous to Tc-99m.

When proteins and peptides of interest are to be used in molecular imaging or therapeutic agents, the bioconjugate and the process that produces it will preferably satisfy a number of requirements, which may include some of the following:

1. The molecular recognition function of the protein or peptide must be preserved. This means the modification should be at a site on the molecule that is well-defined and remote from the target-binding site.
2. The amount of vehicle should be kept to a minimum whilst still incorporating the amount of radioactivity needed for the imaging procedure (i.e. the specific activity of the labelled product should be as high as possible), both to reduce the chances of producing a toxic or undesired physiological effect, for example saturation of the molecular target and thus altering the biological property it is intended to measure, and to reduce costs.
3. The labelling process must be as quick and simple as possible in order to reduce losses of radioactivity by decay, to reduce the opportunity for introduction of microbial contamination, and to reduce the radiation exposure of the operator.
4. The labelling yield should be >95% to avoid the necessity of incorporating additional purification steps to remove non-bound radioactivity
5. The product should as far as possible be homogeneous, that is every radiolabelled molecule should have the same structure, stability and affinity for the target
6. The product should be stable in vivo for the requisite period to perform the imaging procedure

When seeking to address requirements 1-5, the rate of formation of a labelled biomolecular complex (comprising the protein of interest, and a labelling sequence that mediates attachment to the chosen label) is of great importance. Fast labelling, in which only a short incubation time is required for a label to become attached to the biomolecular complex (e.g. by conjugation via the labelling sequence) is highly beneficial.

When seeking to address requirement 6, kinetic stability of the radiolabelled conjugate is of great importance.

Combining these is hard to achieve because design features that increase the rate of complex formation with the radiometal (i.e. reduce the free energy of activation of the labelling reaction) also tend to increase the rate of dissociation.

Achieving fast, high yield/purity labelling is difficult because of requirement 1 which means the concentration of biomolecule or bioconjugate must be minimised, whilst the radionuclide is necessarily present also at very low concentration. It is therefore critical that the highest possible fraction of encounters between radiometal and biomolecule must result in radiolabelling. To achieve this, a well-designed binding site must be incorporated into the biomolecule to bind the radionuclide with the maximum efficiency and fastest kinetics. This will help to ensure that requirements 1-5 above are met. Such a binding site would have much faster labelling and higher affinity for the radiolabel than other parts of the biomolecule, leading to optimal homogeneity (requirement 5), and if this site is remote from the molecular recognition site, this in turn would ensure that the recognition site is preserved (requirement 1). High efficiency and fast kinetics would also mean that the labelling could achieve the required yield (> 95%, removing the need for purification steps, requirement 4), with very low concentration of biomolecule (requirement 2), with the minimum operational complexity and time (requirement 3).

Synthetic binding sites, in the form of bifunctional chelators, can be prepared for attaching radiometals to biomolecules. Bifunctional chelators are molecules that comprise two functional components: a metal-binding moiety that is designed for fast binding of the radiometal combined with slow dissociation of the complex; and a biomolecule-binding moiety, such as an active ester that reacts with amine groups of lysine side chains of the biomolecule. The challenge for achieving site-specific attachment of radiolabels is that the amino acid side chains with which these bifunctional chelators react are rarely unique in the biomolecule. For example, in proteins with multiple lysine residues, it is difficult or impossible to control the bioconjugation conditions to achieve modification at only one chosen site. This means there will generally be a mixture of differently modified protein molecules produced, which have varying biological behaviours. Furthermore, if lysine residues are close to the target recognition site the biological activity will frequently be impaired, as has been demonstrated with a range of biomolecular complexes comprising different proteins of interest.

One approach by which this problem may be overcome is through use of synthetic peptides using non-natural synthetic amino acids with side chains that incorporate metal chelating

agents or other radionuclide binding sites. These can be incorporated site-specifically at any chosen point in the peptide amino acid sequence using the standard solid phase peptide synthesis methodologies. See for example Greenland WEP, Howland K, Hardy J, Blower PJ. Solid phase synthesis of peptide radiopharmaceuticals using Fmoc-N- ϵ -(Hynic-Boc)-Lysine, a technetium-binding amino acid. Application to Tc-99m-labelled salmon calcitonin. *J Med Chem* 2003;46:1751-1757.

In the context of biologically produced recombinant proteins, however, these synthetic amino acids cannot be handled by the cellular translational apparatus. Only the twenty proteogenic amino acids can be used. In this context, the side chains of these amino acids, or the N- or C-terminus, must be used. Often a unique binding site, such as a single cysteine residue with its reactive thiol group, may be used, by coupling to bifunctional chelators containing thiol-specific synthons such as maleimide groups. However, the presence of cysteine residues can introduce problems such as undesirable disulfide bond formation which can alter the protein fold or cause dimerisation, and are not always suitable for the purpose of bioconjugation.

Alternative techniques to address the problems have exploited the effect of a series of arginine residues on the labelling efficiency of peptides with the pentavalent technetium-99m oxo-core (for example in a patent application published as WO2010/076654). The mechanism by which this increased efficiency arises is unclear. Another potential approach is to exploit changes in reactivity arising from proximity of amino acids in a specific sequence. For example, a series of (most commonly six) consecutive histidine residues, the so-called his-tag, can act as an effective metal chelating sequence. This phenomenon is used to good effect in the purification of recombinant his-tagged proteins using nickel chelating solid phases.

For many imaging applications the radioisotope Tc-99m is preferred for reasons identified above. Its periodic congener rhenium can be used analogously by virtue of the availability of beta-emitting radioisotopes, Re-186 and Re-188. These radiometals have complex chemistry with a variety of ligand preferences and structures in different metal oxidation states. Two particularly stable complex types, or "cores", have been widely used by virtue of their stability and synthetic accessibility from pertechnetate and perrhenate, the forms in which the radiometals are most readily available. These are the MO^{3+} core, with the metal in oxidation state +5, and the $\text{M}(\text{CO})_3^+$ core, with the metal in oxidation state +1 (where M may, for example, be Tc or Re). These cores can be chelated by various chelating agents, such

as the chelator-derivatised amino acids referred to above, which may be optimally designed for the particular core. The requirements of the chelator design are different because of the different geometries preferred by the cores: typically the chelator should form the base of a square pyramidal complex for the MO^{3+} core and the trigonal face of an octahedral complex for the $\text{M}(\text{CO})_3^+$ core. Other cores can be considered but have not been exploited to a significant degree hitherto.

Both of these cores can be bound effectively by sequences of amino acids. MO^{3+} is particularly effective in the context of amino acid sequences containing a cysteine thiol. The coordinating atoms in this context are believed to comprise typically the thiolate sulphur and three sequential anionic, deprotonated amide nitrogens of the peptide backbone. Others have found that binding of TcO^{3+} , generated *in situ* by the reduction of pertechnetate with stannous compounds, to peptides is enhanced by the presence of a series of arginine residues. The mechanism of this enhancement is unclear.

By contrast, the $\text{M}(\text{CO})_3^+$ core is particularly effective in the context of proteins containing a his-tag. The coordinating ligands in this setting are believed to comprise two histidine imidazole groups and a third, as yet unknown, ligand. This core is especially attractive for several reasons. It is particularly inert towards ligand substitution and oxidation and hence proteins labelled in this way show excellent *in vivo* stability. In addition, the his-tag is a very common motif in recombinant proteins because of its value in protein purification. The radioactive $\text{M}(\text{CO})_3^+$ synthon (often assumed to be $[\text{M}(\text{CO})_3(\text{H}_2\text{O})_3]^+$) is easily synthesised from MO_4^- by a simple kit-based method.

In many his-tagged proteins the radiolabelling works well and satisfies to a variable extent the six requirements set out above. However, while some his-tagged proteins label efficiently at reasonably low concentration, others require higher protein concentration or higher temperature reaction conditions and/or a subsequent purification step. Thus the presence of a his-tag does not in itself provide the desirable attribute that radiolabelling efficiency should be essentially independent of the particular protein. The factors that dictate the labelling efficiency are unknown but must arise from features of the amino acid sequence in the vicinity of the his-tag. In the absence of a his-tag some proteins can still be labelled albeit with reduced efficiency, therefore it is not certain that the his-tag guarantees site-specificity. It is clear that the presence of a his-tag alone is not sufficient to guarantee efficient labelling and that radiolabelling of each new protein has to be optimised before its biological and

clinical evaluation as a molecular imaging agent can proceed. In the common context of evaluation of large libraries of trial recombinant proteins this is inefficient and unpredictable.

Some progress towards optimising the design of the his-tag to improve labelling efficiency with the $M(CO)_3^+$ synthon has been reported by including a cysteine close to the his-tag. This modification produces a moderate but significant improvement in radiolabelling efficiency (as described in a patent application published as WO 2011/021001).

Despite certain claims made in documents in this field, it is unclear, and indeed, unlikely in light of coordination chemistry knowledge, that the peptides proposed in WO2010/076654 will themselves be suitable for binding of the $M(CO)_3^+$ core. The charge on the synthetic precursor containing the $M(CO)_3^+$ core is not the same as that associated with the TcO^{3+} core complex, and it is known to those skilled in the field of transition metal coordination chemistry that the electronic and geometric requirements of the metal in the two very different oxidation states are very different.

Thus, currently available protein labelling methods do not fully satisfy the requirements listed above in a reliable and predictable way.

It is an aim of certain embodiments of the present invention to provide a solution to this problem of unpredictability and inefficiency associated with use of the his-tag for radiolabelling with $^{99m}Tc(CO)_3^+$ and $^{186/188}Re(CO)_3^+$ cores, by providing an optimised sequence of proteogenic amino acids that can be incorporated into recombinant proteins to give a binding site that offers very high labelling efficiency, site-specifically under mild conditions, regardless of the nature of other domains of the protein. Incorporation of the sequence into recombinant proteins will render them amenable to site-specific radiolabelling in a simple kit-based procedure at very low concentration, and hence to a very high specific activity, in high radiochemical yield removing the need for additional purification steps, to produce a radiopharmaceutical with intact biological recognition activity, high structural homogeneity and excellent resistance to loss of radiolabel *in vivo*.

Summary of the invention

According to a first aspect of the invention there is provided a biomolecular complex comprising a protein of interest and a labelling sequence capable of conjugation to a metal tricarbonyl ($[M(CO)_3]^+$),

wherein the labelling sequence consists of between 6 and 15 amino acid residues
of which between 4 and 6 are histidine residues and
of which at least 2 residues are selected from the group consisting of lysine and/or arginine
residues
and the labelling sequence has an isoelectric point (pI) of at least 9.

According to a second aspect of the invention there is provided a nucleic acid molecule
encoding a biomolecular complex in accordance with the first aspect of the invention.

According to a third aspect of the invention there is provided a method of manufacturing a
biomolecular complex according to the first aspect of the invention, the method comprising
expressing a nucleic acid according to the second aspect of the invention to yield a
biomolecular complex.

According to a fourth aspect of the invention there is provided a biomolecular complex
according to the first aspect of the invention, and further comprising a conjugated metal
tricarboxyl comprising a radionuclide, for use as a medicament in radiotherapy.

According to a fifth aspect of the invention there is provided a method of treatment, the
method comprising providing a therapeutically effective amount of a biomolecular complex
according to the first aspect of the invention, further comprising a conjugated metal
tricarboxyl comprising a radionuclide, to a subject in need thereof.

Suitable methods of treatment in accordance with this aspect of the invention are described
elsewhere in the present application. Merely by way of example, such methods of treatment
may make use of biomolecular complexes in which the protein of interest is one that
associates with a marker associated with the disease to be treated, thus targeting the
radionuclide (associated with the tricarboxyl) to a site where it can achieve a therapeutic
impact upon cells associated with the disease to be treated. The studies reported in more
detail below illustrate that the biomolecular complexes of the invention are capable of
incorporating antibodies such as those directed to prostate specific membrane antigen
(PSMA), a marker expression of which is frequently up-regulated at sites of prostate cancer.

According to a sixth aspect of the invention there is provided a method of molecular imaging,
the method comprising providing a biomolecular complex according to the first aspect of the
invention, further comprising a conjugated metal tricarboxyl comprising, to a subject, and

determining the location of the conjugated metal tricarbonyl within the subject, wherein the location of the conjugated metal tricarbonyl within the subject is indicative of the location of a binding partner of the protein of interest.

The biomolecular complexes of the invention are also suitable for use in a number of screening applications.

Merely by way of example, in a suitable embodiment biomolecular complexes of the invention may be used to identify proteins of interest capable of interacting with a desired binding partner. In such cases, multiple biomolecular complexes incorporating different proteins of interest may be produced, for example, by means of expression libraries in which the proteins of interest are associated with a suitable labelling sequence (thus effectively giving rise to libraries of biomolecular complexes of the invention). A plurality of biomolecular complexes, comprising a plurality of proteins of interest, may then be provided to a sample comprising the desired binding partner (such as a cell expressing a molecule to which it is desired to identify a protein of interest that can be used for cellular targeting). The presence of a protein of interest capable of binding to the desired binding partner can be identified by localisation of the metal tricarbonyl label with the sample. Further analysis may then be undertaken to identify which of the proteins of interest within the plurality of biomolecular complexes is responsible for this labelling (and thus which protein of interest interacts with the binding partner in question).

Alternative screening applications of the biomolecular complexes of the invention may make use of a plurality of biomolecular complexes comprising a plurality of proteins of interest, wherein the proteins of interest have been selected for their ability to interact with a desired binding partner. For example, the plurality of proteins of interest may comprise different antibody fragments or derivatives (e.g. SFv, diabodies, or minibodies) each directed to the same antigen. The protein of interest having most favourable binding characteristics can then be identified by assessing which of the biomolecular complexes exhibits the best binding, and then analysing this biomolecular complex to identify which of the proteins of interest it contains.

As discussed further below, the properties of the biomolecular complexes of the invention are able to prove beneficial in these various important clinical and research applications.

Brief description of the drawings

Figure 1 shows the results of a study investigating radiochemical yield of the His/Cys Tag peptide on the Celluspot™ peptide array

Figure 2 shows the results of a study investigating radiochemical yield of the His/Cys Tag peptide in solution

Figure 3 shows the results of a study investigating the correlation between the radiochemical yield of the His/Cys Tag peptides in solution and on solid phase

Figure 4 shows the results of a study investigating relative radiochemical yields of all 384 peptides on the Celluspot™ array post labelling with $[^{99m}\text{Tc}(\text{CO})_3]^+$. Results obtained after 30 minutes of labelling in PBS buffer at pH 7.4. The peptides have been categorised according to their main characteristics. All peptides other than the controls contain at least 1 histidine residue

Figure 5 shows the results of a comparison study between the labelling of peptide sequence with multiple negatively charged amino acids and positively charged amino acids. Sequences include at least 2 negatively charged amino acids (glutamic acid and/or aspartic acid) or 2 positively charged amino acids (arginine and/or lysine)

Figure 6 shows the results of a study investigating HHHCHHHXLAAAL Sequences where X differs between each sequence. The amino acids varied include positively charged Arg and Lys, negatively charged Glu, Asp and neutral Gly

Figure 7 shows the results of a comparison study between Glutamic acid and Arginine containing sequences. A single amino acid has been changed within the sequences

Figure 8 shows the results of a comparison study between R, G, S, E and D containing sequences. A single amino acid is replaced in the same position within each sequence

Figure 9 shows the results of a study investigating His/Cys Tag sequences in which a single amino acid has been replaced. In the original His/Cys Tag sequence a glutamic acid residue

was included next to the histidines. This has been changed from E to D, S, K, R or no amino acid. The influence of charged amino acids on the labelling of histidines can be easily observed

Figure 10 shows the results of a study investigating radiochemical yield plotted against isoelectric point for all peptide sequences with a His-Tag i.e. 6 consecutive histidines HHHHHH

Figure 11 shows the results of a study investigating radiochemical yield plotted against isoelectric point for all comparator peptide sequences with 3 histidines HXHXHX

Figure 12 shows the results of a study investigating radiochemical yield plotted against isoelectric point for all peptide sequences with 4 consecutive histidines HHHH

Figure 13 shows the results of a study investigating radiochemical yield plotted against isoelectric point for all comparator peptide sequences with 2 histidines HXHX

Figure 14 shows the results of a study investigating radiochemical yield plotted against isoelectric point for all comparator peptide sequences with 2 histidines HXXH

Figure 15 shows the results of a study investigating number of arginine residues plotted against radiochemical Yield and isoelectric point of sequences that contain 6 consecutive histidines (a His-Tag). Optimum number of arginines appears to be between 2-4 arginine residues within a sequence. There is a significant difference ($p < 0.0001$) between the radiochemical yield for 1 arginine and 2 arginines. The pI and the radiochemical yield of the peptide sequences increase in proportion to the number of arginines present in the sequence.

The histidine peptide sequences analysed in these graphs contain a His-Tag i.e. 6 consecutive histidine. There are other arginine sequences with > 4 arginines however these are in sequences where there are only 2/3 histidines. A decrease in histidines = decrease in radiochemical yield therefore it is not possible to directly compare the influence of the arginine residues to sequences containing arginines in combination 6 histidines.

Conclusion: The number of arginine residues present correlates with the pI of the labelling sequence, and with the radiochemical yield attained.

Figure 16 shows the results of a study investigating number of lysine residues in a peptide sequence that contains 6 consecutive histidines (a His-Tag) plotted against isoelectric point and radiochemical yield. The results are very similar to that of arginine however, lysine has a side chain with a lower pKa therefore more lysines are needed to give an equivalent pI to sequences including arginines. This is shown in the top graph where a significant difference in radiochemical yield is observed between sequences with 2 and three lysine residues. Consequently, 3-5 lysine residues are favoured. Again, pI and radiochemical yield of the peptide sequences are proportional to the number of lysine residues present.

Conclusion: The number of lysine residues present correlates with the pI of the labelling sequence, and with the radiochemical yield attained

Figure 17 shows the results of a study investigating combined arginine and lysine containing peptide sequences plotted against pI and radiochemical yield. This highlights the fact that arginine is a more positively charged amino acid and can have a greater influence on the increase in pI of a peptide sequence. Consequently it also has a greater influence on the radiochemical yield. The binding affinities of the $[M(CO)_3]^+$ complexes to histidines adjacent to multiple arginine residues is greater than to histidines adjacent to multiple lysine residues. A larger number of lysines are required in the labelling sequence to provide the same labelling ability of the arginine containing sequences.

Figure 18 shows the results of a comparison study between different positions of the arginine residues in the labelling sequences with respect to the histidines.

Figure 19 shows the results of a study investigating the radiochemical yield of peptide sequences with 6 consecutive histidines compared with histidines in HXHXHX, HXHX, HXXH and HHHH combinations showing that 6 consecutive histidines are better. Also, it confirms the previous observation from the trial Celluspot™ plates that histidines are required for labelling. No histidines = very low radiochemical yield (bottom graph). The exponential relationship between the isoelectric point and radiochemical yield for each type of histidine tag can be seen in the top graph. This clearly shows the superiority of the His-Tag in comparison to other histidine combinations

Figure 20 shows the results of a study investigating radiolabelling efficiencies with $[^{99m}\text{Tc}(\text{CO})_3]^+$ high-throughput screening methodology for His-tag peptide sequences of the invention compared with prior art labelling sequences and comparator sequences.

Column 1 and 2 = LAAALEHHHHHH, CKLAAALEHHHHHH (Tavare et al. Bioconj. Chem. 2009, 20, 2071-81), (and as described in WO 2011/021001)

Column 3 and 4 = KNDVQLHHHHHHSSG, EKNFKNEAEHEHEHM (Tolmachev et al. Bioconj. Chem. 2012, 21, 2013-2022)

Column 5 = CLRRRLAHHHHHH → Preferred labelling sequence on the CelluspotTM peptide array.

Column 6 and 7 = CLDDDLAHHHHHH, CLEEELAHHHHHH → The same sequences as the preferred labelling sequence however the positively charged amino acids (R) have been replaced with negatively charged amino acids (E, D).

Figure 21 shows the results of a study comparing radiochemical yield of the top 10 sequences with and without a cysteine residue

Figure 22 shows the results of a study investigating radiochemical yield for identical peptide sequences in which a cysteine has been replaced with methionine. The difference in labelling sequences containing a methionine rather than cysteine is insignificant. Consequently, methionines can be used instead of cysteine to give an almost identical rate of labelling while avoiding disulfide bond formation. A few comparisons have been made between the cysteine/methionine containing sequences and those without. There is a difference in radiochemical yield when the Met and Cys are removed

Figure 23 shows the results of a study investigating radiochemical yield of all peptide sequences within the assigned categories. All peptides that include an arginine or multiple lysine have been highlighted. This demonstrates that the sequences with the highest radiochemical yield in each category always contain either an arginine or lysine.

Figure 24a. SDS-PAGE and Western Blot of the extraction and purification process of the J591scFvJWT protein from the culture supernatant. Lanes in both left and right hand images are as follows: A is culture supernatant; B is 35mM imidazole wash of the NiNTA column; C is J591scFvJWT elution from NiNTA column (250mM imidazole); D is SEC Purification: Fraction 1 – BSA Protein; E is SEC Purification: Fraction 2 – Non-covalent dimers of J591scFvJWT; F is SEC Purification: Fraction 3 – Purified monomeric J591scFvJWT; and G

is concentrated purified J591scFvJWT – 1.3mg/ml. Left: SDS-PAGE with lanes A-G. Right: Western Blot of the SDS-PAGE with lanes A-G. J591scFvJWT sample runs as a single band corresponding to the size of the monomer, 28kDa.

Figure 24b. SDS-PAGE and Western Blot of the extraction and purification process of the J591scFv protein from the culture supernatant. Lanes in both left and right hand images are as follows: A is culture supernatant; B is 35mM imidazole wash of the NiNTA column; C is J591scFv elution from NiNTA column (250mM imidazole); D is SEC Purification: Fraction 1 – BSA Protein; E is SEC Purification: Fraction 2 – Non-covalent dimers of J591scFv; and F is SEC Purification: Fraction 3 – Purified monomeric J591scFv. Left: SDS-PAGE with rows from A-F. Right: Western Blot of the SDS-PAGE with lanes from A-F. J591scFv sample runs as a single band corresponding to the size of the monomer, 27kDa.

Figure 25. HPLC SEC analysis of the scFv proteins shows elution primarily as a single monomeric species at 9min. The peak at 8min represents the non-covalent dimers of the scFv protein. A) J591scFvJWT protein. B) J591scFv protein. C) huJ591 protein.

Figure 26. Radiolabelling efficiencies of the J591scFvJWT, J591scFv, huJ591, 6C7.1 and 6C7.1-Cys proteins under increasingly dilute conditions expressed as % radiochemical yield against log[protein]. Table 4 reveals the concentration in uM and mg/ml corresponding to the log[protein] data points on the graphs. The labelling efficiency was recorded at 5 different time points: Panel A) 15 minutes, Panel B) 30 minutes, Panel C) 60 minutes, Panel D) 90 minutes and Panel E) 120 minutes.

Figure 27. Comparison between the radiolabelling efficiency of J591scFvJWT at 37°C and 25°C. The protein concentration for both experiments was 14.1uM.

Figure 28. ITLC-SA results for the stability of the $[^{99m}\text{Tc}(\text{CO})_3]^+$ -J591scFvJWT conjugate in human serum for 4 hours at 37°C. The radiochemical purity remains at 98.9%.

Figure 29. Serum stability of $[^{99m}\text{Tc}(\text{CO})_3]^+$ -J591(scFv)JWT by SDS-PAGE and Coomassie staining (A) and autoradiograph (B) for the serum stability analysis of J591scFvJWT for 4 hours at 37°C. Lanes are as follows: A is serum proteins + $[^{99m}\text{Tc}(\text{CO})_3]^+$; B is $[^{99m}\text{Tc}(\text{CO})_3]^+$ -J591(scFv)JWT-; C is $[^{99m}\text{Tc}(\text{CO})_3]^+$ -J591(scFv)JWT in serum at 0 min; D is $[^{99m}\text{Tc}(\text{CO})_3]^+$ -J591(scFv)JWT in serum at 15 min; E is $[^{99m}\text{Tc}(\text{CO})_3]^+$ -J591(scFv)JWT in serum at 30 min; F is $[^{99m}\text{Tc}(\text{CO})_3]^+$ -J591(scFv)JWT in serum at 60 min; G is $[^{99m}\text{Tc}(\text{CO})_3]^+$ -J591(scFv)JWT in

serum at 120 min; H is [$^{99m}\text{Tc}(\text{CO})_3$] $^+$ -J591(scFv)JWT in serum at 240 min; and I is [$^{99m}\text{Tc}(\text{CO})_3$] $^+$.

Table 1 shows a number of sequences suitable for use as labelling sequences in the biomolecular complexes of the invention (as well as a number of comparator sequences),

Table 2 shows a subset of these sequences.

Table 3 sets out the amino acid sequences of the scFv protein fragments: J591scFvJWT, J591scFv, huJ591, 6C7.1scFv, 6C7.1CscFv referred to in Study 2. Labelling sequences have been highlighted in bold.

Table 4 sets out details of the protein concentrations used in Study 2 and shown in Figure 26.

Table 5 sets out details of protein concentrations and incubation times at which J591(scFv)JWT demonstrates a radiochemical yield greater than or equal to 95%.

Detailed description of the invention

The biomolecular complexes of the invention comprise a protein of interest and a labelling sequence. These labelling sequences allow the biomolecular complexes to be conjugated with metal tricarbonyl. In suitable embodiments the biomolecular complexes of the invention may further comprise a metal tricarbonyl conjugated to the labelling sequence. Further details of such embodiments are described in more detail elsewhere in the specification. The metal tricarbonyls are able to serve as labels that can subsequently be detected, for example by detection of a radionuclide incorporated in the metal tricarbonyl, or by infrared-based techniques.

Without wishing to be bound by any hypothesis, the inventors believe that as the isoelectric point (pI) of the labelling sequence increases, so the ability of the labelling sequence to conjugate metal tricarbonyls (a conjugation that occurs via histidine residues in the labelling sequence) increases. As set out in the Examples below, labelling sequences suitable for use in the biomolecular complexes of the present invention, where the labelling sequence has a pI greater than 9, are associated with a significant improvement in labelling efficiency. In a suitable embodiment a biomolecular complex of the invention may comprise a labelling sequence having a pI of at least 9.5, at least 10, or even at least 10.5.

For the purposes of the present disclosure, references to the isoelectric point should be taken as being the isoelectric point as determined using compute pI/Mw by ExPASy Bioinformatics Resource Portal. Compute pI/Mw is a tool which allows the computation of the theoretic pI (isoelectric point) and Mw (molecular weight) for a list of UniProt Knowledgebase (Swiss Prot or TrEMBL) entries or for user entered sequences.

Advantages of new labelling sequences over published sequences are their surprisingly improved labelling efficiency and thus specific activity. As a consequence, the labelling reaction is faster and can occur under mild conditions, the labelling yield is higher and more reliable, the labelling is more site-specific, the labelled product will be more homogenous and the amount of protein of interest can be reduced. The results herein disclosed clearly illustrate that biomolecular complexes of the invention, comprising labelling sequences that meet the criteria set out herein, show a degree of labelling efficiency that is much higher than random sequences or sequences with fewer histidines used as comparators. These beneficial characteristics of the biomolecular complexes of the invention are such that site specificity of labelling is likely to be considerably improved.

The biomolecular complexes of the invention comprise labelling sequences having a length of between 6 and 15 amino acid residues. For example, a suitable biomolecular complex may comprise a labelling sequence consisting of 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues. In suitable embodiments of the biomolecular complexes of the invention, the labelling sequence consists of between 12 and 15 amino acid residues.

The biomolecular complexes of the invention comprise between 4 and 6 histidine residues. Thus suitable embodiments include those wherein 4, 5 or 6 histidine residues are present.

In embodiments of the biomolecular complexes of the invention where 6 histidine residues are present a suitable embodiment comprises the 6 residues arranged as two groups of three contiguous histidine residues, the two groups being separated by a single non-histidine residue. In alternative embodiments, the 6 histidine residues are contiguous with one another.

Biomolecular complexes of the invention may comprise a labelling sequence wherein the histidine residues are located at an end of the labelling sequence. In the context of the present disclosure, "an end" should be construed as requiring that the histidine residues

provide the initial or final amino acids of the labelling sequence. In certain embodiments the histidine residues may be provided solely at an end of the labelling sequence. In other embodiments one or more of the histidine residues are provided at an end of the labelling sequence, and further histidine residues are provided elsewhere in the labelling sequence.

In certain suitable embodiments of the biomolecular complexes of the invention all of the histidine residues are embedded within the labelling sequence. For the purposes of the present disclosure reference to amino acid residues being “embedded within” a sequence should be construed as requiring that the residues referred to are not present at an end of the sequence.

Biomolecular complexes of the invention comprise labelling sequences in which at least 2 of the amino acid residues present are selected from the group consisting of lysine and/or arginine residues. Accordingly, it will be recognised that in suitable embodiments the biomolecular complexes of the invention may make use of labelling sequences that comprise a single lysine residue and a single arginine residue. In suitable embodiments the biomolecular complexes of the invention may make use of labelling sequences that comprise two or more lysine residues. In suitable embodiments the biomolecular complexes of the invention may make use of labelling sequences that comprise two or more arginine residues.

Biomolecular complexes of the invention may utilise labelling sequences in which both lysine and arginine residues are present. Alternatively, suitable biomolecular complexes include those that comprise labelling sequences in which lysine residues are present, but arginine residues are absent. In a further alternative, suitable biomolecular complexes of the invention include those that comprise labelling sequences in which arginine residues are present, but lysine residues are absent.

Certain embodiments of the biomolecular complexes of the invention may comprise a labelling sequence comprising at least 3 lysine residues. In particular, the biomolecular complexes of the invention may make use of a labelling sequence that comprises between 3 and 5 lysine residues. For example, a biomolecular complex of the invention may incorporate a labelling sequence that comprises 3 or 4 lysine residues, such as a labelling sequence in which 4 lysine residues are present.

In suitable embodiments the biomolecular complexes of the invention may include a labelling sequence that comprises between 2 and 5 arginine residues. For example, a suitable

biomolecular complex of the invention may include a labelling sequence that comprises between 2 and 4 arginine residues, such as a labelling sequence in which 3 arginine residues are present.

In suitable embodiments the biomolecular complexes of the invention may comprise a labelling sequence that does not contain aspartic acid residues. In suitable embodiments the biomolecular complexes of the invention may comprise a labelling sequence that does not contain glutamic acid residues. Indeed, in suitable embodiments the biomolecular complexes of the invention may comprise a labelling sequence that contains neither aspartic acid nor glutamic acid residues.

The biomolecular complexes of the invention may include a labelling sequence that comprises a cysteine or methionine residue. The inclusion of a cysteine or methionine residue in accordance with this embodiment may be preferred in examples of biomolecular complexes comprising one or more arginine residues. Indeed, biomolecular complexes comprising labelling sequences that comprise arginine residues in combination with a methionine residue constitute preferred embodiments of the present invention.

Cysteine residues have been shown to improve the conjugation of histidine tagged peptides (such as labelling sequences) with metal tricarbonyls. The inventors have surprisingly found that similar advantages are also provided by the provision of one or more methionine residues within the labelling sequence.

Examples of specific suitable labelling sequences that may be used in accordance with the present invention are set out elsewhere in the specification. In the case of such examples that comprise one or more cysteine residues, the disclosure of the present application should also be taken as encompassing corresponding labelling sequences in which one or more of these cysteine residues has been replaced by a methionine residue.

In the case of a biomolecular complex of the invention in which cysteine is present in the labelling sequence, it may be preferred that such a labelling sequence comprises a maximum of 1 cysteine residue.

In an alternative embodiment, a biomolecular complex of the invention may utilise a labelling sequence that does not contain cysteine.

As set out above, the biomolecular complexes of the invention optionally comprise a metal tricarbonyl. Thus in certain embodiments such a metal tricarbonyl core, conjugated to the labelling sequence, forms part of the biomolecular complex.

The metal tricarbonyl may comprise a complex or fragment wherein the metal has a d^{10} electron configuration (e.g. Tc(I), Re(I), Cr(0), Mo(0), W(0)) and three carbonyl ligands coordinated mutually cis to each other (i.e. facially).

In suitable embodiments, the metal tricarbonyl is selected from the group consisting of: $[Mo(CO)_3]$; $[Cr(CO)_3]$; $[W(CO)_3]$; $[Tc(CO)_3]^+$; and $[Re(CO)_3]^+$.

The biomolecular complexes of the invention may include a metal tricarbonyl that comprises a radionuclide. Such complexes may be of use in applications such as radiotherapy (particularly targeted radionuclide therapy), or radioimaging, as discussed elsewhere in the specification. In suitable embodiments the radionuclide may comprise technetium, and the technetium may be selected from the group consisting of: Tc-99; Tc-99m; and Tc-94m.

In suitable embodiments the radionuclide may comprise rhenium, and the rhenium may be selected from the group consisting of: Re-188; and Re-186.

Alternatively, biomolecular complexes in accordance with the present invention may make use of a non-radioactive metal tricarbonyl comprising rhenium, chromium, molybdenum or tungsten. By way of example, a suitable metal tricarbonyl for use in such embodiments may comprise rhenium selected from the group consisting of: Re-185; and Re-187.

As referred to above, the protein of interest incorporated in biomolecular complexes of the invention will generally be selected with reference to a binding partner (i.e. another molecule, such as a protein or other biological molecule) with which the protein of interest interacts. Accordingly, the protein of interest can be used to target the metal tricarbonyl (attached to the protein of interest via the labelling sequence) to the location of the binding partner.

Metal tricarbonyls are detectable via infrared techniques. Thus biomolecular complexes of the invention incorporating metal tricarbonyls are suitable for use in techniques where the presence (and optionally location) of the metal tricarbonyl is determined via infrared. Merely by way of example, these may include such techniques as infrared surface enhanced spectroscopy,

Metal tricarbonyls comprising a radionuclide are readily detected (by virtue of their emission of radiation), and thus represent useful agents for use in molecular imaging applications in which it is desired to determine the location of a molecule (which is a binding partner for a protein of interest) within a sample. Biomolecular complexes comprising a metal tricarbonyl with a radionuclide that is a gamma radiation or positron emitter are particularly suitable for use in such embodiments.

Examples of molecular imaging applications of the sort considered above include those described in the sixth aspect of the invention. Accordingly, it will be recognised that metal tricarbonyls comprising a radionuclide that is a gamma radiation or positron emitter are well suited for use in embodiments of such aspects in which the location of the metal tricarbonyl is to be determined by detection of radioactivity.

The biomolecular complexes of the invention are also suitable for use in therapeutic roles. These include use as medicaments for radiotherapy, which may be used in applications such as the methods of treatment of the fifth aspect of the invention. In therapeutic uses of this sort the protein of interest may be selected so that it has a binding partner associated with a disease condition. For example, the binding partner may be a molecule associated with cancer, such as a marker expressed by cancer cells. The interaction of binding partner with the protein of interest can thus target the biomolecular complex, to the site of the disease. Suitably, the biomolecular complex may comprise a metal tricarbonyl incorporating a radionuclide that is able to kill cells associated with the disease condition, such as cancer cells. Radionuclides suitable for use in such embodiments include particle emitters, and it will be appreciated that such radionuclides represent preferred components that may be utilised in metal tricarbonyls utilised in biomolecular complexes for medical use, such as methods of treatment.

In view of the above, the skilled person will recognise the importance of the protein of interest in determining the utility and targeting of the biomolecular complexes of the invention. In suitable embodiments, the protein of interest to be utilised in a biomolecular complex of the invention may be an antibody or antibody fragment, and the binding partner may be the antigen recognised by said antibody or antibody fragment (such as an scFv antibody). Results set out elsewhere in the specification illustrate the suitability of antibodies (or antibody fragments) to serve as proteins of interest in the biomolecular complexes of the invention. This utility is illustrated with (but not limited to) biomolecular complexes

comprising the anti-prostate specific membrane antigen (PSMA) antibody J591, and in particular scFv antibodies derived from this.

In other embodiments, the protein of interest may be an enzyme, and the binding partner may be a substrate of said enzyme. In alternative embodiments, the protein of interest may be a substrate of an enzyme, and the binding partner may be said enzyme. In suitable embodiments the protein of interest and binding partner may be selected from a ligand and receptor known to bind to one another. The skilled person will readily appreciate that other proteins known have an interaction with a binding partner to which it is desired to target a metal tricarbonyl (whether for imaging, therapeutic, or other applications), will represent suitable examples of proteins of interest that may be used in the biomolecular complexes of the invention. Further examples of suitable proteins of interest include: neurotransmitters; hormones; albumin; and amyloid precursors. Suitable proteins of interest include synthetic proteins, which may be designed to bind to a desired target.

Except for where the context requires otherwise, it may not be necessary to use the full length native version of a protein of interest, and a suitable fragment capable of binding to the binding partner may be used instead.

Suitable biomolecular complexes in accordance with the present invention include those comprising labelling sequences selected from the group consisting of: HHHHHHALRRRLC; HHHHHHALRRRLM; CLRRRLAHHHHHH; MLRRRLAHHHHHH; HHHHHHALRRRLKC; HHHHHHALRRRLKM; CKLRRRLAHHHHHH; MKLRRRLAHHHHHH; CRRHHHHHHRRRC; MRRHHHHHHRRM; GRRHHHHHHRRG; HHHHHHRRRAARRC; HHHHHHRRRAARRM; CRRRAARRHHHHHH; MRRRAARRHHHHHH; HHHHHHALRRRL; LRRRLAHHHHHH; RCRGHHHHHHGRCR; RMRGHHHHHHGRMR; GGRHHHRHHHRGG; HHHHHHRGGGRC; HHHHHHRGGGRM; CRGGGRHHHHHH; MRGGGRHHHHHH; HHHHHHRARARC; HHHHHHRARARM; CRARARHHHHHH; MRARARHHHHHH; GKHHHHHHKKG; HHHHHHRARAR; RARARHHHHHH; HHHHHHRGGRC; HHHHHHRGGRM; CRGGRGHHHHHH; MRGGRGHHHHHH; GKHHHHHHKKG; GKHHHHHHKKG; CGKHHHHHHKKG; MGKHHHHHHKKG; HHHHHHRRRAARR; RRAARRHHHHHH; HHHHHHRGGGR; RGGGRHHHHHH; HHHHHHKGGGK; KGGGKHHHHHH; HHHHHHKAKAK; and KAKAKHHHHHH. This group includes sequences set out in Table 1, which provides details of a number of suitable labelling sequences that may be used in the biomolecular complexes of the invention.

Particularly useful examples of biomolecular complexes in accordance with the present invention include those comprising labelling sequences selected from the group consisting of: HHHHHHALRRRLC; HHHHHHALRRRLM; CLRRRLAHHHHHH; MLRRRLAHHHHHH; HHHHHHALRRRLKC; HHHHHHALRRRLKM; CKLRRRLAHHHHHH; MKLRRRLAHHHHHH; CRRHHHHHHRRRC; MRRHHHHHHRRM; GRRHHHHHHRRG; HHHHHHRRRAARRC; HHHHHHRRRAARRM; CRRRAARRHHHHHH; MRRRAARRHHHHHH; HHHHHHALRRRL; LRRRLAHHHHHH; RCRGHHHHHHGRCR; RMRGHHHHHHGMR; GGRHHHHHHRRGG; HHHHHHRRGGGRC; HHHHHHRRGGGRM; CRGGGRHHHHHH; and MRGGGRHHHHHH. This group includes sequences set out in Table 2, which provides details of a number of suitable labelling sequences that may be used in the biomolecular complexes of the invention (along with a comparator sequence).

Particularly preferred biomolecular complexes of the invention include those comprising the labelling sequence LRRRLAHHHHHH, or CLRRRLAHHHHHH, or HHHHHHALRRRLC. Further suitable examples of preferred biomolecular complexes include those comprising the labelling sequence MLRRRLAHHHHHH or HHHHHHALRRRLM.

As set out above, the invention also provides a nucleic acid molecule encoding a biomolecular complex in accordance with the first aspect of the invention, and the translation of such nucleic acids to yield a biomolecular complex represents a suitable method of manufacturing a biomolecular complex of the invention. Such expression may be accomplished by cellular expression, or *in vitro* translation/transcription approach. It will be appreciated that biomolecular complexes of the invention may also be produced by other means, including *de novo* synthesis. Suitable means of synthesis will be apparent to those skilled in the art.

Methods in accordance with the third aspect of the invention may optionally further comprise a step of conjugating the biomolecular complex (produced on expression of the nucleic acid) with a metal tricarbonyl (of any sort considered herein).

As discussed further in the Studies set out elsewhere in this disclosure, the biomolecular complexes of the invention provide notable advantages in terms of their efficiency in conjugating with metal tricarbonyls. These advantages give rise to certain beneficial embodiments of the methods of the invention by which such conjugated biomolecular complexes of the invention can be manufactured. These methods may be used to achieve

levels of conjugation of 95% or more, levels which are considered useful in both clinical and pre-clinical applications.

In a suitable embodiment, a method of manufacturing a biomolecular complex comprising a metal tricarbonyl (optionally comprising a radionuclide) as considered elsewhere in this disclosure, may utilise a concentration of the biomolecular complex, at the time that the conjugation is performed, of between approximately 5 μ M and approximately 30 μ M. In a suitable embodiment, the concentration of the biomolecular complex at conjugation is selected from the group consisting of: approximately 28 μ M; approximately 14 μ M; and approximately 7 μ M.

The methods of the invention are able to achieve useful levels of conjugation while making use of advantageously low concentrations of the biomolecular complexes of the invention. In a suitable embodiment, a method of the invention may utilise a concentration of the biomolecular complex (at the time that conjugation is performed) of below approximately 15 μ M, or even below, 10 μ M, 9 μ M, or 8 μ M. The efficiency of labelling of the biomolecular complexes of the invention is so high that useful levels of metal tricarbonyl conjugated biomolecular complexes can even be achieved using concentrations of the biomolecular complexes as low as 7 μ M.

Furthermore, the efficiency of labelling of biomolecular complexes of the invention is so high that useful levels of conjugation may be achieved using only short periods of incubation between the biomolecular complexes and metal tricarbonyls. As described elsewhere, rapid labelling methods of this sort provide considerable practical advantages.

In a suitable embodiment of such a method, conjugation may be conducted over a period of between about 25 and 130 minutes. As exemplified by the Studies set out herein, such a method may be one wherein the conjugation is conducted over a period of between about 30 and 120 minutes.

Indeed the inventors have surprisingly found that useful levels of tricarbonyl labelling may be achieved in methods in which the conjugation is conducted over a period of less than 90 minutes, for example a period of 60 minutes or less. Suitable useful levels of labelling may even be achieved using methods wherein the conjugation is conducted over a period of approximately 30 minutes.

Furthermore, the efficiency of labelling of biomolecular complexes of the invention is so high that the threshold labelling efficiency of 95% which typically would obviate the need for post-labelling purification, is reached at low protein concentrations in a short time. Elimination of post-labelling purification considerably simplifies the procedure.

Furthermore, the efficiency of labelling of the sequences of the invention in the biomolecular complexes is so high compared to other alternative labelling sequences known from the prior art (such as sequences containing one or two histidines) that such a high level of site-specificity can be achieved that the binding affinity of the protein of interest to its target should not be adversely affected.

As the results set out below describe in more detail, the biomolecular complexes of the invention, conjugated to metal tricarbonyl groups, are readily able to achieve very high levels of labelling efficiency, at or above the 95% degree that is considered important for clinical or other biological uses. These very high levels of labelling efficiency obviate the need for a purification step prior to use, which in turn provides substantial benefits in terms of the simplicity, quality, and speed of the labelling procedure.

Finally, the inventors have found that methods of producing biomolecular complexes in accordance with the invention may be carried out by performing the conjugation step at approximately room temperature. Such methods may be of particular utility when it is wished to use high concentrations of the biomolecular complexes during conjugation.

Examples

Study 1

The following study was undertaken to investigate and illustrate the effectiveness of biomolecular complexes in accordance with the present invention, and particularly the labelling sequences to be used in such biomolecular complexes.

1 *Validation of the model*

The first study undertaken illustrated the validity of the experimental model used. As described further below, this study demonstrated an excellent correlation between the performance of labelling sequences in solution and the performance of labelling sequences on solid phase (Celluspot) arrays used in the other studies outlined below.

Various labelling sequences suitable for use in the biomolecular complexes of the invention were produced, as set out in Figures 1 and 2. These labelling sequences, were incubated with metal tricarbonyls comprising radionuclides, and conjugation of metal tricarbonyls to labelling sequences was demonstrated by increasing radiochemical yield.

Figure 1 shows the labelling efficiencies of the various labelling sequences when provided on solid phase arrays (i.e. conjugated to CelluspotTM peptide array), while Figure 2 shows labelling efficiency when the labelling sequences are provided as individual peptide chains in solution. As can be seen from Figure 3, it is possible to make a direct correlation between the results achieved in solution and those achieved in the solid phase, illustrating that the model used is of high quality. This proves that labelling efficiencies observed in the studies set out below (in which labelling efficiency is investigated through analysis of [^{99m}Tc(CO)₃]⁺ labelled CelluspotTM arrays) can be considered an accurate reflection of the situation in solution.

2 *Influence of isoelectric point of labelling sequence on labelling efficiency*

The inventors investigated the influence of isoelectric point (pI) of labelling sequences on conjugation of metal tricarbonyls ([M(CO)₃]⁺) via histidine residues contained within the labelling sequences. The studies undertaken illustrated that labelling sequences with high pI show remarkably enhanced radiolabelling efficiencies – indicating increased conjugation of metal tricarbonyls as compared to labelling sequences having lower pI.

The isoelectric point (pI), is the pH at which a protein carries no net charge. At pH values below the isoelectric point proteins carry a net positive charge, above it a net negative charge. For the purposes of the present disclosure, pI has been calculated using compute pI/Mw by ExPASy Bioinformatics Resource Portal. Compute pI/Mw is a tool which allows the computation of the theoretic pI (isoelectric point) and Mw (molecular weight) for a list of UniProt Knowledgebase (Swiss Prot or TrEMBL) entries or for user entered sequences. The presence of positively charged amino acids in a peptide sequence will increase the pI and peptides with high pI are also associated with high radiochemical yields.

Figures 10 to 14 set out 5 graphs in which pI is plotted against the radiochemical yields of peptide sequences that have

- i. His-Tag → 6 consecutive histidines
- ii. HHHH → 4 consecutive histidines
- iii. HXHXHX → 3 histidines with an alternate spacer
- iv. HXHX → 2 histidines with an alternate spacer
- v. HXXH → 2 histidines with 2 spacers

In every graph there is a positive correlation between high pI and high radiochemical yield. In particular there is an exponential relationship observed in respect of labelling sequences suitable for use in the biomolecular complexes of the invention. Here it can be seen that a marked increase in radiochemical yield is observed in respect of sequences having a pI greater than 9. Black dotted lines on the graphs demonstrate pIs of 9 and 9.5.

It is worth noting that among the sequences having 4 consecutive histidines investigated in this study, the highest pI achieved is 8.5 – which is below the level of 9 required by the linker sequences that may be used in biochemical complexes of the invention. Accordingly, these linker sequences constitute comparator peptides for the sake of the present disclosure. In keeping with this, it will be observed that there are no peptides in this group which achieve high radiochemical yields (as compared to the yields that result when labelling sequences suitable for inclusion in biomolecular complexes of the invention are used). That said, a trend towards increased labelling correlated with increased pI can be observed to some extent within the members of this group.

The inventors' studies further illustrated that the presence of 2-4 arginine residues, or 3-5 lysine residues, in labelling sequences of the invention (which will typically be sufficient to

ensure that the pI of such labelling sequences is greater than 9) provides notable advantages.

Supporting results are set out in Figures 15 to 17. Here, all peptide sequences with a high pI and high radiochemical yield comprise multiple arginine and/or lysine residues. The graphs indicate that, in labelling sequences with a pI of 9 or above, the best radiolabelling efficiency is achieved in labelling sequences that comprise 2-4 arginine residues, or 3-5 lysine residues.

3 *Influence of amino acid residue charge on labelling efficiency*

The inventors' studies further illustrated that the presence of positively charged amino acid residues in labelling sequences increases the ability of histidine residues within these sequences to conjugate metal tricarbonyls.

Figures 4 and 23 plot the radiochemical yield of a number of peptide sequences (comprising labelling sequences suitable for use in the biomolecular complexes of the invention, and comparator sequences) after 15 minutes of incubation with $[^{99m}\text{Tc}(\text{CO})_3]^+$. Figure 23 provides further information regarding the presence of lysine and/or arginine residues over all peptide sequence categories. The results achieved clearly highlight that the sequences that contain positively charged amino acids are the ones that have the highest rate of labelling. Peptide sequences can be placed into categories based on the amino acids that they contain and the arrangement of the amino acids with regards to the histidines. Figure 4 and Figure 23 demonstrate the importance of positively charged amino acids in a labelling sequence for conjugation with $[\text{M}(\text{CO})_3]^+$.

In contrast, negatively charged amino acids are detrimental to the ability of histidines within labelling sequences to conjugate metal tricarbonyls.

Figure 5 compares labelling of sequences comprising positively charged amino acid residues with those comprising negatively charged amino acid residues. There is a significant difference ($p < 0.005$) between the average labelling efficiency of the sequences containing negatively charged amino acids and the sequences containing positively charged amino acids.

Figures 6 to 9 demonstrate results of binding studies using sequences in which a single amino acid is changed in order to compare direct effects on the labelling of the histidines.

The amino acids that are varied include glutamic acid (E), aspartic acid (D), lysine (K), arginine (R), serine (S), glycine (G). The sequences containing aspartic acid and glutamic acid have the lowest labelling efficiency due to the negatively charged amino acids. Sequences containing lysine and arginine have the highest labelling efficiency due to the presence of positively charged amino acids

These results indicated that positively charged amino acids (e.g. lysine and arginine) increase labelling efficiency whereas negatively charged amino acids (e.g. aspartate and glutamate) have the opposite influence.

Accordingly, the presence of positively charged amino acids in the labelling sequences of the invention provides significant benefits by improving the ability of such labelling sequences to bind metal tricarbonyls via their conjugation with histidine residues present in the labelling sequence.

4 Preferred arrangements of requisite amino acid residues

The labelling sequences of biomolecular complexes of the invention must contain between 4 and 6 histidine residues, and at least 2 residues are selected from the group consisting of lysine and/or arginine residues. A study was undertaken to identify preferred arrangements of these requisite amino acid residues within the labelling sequences of biomolecular complexes of the invention.

In accordance with this aim, a study was undertaken to determine preferred arrangements of histidine residues that confer beneficial characteristics in terms of the ability of the labelling sequences to conjugate metal tricarbonyls. As set out below, this study illustrated that labelling sequences comprising six contiguous histidine residues achieve the highest levels of conjugation (indicated by radiochemical yield).

The results set out in Figure 19 illustrate that the radiochemical yield that may be achieved by peptide sequences comprising 6 contiguous histidine residues is better than that which may be achieved in sequences where histidines are arranged in HXHXHX, HXHX, HXXH and HHHH combinations. Furthermore, these results confirm the inventors' previous observation that histidines are required in the labelling sequences in order for these sequences to be able to conjugate with metal tricarbonyls. As can be seen, comparator sequences containing no histidine residues achieved very low, or no, radiochemical yield (bottom graph).

The exponential relationship between the isoelectric point and radiochemical yield for each type of histidine tag can be seen in the top graph. This clearly shows the benefits of sequences comprising six contiguous histidine residues, in comparison to other arrangements of histidine residues.

A further study investigated the preferred relationships between the positioning in arginine and histidine residues within labelling sequences. This identified that arginines are preferably not positioned between histidine residues, and yielded a preferred positioning of arginine residues in relation to histidine residues.

The results shown in Figure 18 illustrate that, in general, sequences in which arginine residues separate the histidine residues (e.g. HRHRHR) have the lowest radiochemical yields. Sequences in which the histidines are separated by two arginines are least favourable. All other positional arrangements of the arginine with respect to the histidines have similar radiochemical yields. This includes sequences in which there are arginines on either side of the histidines. The preferred labelling sequence identified in Figure 18 is as follows HHHHHHXXRRRX, and this may be a preferred labelling sequence for use in the biomolecular markers of the invention.

5 Influence of cysteine and/or methionine on effectiveness of labelling sequences

Many of the preferred labelling sequences identified by the inventors (indeed about 70% of such sequences) contain a cysteine residue. However, while this indicates the benefits that may derive from inclusion of cysteine within the labelling sequences of biomolecular complexes of the invention, it is not the most influential factor in determining their effectiveness.

Previously published reports have suggested that the addition of cysteine residues to peptide sequences improved labelling efficiencies (labelling with $[M(CO)_3]^+$) that may be achieved in connection with histidine residues present in such sequences. A sequence, CKLAAALEHHHHHH, identified in previous publications as of particular utility had been used to label the C2A protein with $[^{99m}Tc(CO)_3]^+$.

Further studies were undertaken to investigate the extent to which the presence or absence of cysteine residues plays a major role in the coordination of $[M(CO)_3]^+$ to histidines in a

labelling sequence, and to investigate distances of cysteine residues from histidine residues that may be of benefit in increasing conjugation metal tricarbonyls to the labelling sequences.

In this study the sequences set out in Table 2, all of which demonstrate beneficial labelling characteristics (and which, with the exception of comparator sequence RHRHRHGRGGRC constitute labelling sequences suitable for use in the biomolecular complexes of the invention), were included on experimental plates in forms with and without a cysteine residue. Figure 21 illustrates a direct comparison between the labelling of these sequences in forms with and without the presence of a cysteine residue.

As shown in Figure 21, there is a significant difference between three of the sequences with and without cysteine. The majority of sequences appear to have a better radiochemical yield with a cysteine than without. However for the GGRHHHRHHHRGGC, the preferred labelling sequence is actually the one without a cysteine residue. Cysteine may contribute slightly towards a labelling efficiency increase however, it is not the most influential factor.

While the potential benefits of incorporating cysteine residues in sequences for conjugation with metal tricarbonyls have previously been recognised, the inventors surprisingly found that similar benefits can be provided by the use of methionine and that methionine can be used as a replacement for cysteine (see results in Figure 22). The use of methionine residues rather than cysteine residues may provide advantages, in that the methionine residues are not associated with the formation of disulphide bonds in biomolecular complexes of the invention.

6 Identity of a preferred labelling sequence suitable for use in the biomolecular complexes of the invention

The inventors have identified a particularly effective version of a labelling sequence that may be used in the biomolecular complexes of the invention. This has the amino acid sequence CLRRRLAHHHHHH (or HHHHHHALRRRLC if the histidine sequence is provided at the other terminus).

The ability of this preferred labelling sequence to conjugate metal tricarbonyls was compared with that of prior art sequences and with comparator sequences. The results of this study are shown in Figure 20.

As shown in Figure 20, the present study shows that labelling sequences for use in the biomolecular complexes of the present invention (such as the preferred sequence CLRRRLAHHHHHH) are able to provide a degree of labelling that is has a significant 8 fold higher labelling efficiency at 15 minutes ($p < 0.00001$) than the prior art labelling sequence (CKLAAALEHHHHHH). This prior art sequence had previously been identified as having beneficial labelling properties, and these results show that the biomolecular complexes of the invention, utilising labelling sequences as described herein, will provide practical advantages over such complexes of the prior art.

7 **Materials and methods**

- CelluspotTM Plate synthesised (containing 384 peptides in duplicate) to contain specially designed His-tagged peptides.
- CelluspotTM plate added to 50ml of PBS buffer at pH 7.4 containing 7-8MBq of $[^{99m}\text{Tc}(\text{CO})_3]^+$
- $[^{99m}\text{Tc}(\text{CO})_3]^+ \rightarrow$ Synthesised using Isolink Kits. $^{99m}\text{TcO}_4^-$ (~1000MBq) added to the Isolink kit and heated at 100°C for 30 minutes. Once heated the kit is cooled to room temperature and neutralised to pH 7.4 with 1M HCl. 7-8MBq aliquoted into 50ml of PBS at pH 7.4.
- CelluspotTM incubated with $[^{99m}\text{Tc}(\text{CO})_3]^+$ in PBS for 15 minutes at 37°C with gentle shaking. The plate is completely immersed.
- After 15 minutes the plate is placed in 50 ml of PBS for 3 seconds (to wash off any unbound $[^{99m}\text{Tc}(\text{CO})_3]^+$) and blotted dry with filter paper. (There is no resulting activity on the filter paper)
- The plate is exposed to the phosphor film for 5 minutes and the film is placed in the phosphor imager to generate an image.
- The CelluspotTM plate is re-immersed in the PBS buffer containing the $[^{99m}\text{Tc}(\text{CO})_3]^+$ complex and left for another 15 minutes (incubation total = 30 minutes). The process of washing and imaging is repeated again to obtain results at a 30 minute time point.
- Image processing is done using the Opti-quant programme associated with the phosphor imager. A grid is placed around the image with each square surrounding a single spot. The intensity of each black spot is then record as DLU (digital light units), arbitrary units that can be associated with radiochemical yield.

Study 2

A further study was conducted to illustrate the utility of the biomolecular complexes of the invention in the labelling of antibody fragments. As discussed in more detail below, this study involved:

1. Synthesis of a J591 single chain antibody with new Arg/His tag: LRRRLAHHHHHH (designated "JWT" for the purposes of this study)
2. Synthesis of J591 single chain (designated J591scFv) and a humanised version of the J591 scFv (designated huJ591scFv) to act as controls. These proteins contain His-tags with fewer nearby Arg groups (1 or 0 respectively).
3. Radiolabelling of all biomolecular complexes at different concentrations and comparison of data obtained at 30, 60, 90 and 120 minute labelling time points.
4. Assessment of serum stability of J591JWT radiolabelled protein.

Introduction

As reported in the Study above, radiolabelling the Celluspot™ peptide array identified Arg containing His-Tag (Arg/His) sequences as having the highest affinity for the $[^{99m}\text{Tc}(\text{CO})_3]^+$. Based on these results, these sequences should provide an efficient method for the fast labelling of proteins at low concentrations. To prove this concept a preferred Arg/His labelling sequence of the invention referred to in the study above (designated "JWT" for the purposes of this second study) was engineered into the J591 single chain antibody (scFv) at the C-terminal. J591(scFv) is an antibody derived from the monoclonal antibody J591 which recognises an extracellular epitope of prostate specific membrane antigen (PSMA). PSMA is a well-established marker for prostate carcinoma with elevated expressions detected in virtually all prostate cancers. For hormone refractory and metastatic disease, a further increase in levels of PSMA expression is observed. A deimmunised version of the mAb J591 has been characterised in a number of different clinical studies including: phase I combined radioimmunotherapy and imaging trials with ^{111}In -J591/ ^{90}Y -J591 and ^{177}Lu -J591 and imaging studies with ^{111}In labelled J591. Excellent targeting properties were observed for the mAb J591 however, due to the use of a full length antibody conjugate a long circulation time was observed and images had to be performed days after the injection of the tracer in order to obtain a sufficient contrast. The engineering of an antibody into smaller fragments is possible whilst largely retaining the antigen binding properties. Smaller antibody fragments are beneficial for applications in imaging as a result of their rapid pharmacokinetics, potentially

enabling the imaging to occur at 1hr to 8hr time points post tracer injection. The proteins used in this study are single chain antibody fragments, scFv.

An Arg/His sequence, LRRRLAHHHHHH (JWT), was engineered at the C-terminal of the J591scFv antibody producing the J591scFvJWT antibody fragment (a biomolecular complex in accordance with the present invention). Alternative fragments derived from mAb J591 were also produced, to act as comparators for the biomolecular complex of the invention. Both fragments, J591scFv and huJ591scFv, contained C-terminal (His)6-Tags. In addition, scFv fragments of an antibody directed against mouse vascular cell adhesion molecular 1 (VCAM-1) were obtained for further comparative experiments. These antibodies referred to as 6C7.1 and 6C7.1-Cys contain a (His)6-Tag at the C-terminal and will determine whether the Arg/His system can demonstrate a superior labelling efficiency over any His-Tagged protein independently of the protein sequence. For the 6C7.1-C antibody, a Cys residue is present at the C-terminal side of the His-Tag. This provides a direct comparison between the previously successful Cys/His-Tag system and the newly developed Arg/His-Tag combinations of the invention for labelling proteins with $[^{99m}\text{Tc}(\text{CO})_3]^+$. The range of control proteins used in the comparative studies against the J591scFvJWT has been designed to provide sufficient evidence to conclude whether the presence of Arg residues in combination with a His-Tag in a protein mimics the behaviour of the Arg/His peptides from the His-Tagged CelluspotTM array. Radiolabelling efficiencies for all proteins were achieved at 6 different concentrations ranging from 0.88uM to 28.2uM in a 1 to 2 dilution series. Comparisons were made at 30, 60, 90 and 120 minute total incubation time points. The viability of the radiolabelled J591scFvJWT for use in preclinical imaging was assessed with a serum stability assay.

A Generation of biomolecular complexes comprising antibodies and labelling sequences

A1 Methods and experimental

A1.1 Antibody construction and expression

A single chain fragment variable (scFv) of J591 in VH-VL orientation was PCR amplified from the SFG P28z vector and subcloned into a hybrid expression vector based on Psectag2 (Life Technologies) and Pires-Egfp (clonotech) sequences. J591JWT was generated by insertion of annealed overlapping oligonucleotides (Integrated DNA Technologies) with NotI/EcoRI overhangs replacing the original (His)6 sequence in the expression vector.

Oligo sequences:

JWT_forward:

5' ggccgcACTGAGAAGAAGGCTGGCCCACCACCACCACCACTGAG 3'

JWT_reverse:

5' gcACTGAGAAGAAGGCTGGCCCACCACCACCACCACCACTGAGAATT 3'

A published, humanised sequence of the J591 scFv was synthesised by Geneart and subcloned into the target expression vector.

The sequence coding for the 6C7.1 scFv was kindly provided by Prof S. Duebel, University of Braunschweig, Germany. The 6C7.1 scFv sequence was PCR amplified from a source vector and subcloned into the target expression vector. 6C7.1 scFv with a C-terminal cysteine (6C7.1-Cy(scFv)) was used as an additional control. All sequences were verified by DNA sequencing.

For protein production, HEK393T cells were transfected with the respective expression vector and transfected cells were selected with 100ug/ml of Zeocin. Cells were then expanded to triple layer flasks and culture supernatants containing the recombinant protein were collected.

A1.2 Antibody purification

Purification was achieved by a combination of immobilised metal ion chromatography and size exclusion chromatography. The scFv antibodies were extracted from HEK293T culture supernatant using Ni-NTA chromatography with a 5ml or 1ml Ni-NTA column (Superflow cartridge, Qiagen). A gel filtration step (Superdex 75 HR 10/30) using the AKTA-FPLC further purified the antibody fragments separating the residual BSA protein and dimerised scFv fragments from the monomeric scFv. The purified monomeric scFv proteins in PBS at pH 7.4 were concentrated using VivaSpin molecular weight cut off filter columns (Sartorius). The proteins were concentrated to the following concentrations: 1.3mg/ml for J591scFvJWT, 1.35mg/ml for J591scFv, 1.9mg/ml for huJ591scFv, 1mg/ml for 6C7.1 and 1.1mg/ml for 6C7.1C. Protein concentration was measured by UV spectrometry with a UV absorption of 280nm using a Nanodrop device. A molar extinction coefficient and molecular weight of the respective protein was determined from the primary aminoacid sequence using the ProtParam online tool, assuming all cysteines are present as cystines: for J591scFvJWT E_{280nm} : 50880 M⁻¹ cm⁻¹, MW monomer: 28.33kDa; for J591scFv E_{280nm} : 50880 M⁻¹ cm⁻¹, MW monomer: 27.70kDa; for huJ591scFv E_{280nm} : 50880 M⁻¹ cm⁻¹, MW monomer: 27.28kDa; for

6C7.1 E_{280nm} : 48610 $M^{-1} cm^{-1}$, MW monomer: 28.78kDa; and for 6C7.1C E_{280nm} : 48610 $M^{-1} cm^{-1}$, MW monomer: 28.89kDa. Aliquots were stored at $-80^{\circ}C$.

A1.3 Protein Quality Control: SDS Page, HPLC and Western Blot

Purity of the scFv protein fragments was assessed by SDS-PAGE/Coomassie brilliant blue staining, analytical size exclusion HPLC (BioSep SEC-s2000, Phenomenex) and a Western blot. Proteins were separated using NuPAGE 12% gels and MOPS buffer (Life Technologies). Gels were either stained with Coomassie brilliant blue or proteins were transferred to nitrocellulose membranes for subsequent Western blot detection using antiPentaHis (Qiagen) as primary antibody, Gam:HRP (Millipore) as secondary antibody and SigmaFastDAB (SigmaAldrich) as HRP substrate. Size exclusion HPLC was performed with PBS, pH 7.4 as mobile phase with a flow rate of 1ml/min.

A2 Results

A2.1 scFv Protein Sequences

All scFv antibody fragments were successfully constructed, expressed and purified. Table 3 provides the DNA sequencing data for each scFv antibody. Sequence analysis revealed that each antibody contained a His-Tag at the C-terminal. For J591JWT the labelling sequence contained a His-Tag in addition to 3 Arg residues which gave a calculated local pI of 12.4, and thus the proteins comprising this sequence constituted biomolecular complexes of the present invention. J591scFv has an identical amino acid composition as that of the J591scFvJWT antibody; except that J591scFvJWT has an additional 4-amino acids, LRRR, before the His-Tag sequence. For J591 scFv the labelling sequence, RAAALEHHHHHH, has a pI of 7.21, and thus the proteins comprising this sequence did not constitute biomolecular complexes of the present invention. Despite having a pI < 9.5, the J591 scFv control has an Arg 6 amino acids away from the His residues. An Arg in close proximity to His residues has shown a strong influence on the labelling efficiencies and therefore to compensate for the presence of an Arg in the J591 scFv control, an alternative huJ591scFv was synthesised with a labelling sequence containing no Arg residues. The huJ591scFv has a labelling sequence KLAAALEHHHHHH with a pI of 7.21. The 6C7.1 and 6C7.1-Cys scFvs have identical amino acid composition to each other except for an additional Cys residue at the C-terminal of the His-Tag in the 6C7.1C scFv. The labelling sequences for 6C7.1 and 6C7.1-Cys are TAAALEHHHHHH and TAAALEHHHHHHC respectively and the pI is 6.53 for both. Full details of the various proteins produced are set out in Table 3, in which all labelling sequences have been highlighted in bold.

A2.2 J591scFvJWT and J591scFv Protein Expression & Purification

The scFv proteins were produced in HEK239T cells with yields of purified protein (purity >95%) of 2-6mg/L culture supernatant. The production and purification of the J591scFvJWT and J591scFv proteins were recorded by SDS PAGE and Western blot on nitrocellulose membrane and are displayed in Figure 24a and 24b respectively. The J591scFvJWT (Figure 24a) appears as a monomer of 28kDa and can be seen in every sample added to the NuPage gel. Lanes A-C in the SDS-PAGE monitor the progress of extracting J591scFvJWT from the culture supernatant. (His)₆ recombinant proteins such as J591scFvJWT have a high affinity and selectivity for the Ni-NTA and as a result, the majority of the protein impurities present in the supernatant, lane A, are discarded in the flowthrough of the Ni-NTA purification system. Other protein impurities, principally BSA, unspecifically bound to the Ni-NTA are removed in the 35mM imidazole wash of the Ni-NTA column, lane B. J591scFvJWT was eluted from the column by competition with a 250mM imidazole solution, lane C. Further purification of the J591scFvJWT was required and was achieved with size exclusion chromatography. Three peaks appeared in the SEC purification chromatogram and samples from each were added to the SDS PAGE and can be seen in lanes D-F. The first peak corresponded to BSA, lane D, whilst the second peak corresponded to dimerised J591scFvJWT protein, lane E. The dimers formed were non-covalent dimers and on the SDS-PAGE appear as bands in the 28kDa monomeric region for the J591scFvJWT. The process of producing an SDS-PAGE destroys any aggregates or non-covalent dimers present. J591scFvJWT was eluted as a purified protein in the third peak, lane F, and concentrated to 1.3mg/ml, lane G. The corresponding Western blot reveals all the proteins present with a (His)₆ tag which confirms the appearance of the J591scFvJWT as a monomeric band at 28KDa on the SDS-PAGE. The primary antibody marker used in the Western blot, antiPentaHis, is specific for (His)₆ targeting.

SDS-PAGE and Western Blot monitoring for the process of extracting and purifying the J591scFv protein is shown in Figure 24b. Again, lane A-C represents the extraction of the protein from the culture supernatant and lane D-F represents the purification fractions from the SEC. The J591scFv appears as a monomeric protein at 27kDa and the purified (purity > 95%) sample can be seen in lane F concentrated to 1.35mg/ml. Confirmation for the presence of J591scFv is given by the Western Blot which highlights the position of the (His)₆ containing proteins on the SDS-PAGE. (Figure 24b) Purified (purity > 95%) 6C7.1, 6C7.1-Cys and huJ591 were provided by Dr Florian Kampmeier. The same procedures for production and purification were used for these scFv proteins and assessed by SDS-PAGE and Western blot. SEC HPLC analysis confirms that for J591scFvJWT, J591scFv and

huJ591scFv, the protein exists as a monomer, $t_r = 9\text{min}$, with greater than 95% purity (as shown in Figure 25) The peak observed at 8min, represents the formation of non-covalent dimers and accounts for less than 5% of the protein samples.

B Radiolabelling Efficiencies with $[^{99m}\text{Tc}(\text{CO})_3]^+$

B1 Introduction

All scFv protein fragments were radiolabelled by site-specific chelation of $[^{99m}\text{Tc}(\text{CO})_3]^+$ by the C-terminal (His)6-tag. To compare the relative labelling efficiencies, the proteins were radiolabelled at a range of 6 different concentrations in a 2:1 dilution series. Protein concentrations were calculated post addition of the $[^{99m}\text{Tc}(\text{CO})_3]^+$ radiolabelling solution. The highest concentration achieved for the protein in the $[^{99m}\text{Tc}(\text{CO})_3]^+$ radiolabelling solution was 28.2uM which for the J591scFvJWT, J591scFvJWT and huJ591scFv proteins is equivalent to 0.8mg/ml. The subsequent 5 protein concentrations in the dilution series were 14.1uM, 7uM, 3.5uM, 1.76uM and 0.88uM. Concentrations of the purified 6C7.1 and 6C7.1-Cys were not high enough to enable the protein concentration to reach 28.2uM in the $[^{99m}\text{Tc}(\text{CO})_3]^+$ labelling solution. Consequently, for 6C7.1 and 6C7.1-Cys the highest protein concentrations achieved in the labelling reaction were 14.1uM and 7uM respectively.

In the literature, the majority of (His)6-Proteins are incubated with $[^{99m}\text{Tc}(\text{CO})_3]^+$ for at least 60 minutes and this often yields a radiochemical yield of between 75-90%. In order to use the protein- $[^{99m}\text{Tc}(\text{CO})_3]^+$ conjugate in pre-clinical studies, the radiochemical yield must be greater than 95%. To achieve this, further incubation with $[^{99m}\text{Tc}(\text{CO})_3]^+$ is a possibility however the rate of labelling decreases and it can take at least a total of 2 hours to reach the required 95% labelling efficiency. This is a time consuming process and is not a suitable method for the production of the radiolabelled protein in clinical or pre-clinical applications. The specific activity of the labelled protein and the $[^{99m}\text{Tc}(\text{CO})_3]^+$ solution decreases with radioactive decay and ^{99m}Tc has a relatively short half life of 6 hours. Another possibility is a purification step using a PD-10 column which is commonly used in the production of $[^{99m}\text{Tc}(\text{CO})_3]^+$ -(His)6-Proteins before in vivo experiments. Not only is a purification step time consuming, but also it often involves the loss of protein and reduces the concentration of the protein. Loss of protein can be costly depending on the type of protein used and the ease at which it can be produced. Procedures involving a loss of protein are preferably avoided when preparing radiolabelled protein conjugates for clinical use.

Comparative [$^{99m}\text{Tc}(\text{CO})_3$] $^+$ labelling studies for the J591scFvJWT and control proteins analysed the radiochemical yield at 15, 30, 60, 90 and 120 minute time points for all the different protein concentrations prepared. An ideal situation for pre-clinical and clinical applications would be the identification of a protein that demonstrates a high radiochemical yield, greater than 95% to avoid the need for post-labelling purification, at a low protein concentration in the fastest time possible, preferably below 60 minutes. Plotting the radiochemical yield data from these experiments against the increasingly diluted protein concentration at different time points should reveal whether the Arg/His combination sufficiently improves the radiolabelling efficiency and can become a standardised method for the site-specific labelling of proteins with ^{99m}Tc .

B2 Method

B2.1 Conversion of $^{99m}\text{TcO}_4^-$ to [$^{99m}\text{Tc}(\text{CO})_3$] $^+$

The J591scFvJWT, J591scFv, huJ591scFv, 6C7.1 and 6C7.1-Cys, proteins were labelled with [$^{99m}\text{Tc}(\text{CO})_3$] $^+$ via the C-terminal (His)6-tag for the comparative radiolabelling studies. Preparation of the [$^{99m}\text{Tc}(\text{CO})_3$] $^+$ was achieved using the conventional IsoLink kits kindly provided by Covidien. The Isolink kit was reconstituted with 2200-2500MBq of $^{99m}\text{TcO}_4^-$ (^{99m}Tc pertechnetate) in 400ul of saline and heated to 97°C for 30min. The kit was neutralised with 1M HCl to pH 7.5 (approximately 160ul) and quality control performed by thin layer chromatography (TLC) to verify the conversion rate of $^{99m}\text{TcO}_4^-$ to [$^{99m}\text{Tc}(\text{CO})_3$] $^+$. Glass backed silica gel 60 (Merck) TLC plates were used with a mobile phase of 1% HCl in methanol for the quality control.

B2.2 Preparation of ScFv Proteins

ScFv proteins in PBS at pH 7.4 were prepared to a concentration of: 42uM (1.2mg/ml) for J591scFvJWT, J591scFv and huJ591scFv; 21uM (0.6mg/ml) for 6C7.1; and 10.5uM (0.3mg/ml) for 6C7.1-Cys. Protein concentrations were determined by UV absorption at 280nm using a Nanodrop spectrophotometer. For J591scFvJWT, J591scFv and huJ591scFv, 20ul samples were aliquotted and a 2:1 dilution series performed to give 6 different protein concentrations: 42uM, 21uM, 10.5uM, 5.25uM, 2.625uM and 1.312uM. The same procedure was carried out for 6C7.1 and 6C7.1-Cys proteins starting from a concentration of 21uM and 10.5uM respectively. There were 5 6C7.1 samples and 4 6C7.1-Cys samples. Once diluted with [$^{99m}\text{Tc}(\text{CO})_3$] $^+$ the desired protein concentration, reported in the results, was reached.

B2.3 [$^{99m}\text{Tc}(\text{CO})_3$] $^+$ labelling of scFv proteins

To the scFv proteins, 10ul of approximately 50MBq of $[^{99m}\text{Tc}(\text{CO})_3]^+$ was added to give 2MBq/ug for the protein samples with the highest concentration. The $[^{99m}\text{Tc}(\text{CO})_3]^+$ -protein solutions were incubated at 37°C for up to 2 hours. Aliquots were taken at 15, 30, 60, 90 and 120 minutes for analysis by TLC. iTLC-silicic acid paper (iTLC-SA, 0.75 x 9 cm, Varian Medical Systems UK) was used with the origin at 1cm from the bottom of the strip and solvent front at 8cm. 1.5ul of the $[^{99m}\text{Tc}(\text{CO})_3]^+$ -protein solutions were spotted at the origin, and the strip allowed to air-dry before development in a mobile phase of 0.1M citrate buffer at pH 5. The iTLC-SA strips were cut in half and the radioactivity in each half recorded using a gamma counter (Flow-Count, LabLogic) as counts per minute (cpm). Through visualisation using the PhosphorImager and the TLC reader it had previously been determined that the $[^{99m}\text{Tc}(\text{CO})_3]^+$ conjugated proteins had an $R_f = 0$ and remained at the baseline whereas any unbound $[^{99m}\text{Tc}(\text{CO})_3]^+$ or unreduced $^{99m}\text{TcO}_4^-$ would move to the solvent front, $R_f = 1$. The percentage radiochemical yield was calculated by dividing the cpm recorded from the lower half of the strip with the total cpm for the whole strip, that is the cpm for the top and bottom halves of the strip. (Equation 1)

Equation 1. Calculating percentage radiochemical yield from the TLC data recorded by the gamma counter.

$$\text{Radiochemical yield (\%)} = \left[\frac{\text{Cpm from lower half of TLC strip}}{\text{Total cpm from TLC strip}} \right] \times 100$$

The comparative radiolabelling study for the scFv proteins was performed in duplicate.

B3 Results

All scFv proteins demonstrated an ability to be radiolabelled with $[^{99m}\text{Tc}(\text{CO})_3]^+$ via the (His)6 Tag at the C-terminal. To determine the relative efficiency of radiolabelling, the radiochemical yield was plotted against protein concentration ($\log[\text{protein}]$) for each time point (Figure 26). J591scFvJWT, the biomolecular complex of the invention with the Arg/His labelling sequence, clearly shows a superior labelling efficiency in comparison to all other comparators with labelling sequences that do not meet the requirements of the invention. This can be seen at 15, 30, 60, 90 and 120 minutes. Other scFv proteins (J591scFv, huJ591, 6C7.1 and 6C7.1-Cys) have lower labelling efficiencies and are similar to each other according to the graphs in Figure 26. Among these, a slight increase in the radiochemical yield was observed for the J591scFv. This is probably due to the labelling sequence of

J591scFv that contains an Arg residue 6 amino acids away from the (His)6. For huJ591scFv, 6C7.1 and 6C7.1-Cys, the Arg amino acid has been replaced by a Lys or Leu residue and these proteins displayed the lowest radiochemical yield at all time points.

When analysing the data obtained from the comparative radiolabelling studies, an important consideration is the concentration and time at which a threshold radiochemical yield of 95% has been reached. This is typically the minimum radiochemical purity required for pre-clinical and clinical applications. Table 5 reveals the incubation time and protein concentration at which the scFv proteins were able to reach a 95% radiochemical yield.

Again, it is obvious that by this criterion J591scFvJWT exceeds the efficiency shown by the other proteins with standard (His)6 sequences. From Table 5 it is clear that J591scFvJWT achieved a radiolabelling efficiency greater than 95% at the lowest protein concentration, 7uM, after incubation for 90minutes. For the non Arg/His containing proteins, the lowest protein concentration at which a radiochemical yield greater than 95% was achieved was 28.2uM after 90 minutes, J591scFv and huJ591scFv. This reveals that an Arg/His Tag has demonstrated an identical radiolabelling efficiency to that of a generic (His)6 tag with a significant 4 fold decrease in protein concentration at 90 minutes.

Often for (His)6 tagged proteins incubation with $[^{99m}\text{Tc}(\text{CO})_3]^+$ is standardised to 60 minutes followed by a PD-10 purification in order to yield a $[^{99m}\text{Tc}(\text{CO})_3]^+$ conjugated protein for in vivo use. In the comparative radiolabelling experiments, the non-Arg containing proteins did not reach a radiochemical yield of greater than 95% after 60 minutes. This was only achieved after a minimum of 90 minutes for the highest protein concentration. However, J591scFvJWT after 60 minutes demonstrated a greater than 95% radiochemical yield for both the highest and second highest protein concentrations. In addition, after 90 minutes, the third highest protein concentration also reached the minimum requirement of 95% radiochemical yield.

The concentrations at which the scFv proteins were radiolabelled were not high enough to give a radiolabelling efficiency greater than 95% after 15 minute incubation times. However, at 30 minutes J591scFvJWT at the highest protein concentration, 28.2uM, has a radiochemical yield of 96% which enables it to be directly used for in vivo experimentations without a purification procedure. Consequently for J591scFvJWT protein, with a concentration of 28.2uM or higher in the $[^{99m}\text{Tc}(\text{CO})_3]^+$ labelling solution, the total synthesis time would be 30 minutes or less. To achieve the same outcome with non Arg/(His)6

containing proteins, total incubation time required would be 90 minutes. This is an extra 60 minute incubation time which for ^{99m}Tc corresponds to an approximate 11% decrease in radioactivity.

For high concentrations and long incubation times, it was observed that J591scFvJWT precipitates in solution when heated to 37°C. To overcome this issue, the radiolabelling of J591scFvJWT was carried out at room temperature and the labelling efficiency monitored at 15, 30, 60, 90 and 120 minutes. The protein concentration was 14.1 μM and consequently the radiochemical yield was compared to that of the J591scFvJWT radiolabelling experiment at 14.1 μM when heated to 37°C. Figure 27 demonstrates that performing the $[\text{}^{99m}\text{Tc}(\text{CO})_3]^+$ radiolabelling at room temperature does not affect the efficiency but avoids the problem of precipitation.

The experimental conditions described herein may be used as a guide to suitable conditions that may be employed in methods of manufacturing and radiolabelling the biomolecular complexes of the invention.

The results set out here clearly illustrate that the biomolecular complexes of the invention provide a number of important advantages as compared to comparators incorporating labelling sequences known from the prior art. These advantages (which include the ability to use lower concentrations and/or shorter incubation times, and to achieve higher levels of labelling) have significant utility in clinical, diagnostic and research settings.

C Serum stability of radiolabelled J591scFvJWT

C1 Introduction

To verify the suitability of J591scFvJWT for in vivo experiments, the stability of the radiolabelled protein in serum was assessed. Incubation of the $[\text{}^{99m}\text{Tc}(\text{CO})_3]^+$ conjugated J591scFvJWT in serum can lead to two possibilities in which the $[\text{}^{99m}\text{Tc}(\text{CO})_3]^+$ complex does not remain bound to the (His)6 tag of the protein. Firstly, the $[\text{}^{99m}\text{Tc}(\text{CO})_3]^+$ could potentially dissociate from the (His)6 when diluted with serum and remain as $[\text{}^{99m}\text{Tc}(\text{CO})_3]^+$ or related small molecular species in solution. Another option is that the $[\text{}^{99m}\text{Tc}(\text{CO})_3]^+$ dissociates from the (His)6 due to competition from the serum proteins and subsequently coordinates to serum proteins. In order to check for both possibilities, serum stability was analysed by iTLC-SA and SDS-PAGE.

C2 Materials and Methods

J591scFvJWT was radiolabelled with $[^{99m}\text{Tc}(\text{CO})_3]^+$ as previously described (B2.3). Once the radiochemical yield had reached 95%, the $[^{99m}\text{Tc}(\text{CO})_3]^+$ -J591scFvJWT conjugate was incubated in a 1:1 (v/v) ratio with fresh human serum at 37°C. Aliquots were taken at 0, 15, 30, 60, 120 and 240 minutes for TLC analysis using ITLC-SA chromatography paper and a mobile phase of 0.1M citrate buffer at pH 5. In addition, samples were obtained at the same time points and immediately frozen in liquid nitrogen. Once all samples were collected, they were separated by SDS-PAGE (12% NuPAGE, Invitrogen) which was analysed by Coomassie blue staining and autoradiography using a phosphor imager (Cyclone Plus, PerkinElmer).

C3 Results

Stability of the $[^{99m}\text{Tc}(\text{CO})_3]^+$ radiolabelled J591scFvJWT upon incubation in human serum was analysed by ITLC-SA and SDS-PAGE. The ITLC-SA method has previously been used in analysing the radiolabelling efficiencies of the J591scFvJWT through comparisons between the percentage of $[^{99m}\text{Tc}(\text{CO})_3]^+$ labelled J591scFvJWT and the unbound $[^{99m}\text{Tc}(\text{CO})_3]^+$ and unreduced $[^{99m}\text{TcO}_4]^-$. Once developed in the citrate buffer mobile phase, the unbound $[^{99m}\text{Tc}(\text{CO})_3]^+$ complex and unreduced $^{99m}\text{TcO}_4^-$ move to the solvent front with an $R_f = 0$. The $[^{99m}\text{Tc}(\text{CO})_3]^+$ radiolabelled J591scFvJWT remains at the baseline with $R_f=0$. The same principles apply when analysing the serum stability and it is possible to observe whether the $[^{99m}\text{Tc}(\text{CO})_3]^+$ is dissociated and released as free $[^{99m}\text{Tc}(\text{CO})_3]^+$. According to Figure 28, results from the serum stability demonstrate that there is no loss of radiolabel from the $[^{99m}\text{Tc}(\text{CO})_3]^+$ -J591scFvJWT conjugate over a 4 hour period at 37°C. The radiochemical purity remains at 99.8% throughout. It must be taken into consideration that the $[^{99m}\text{Tc}(\text{CO})_3]^+$ can coordinate to serum proteins. Similarly to the $[^{99m}\text{Tc}(\text{CO})_3]^+$ -J591scFvJWT conjugate, the serum proteins remain at the baseline of the ITLC-SA paper once developed in 0.1M citrate buffer at a pH 5. Consequently, any radiolabelled serum proteins present in the sample will be recorded at the baseline in the same position as the radiolabelled J591scFvJWT, $R_f = 0$.

To discriminate between the serum-bound and protein-bound radioactivity, an SDS-PAGE was carried out on all the samples collected at the 0, 15, 30, 60, 120 and 240 minute time points. In addition control samples were included on the SDS-PAGE gel as references for the individual components: $[^{99m}\text{Tc}(\text{CO})_3]^+$, serum proteins and J591scFvJWT. The control samples were $[^{99m}\text{Tc}(\text{CO})_3]^+$ only, $[^{99m}\text{Tc}(\text{CO})_3]^+$ -serum protein conjugates and $[^{99m}\text{Tc}(\text{CO})_3]^+$ -

J591scFvJWT conjugate. The results of the SDS-PAGE can be seen in Figure 29 with the Coomassie staining image on the left (A) and autoradiograph on the right (B).

Lanes C-H in the autoradiograph confirm the presence of the radiolabelled J591scFvJWT as single black bands at 28kDa corresponding to the monomeric protein. Lane A is a control and in the autoradiograph the black band corresponds to $[^{99m}\text{Tc}(\text{CO})_3]^+$ conjugated to serum proteins. In lane I, it is not possible to observe any radioactivity which is understandable as the stable loaded in this row was $[^{99m}\text{Tc}(\text{CO})_3]^+$ which is small and highly charged. It is very likely that it has travelled to the end of the NuPAGE gel and is no longer registered on the SDS-PAGE. The corresponding Coomassie blue stained image (Figure 29, A), identifies the location of the serum proteins within the serum containing samples. Once the serum proteins have been visualised it is possible to confirm that the serum proteins are not conjugated to $[^{99m}\text{Tc}(\text{CO})_3]^+$ as they do not appear as black bands in the autoradiograph. Due to the extremely low concentrations of J591scFvJWT protein, it is not possible to observe the protein in the SDS-PAGE gel.

To conclude, once radiolabelled J591scFvJWT is stable in serum for at least 4 hours at 37°C. The other four proteins (J591scFv, huJ591scFv, 6C7.1 and 6C7.1-Cys) have previously been analysed for serum stability by Dr Florian Kampmeier and they demonstrate an identical behaviour.

Table 1

Peptide Sequences	DLU	High DLU --> Low DLU	pI
HHHHHHHALRRRLC	15049.38	1	11.7
HHHHHHHALRRRLKC	12307.98	2	11.71
GRHHHHHHHRRRC	12028.73	3	11.53
GRRHHHHHHHRRG	11857.98	4	12.48
HHHHHHHRRRAARRC	10893.63	5	12
HHHHHHHALRRPL	10655.66333	6	12.3
RCRGHHHHHHHGRCF	9419.363333	7	11.35
GGRHHHHHHHRRGG	9107.196667	8	12.3
HHHHHHHRGGGRC	8897.08	9	10.35
RHRHRHGRGGRC	8766.78	10	12.18
HHHHHHHRAARAC	8737.263333	11	11.7
GKKHHHHHHHKKG	8678.58	12	10.48
HHHHHHHRAARAR	8481.663333	13	12.3
HHHHHHHGRGGRC	8454.23	14	10.35
RHRHRHRLAAAL	8446.196667	15	12.48
GKKHHHHHHHKKGC	8382.596667	16	10.04
HHHHHHHRRRAARR	8225.43	17	12.48
HHHHHHHRGGGR	7598.73	18	12
HHHHHHKGGGK	7503.88	19	10
HHHHHHKAKAK	7040.996667	20	10.3

Table 2

AA sequence
HHHHHHALRRRLC
HHHHHHALRRRLKC
CRRHHHHHHHRRRC
GRRHHHHHHHRRRG
HHHHHHHRRRAARRC
HHHHHHALRRRL
RCRGHHHHHHHGRGR
GGRHHHRHHHRGG
HHHHHHRGGGRC
RHRHRHGRGGRC

Table 3

Antibody/labelling sequence designated	Sequence (labelling sequence in bold)
J591scFvJWT	DAAQPAMAEVQLQQSGPELVKPGTSVRISCKTSGYTFTEYTIHWVK QSHGKSLEWIGNINPNNGGTTYNQKFEDKATLTVDKSSSTAYMELR SLTSEDSAVYYCAAGWNFDYWGGGTTVTVSSGGGGSGGGGSGG GGSDIVMTQSHKFMSTSVGDRVSIICKASQDVGTAVDWYQQKPGQ SPKLLIYWASTRHTGVPDRFTGSGSGTDFTLTITNVQSEDLADYFC QQYNSYPLTFGAGTMLDLKRAAALRRRLAHHHHHH
J591scFv	DAAQPAMAEVQLQQSGPELVKPGTSVRISCKTSGYTFTEYTIHWVK QSHGKSLEWIGNINPNNGGTTYNQKFEDKATLTVDKSSSTAYMELR SLTSEDSAVYYCAAGWNFDYWGGGTTVTVSSGGGGSDIVMTQSH KFMSTSVGDRVSIICKASQDVGTAVDWYQQKPGQSPKLLIYWAST RHTGVPDRFTGSGSGTDFTLTITNVQSEDLADYFCQQYNSYPLTFG AGTMLDLKRAAALEHHHHHH
huJ591scFv	DAAQPAMAEVQLVQSGAAEVKKPGASVKISCKTSGYTFTEYTIHWV KQASGKGLEWIGNINPNNGGTTYNQKFEDRATLTVDKSTSTAYMEL SSLRSEDTAVYYCAAGWNFDYWGGGTTVTVSSGGGGSGGGGSGG GGSDIVMTQSPSSLSASVGDRVITICKASQDVGTAVDWYQQKPG KAPKLLIYWASTRHTGVPDRFTGSGSGTDFTLTISLQPEDFADYFC QQYNSYPLTFGGGKLEIKAAALEHHHHHH
6C7.1scFv	DAAQPAQVQLQESGGGLMQPGRSLNLSCVASGFTFNYYWMTWIR QAPGKGLEWVASVSFTGGSTYYPDVSKGRFTISRDNASTLYLQM NSLRSDDTATYYCTRGGYSSPFAYWGQGLTVTVSSAETTPKLEEG EFSEARVDIQMTQRPASLSASLGETVSIIECLASEDISNSLAWYQQK PGKSPQLLINGASSLQDGVPSRFSGSGSGTQYSLKISGMQPEDEG VYYCQQGYKYPPTFGGGTKLELKRADAAPTAAALEHHHHHH

6C7.1CscFv	DAAQPAQVQLQESGGGLMQPGRSLNLSCVASGFTFNNYWMTWIR QAPGKGLEWVASVSFTGGSTYYPDsvKGRFTISRDNakSTLYLQM NSLRsDDTATYYCTrGGYSSPFAYWGQGTlVTVSSAETTPKLEEG EFSEARVDIQMTQRPASLSASLGETVSIECLASEDISNSLAWYQQK PGKSPQLLINGASSLQDGVPSRFSGSGSGTQYSLKISGMQPEDEG VYYCQQGYKYPPTFGGGTKLELKRADAAPT TAAALEHHHHHC
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Table 4

[Protein] (uM)	[Protein] (mg/ml)	Log[protein] (uM)
28.2	0.8	1.45
14.1	0.4	1.15
7	0.2	0.85
3.5	0.1	0.54
1.76	0.05	0.25
0.88	0.025	-0.06

Table 5

	15min	30min	60min	90min	120min
28.2uM		J591scFvJWT – 96%	J591scFvJWT – 99%	J591scFvJWT – 99% J591scFv – 97% huJ591 – 96%	J591scFvJWT – 100% J591scFv – 99% huJ591 – 98%
14.1uM			J591scFvJWT – 95%	J591scFvJWT – 98%	J591scFvJWT – 99%
7uM				J591scFvJWT – 95%	J591scFvJWT – 99%
3.5uM					
1.76uM					
0.88uM					

CLAIMS

1. A biomolecular complex comprising a protein of interest and a labelling sequence capable of conjugation to a metal tricarbonyl $[M(CO)_3]^+$,
wherein the labelling sequence consists of between 6 and 15 amino acid residues
of which between 4 and 6 are histidine residues and
of which at least 2 residues are selected from the group consisting of lysine and/or arginine residues
and the labelling sequence has an isoelectric point (pI) of at least 9.
2. The biomolecular complex of claim 1, wherein the labelling sequence has a pI of at least 9.5.
3. The biomolecular complex of claim 1 or claim 2, wherein the labelling sequence consists of between 12 and 15 amino acid residues.
4. The biomolecular complex of any preceding claim wherein 6 histidine residues are present.
5. The biomolecular complex of claim 4, wherein the 6 histidine residues are arranged as two groups of three contiguous histidine residues, the two groups being separated by a single non-histidine residue.
6. The biomolecular complex of claim 4, wherein the 6 histidine residues are contiguous with one another.
7. The biomolecular complex of any preceding claim, wherein the histidine residues are located at an end of the labelling sequence.
8. The biomolecular complex of any of claims 1 to 6, wherein the histidine residues are embedded within the labelling sequence.
9. The biomolecular complex of any preceding claim, wherein the labelling sequence comprises at least 3 lysine residues.

10. The biomolecular complex of any preceding claim, wherein the labelling sequence comprises between 3 and 5 lysine residues.
11. The biomolecular complex of any preceding claim, wherein the labelling sequence comprises 3 or 4 lysine residues.
12. The biomolecular complex of any preceding claim, wherein 4 lysine residues are present.
13. The biomolecular complex of any preceding claim, wherein the labelling sequence comprises at least 2 arginine residues.
14. The biomolecular complex of any preceding claim, wherein the labelling sequence comprises between 2 and 5 arginine residues.
15. The biomolecular complex of any preceding claim, wherein the labelling sequence comprises between 2 and 4 arginine residues.
16. The biomolecular complex of any preceding claim, wherein 3 arginine residues are present.
17. The biomolecular complex of any preceding claim, wherein the labelling sequence does not contain aspartic acid residues.
18. The biomolecular complex of any preceding claim, wherein the labelling sequence does not contain glutamic acid residues.
19. The biomolecular complex of any preceding claim, wherein the labelling sequence does not contain aspartic acid or glutamic acid residues.
20. The biomolecular complex of any preceding claim, wherein the labelling sequence comprises a cysteine or methionine residue.
21. The biomolecular complex of claim 20, wherein the labelling sequence comprises a maximum of 1 cysteine residue.

22. The biomolecular complex of any preceding claim, wherein the labelling sequence does not contain cysteine.

23. The biomolecular complex of claim 1 selected from the group consisting of: HHHHHHALRRRLC; HHHHHHALRRRLM; CLRRRLAHHHHHH; MLRRRLAHHHHHH; HHHHHHALRRRLKC; HHHHHHALRRRLKM; CKLRRRLAHHHHHH; MKLRRRLAHHHHHH; CRRHHHHHHRRRC; MRRHHHHHHRRM; GRRHHHHHHRRG; HHHHHHRRRAARRC; HHHHHHRRRAARRM; CRRRAARRHHHHHH; MRRRAARRHHHHHH; HHHHHHALRRRL; LRRRLAHHHHHH; RCRGHHHHHHGRCR; RMRGHHHHHHGRMR; GGRHHHRHHHRGG; HHHHHHRGGGRC; HHHHHHRGGGRM; CRGGGRHHHHHH; MRGGGRHHHHHH; HHHHHHRARARC; HHHHHHRARARM; CRARARHHHHHH; MRARARHHHHHH; GKHHHHHHKKKG; HHHHHHRARAR; RARARHHHHHH; HHHHHHGRGGRC; HHHHHHGRGGRM; CRGGRGHHHHHH; MRGGRGHHHHHH; GKHHHHHHKKGC; GKHHHHHHKKGM; CGKHHHHHHKKG; MGKHHHHHHKKG; HHHHHHRRRAARR; RRAARRHHHHHH; HHHHHHRGGGR; RGGGRHHHHHH; HHHHHHKGGGK; KGGGKHHHHHH; HHHHHHKAKAK; and KAKAKHHHHHH .

24. The biomolecular complex of any preceding claim, comprising a protein of interest selected from the group consisting of: an antibody, or a fragment thereof; an enzyme; a substrate of an enzyme; a receptor or portion thereof; or a receptor ligand; a neurotransmitter; a hormone; an amyloid precursor; and albumin.

25. The biomolecular complex of claim 24, wherein the antibody, or antibody fragment, binds specifically to prostate specific membrane antigen (PSMA).

26. The biomolecular complex of claim 24, wherein the antibody is J591, or a derivative thereof, such as a humanised form of J591, or an scFv antibody derived from J591.

27. The biomolecular complex of any preceding claim, further comprising a metal tricarbonyl.

28. The biomolecular complex of claim 27, wherein the metal tricarbonyl is selected from the group consisting of: $[\text{Mo}(\text{CO})_3]$; $[\text{Cr}(\text{CO})_3]$; $[\text{W}(\text{CO})_3]$; $[\text{Tc}(\text{CO})_3]^+$; and $[\text{Re}(\text{CO})_3]^+$.

29. The biomolecular complex of any preceding claim, wherein the metal tricarbonyl comprises a radionuclide.

30. The biomolecular complex of claim 29, wherein the radionuclide is selected from the group consisting of: Tc-99; Tc-99m; and Tc-94m.
31. The biomolecular complex of claim 29, wherein the radionuclide is selected from the group consisting of: Re-188; and Re-186.
32. The biomolecular complex of claim 28, wherein the rhenium is selected from the group consisting of: Re-185; and Re-187.
33. A nucleic acid encoding a biomolecular complex according to any one of claims 1 to 26.
34. A method of manufacturing a biomolecular complex according to any one of claims 1 to 26, the method comprising expressing a nucleic acid according to claim 33 to yield a biomolecular complex.
35. A method of manufacturing a biomolecular complex according to any one of claims 27 to 32, the method comprising expressing nucleic acid according to claim 33 to yield a biomolecular complex, and conjugating the biomolecular complex with a metal tricarbonyl.
36. A method according to claim 35, wherein the metal tricarbonyl is as defined in any of claims 28 to 32.
37. A method according to claim 35 or claim 36, wherein the concentration of the biomolecular complex at conjugation is between approximately 5 μ M and approximately 30 μ M.
38. A method according to claim 37, wherein the concentration of the biomolecular complex at conjugation is selected from the group consisting of: approximately 28 μ M; approximately 14 μ M; and approximately 7 μ M.
39. A method according to any of claims 34 to 38, wherein the concentration of the biomolecular complex at conjugation is below approximately 15 μ M.

40. A method according to any of claims 34 to 39, wherein the conjugation is conducted over a period of between about 25 and 130 minutes.
41. A method according to claim 40, wherein the conjugation is conducted over a period of between about 30 and 120 minutes.
42. A method according to any of claims 34 to 41, wherein the conjugation is conducted over a period of less than 90 minutes.
43. A method according to any of claims 34 to 42, wherein the conjugation is conducted over a period of 60 minutes or less.
44. A method according to claim 43, wherein the conjugation is conducted over a period of approximately 30 minutes.
45. A method according to any of claims 34 to 44, wherein the conjugation is performed at approximately room temperature.
46. A biomolecular complex according to any of claims 27 to 32, for use as a medicament in targeted radionuclide therapy.
47. A method of treatment, the method comprising providing a therapeutically effective amount of a biomolecular complex according to any of claims 27 to 32 to a subject in need thereof.
48. A method of molecular imaging, the method comprising providing a biomolecular complex according to any of claims 27 to 32, and determining the location of the conjugated metal tricarbonyl within the subject, wherein the location of the conjugated metal tricarbonyl within the subject is indicative of the location of a binding partner of the protein of interest.
49. The use of a biomolecular complex according to any of claims 1 to 32 in a method of screening to identify proteins of interest capable of interacting with a desired binding partner.
50. The use of a biomolecular complex according to any of claims 1 to 32 in a method of screening proteins of interest selected for their ability to interact with a desired binding partner, in order to identify proteins of interest having favourable binding characteristics.

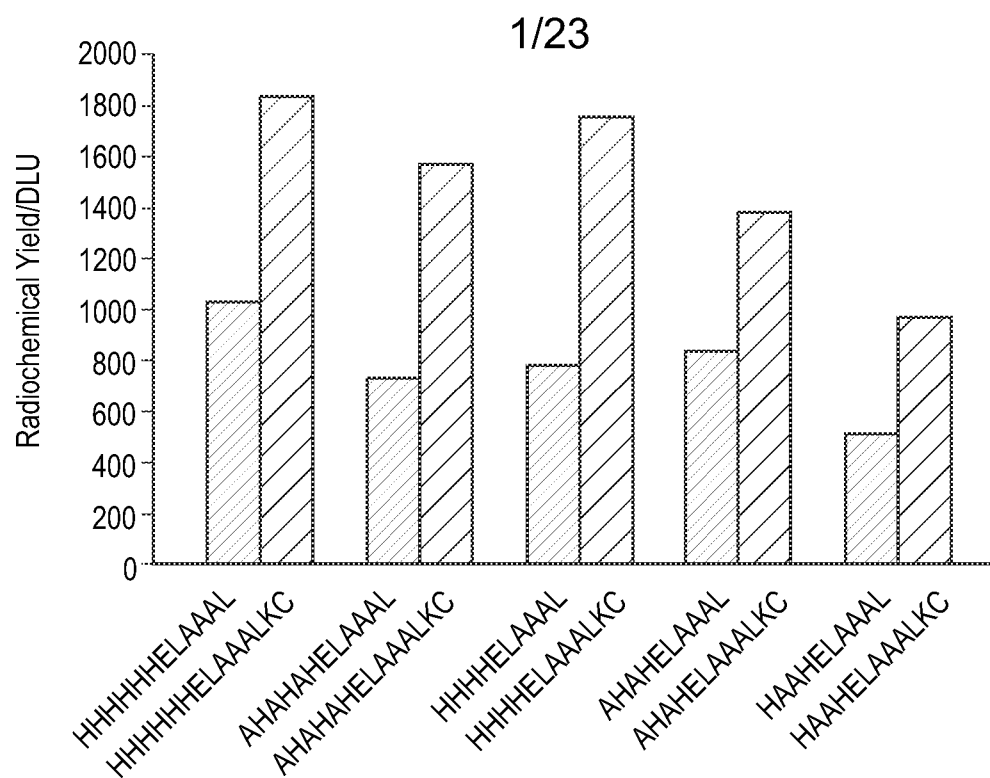


Fig. 1

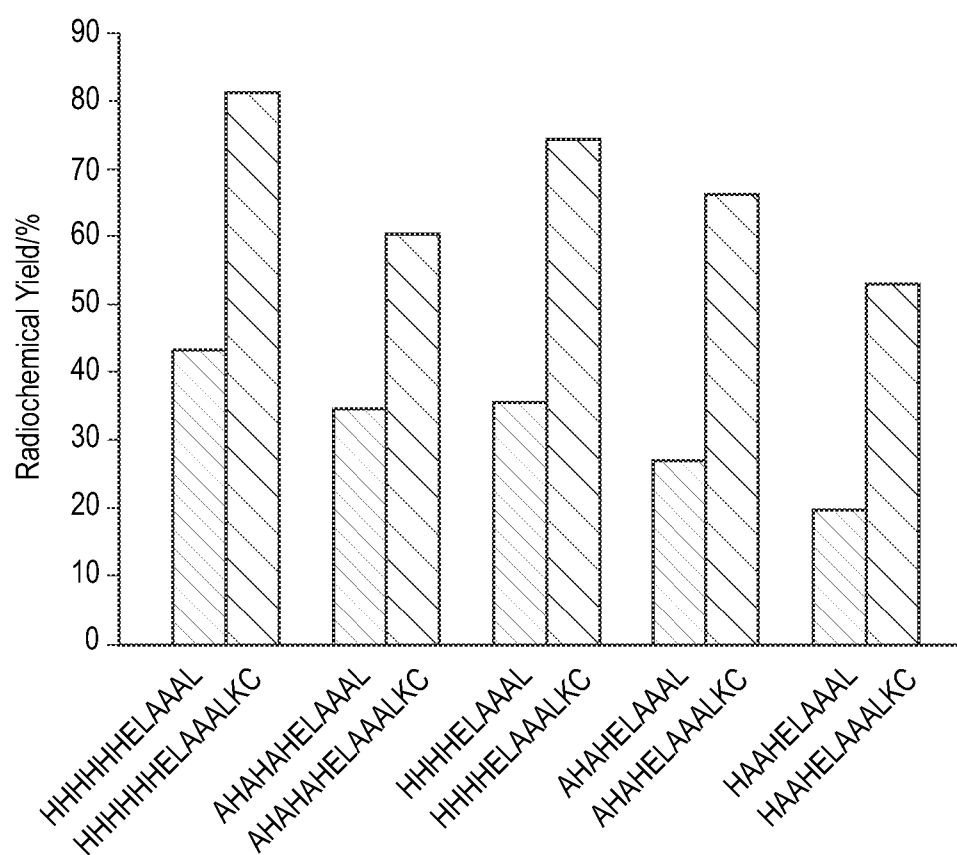


Fig. 2

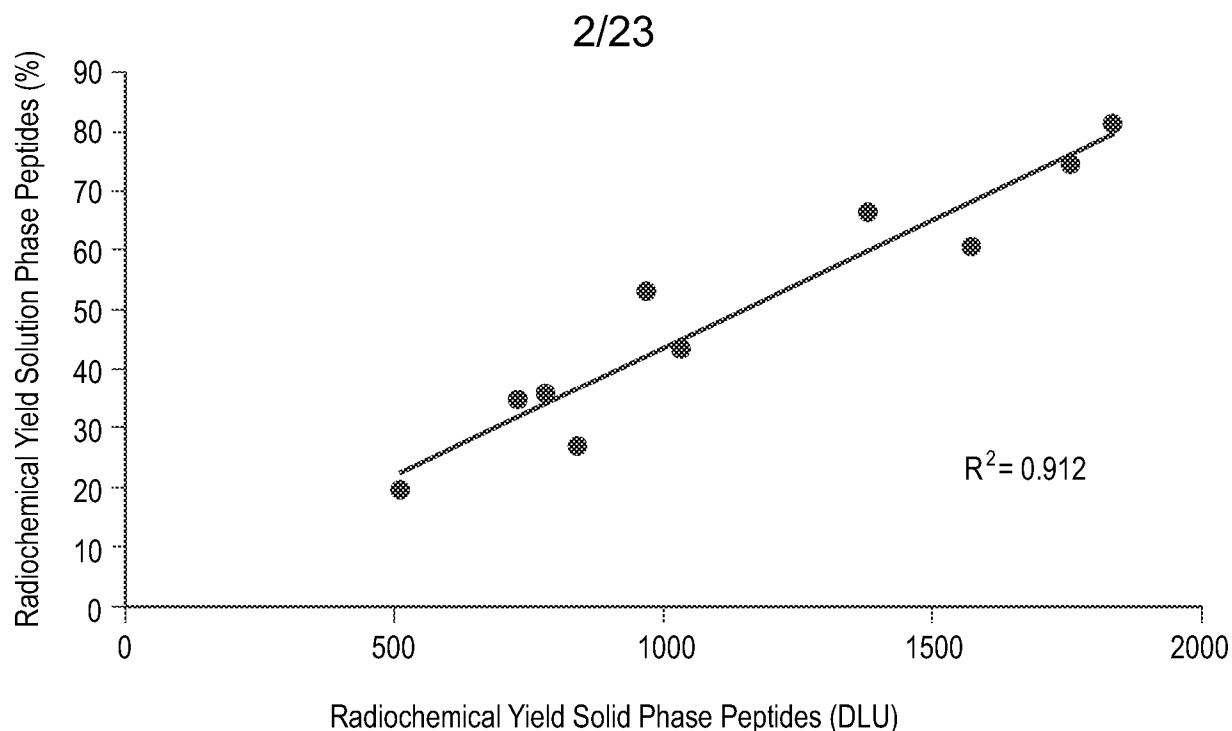


Fig. 3

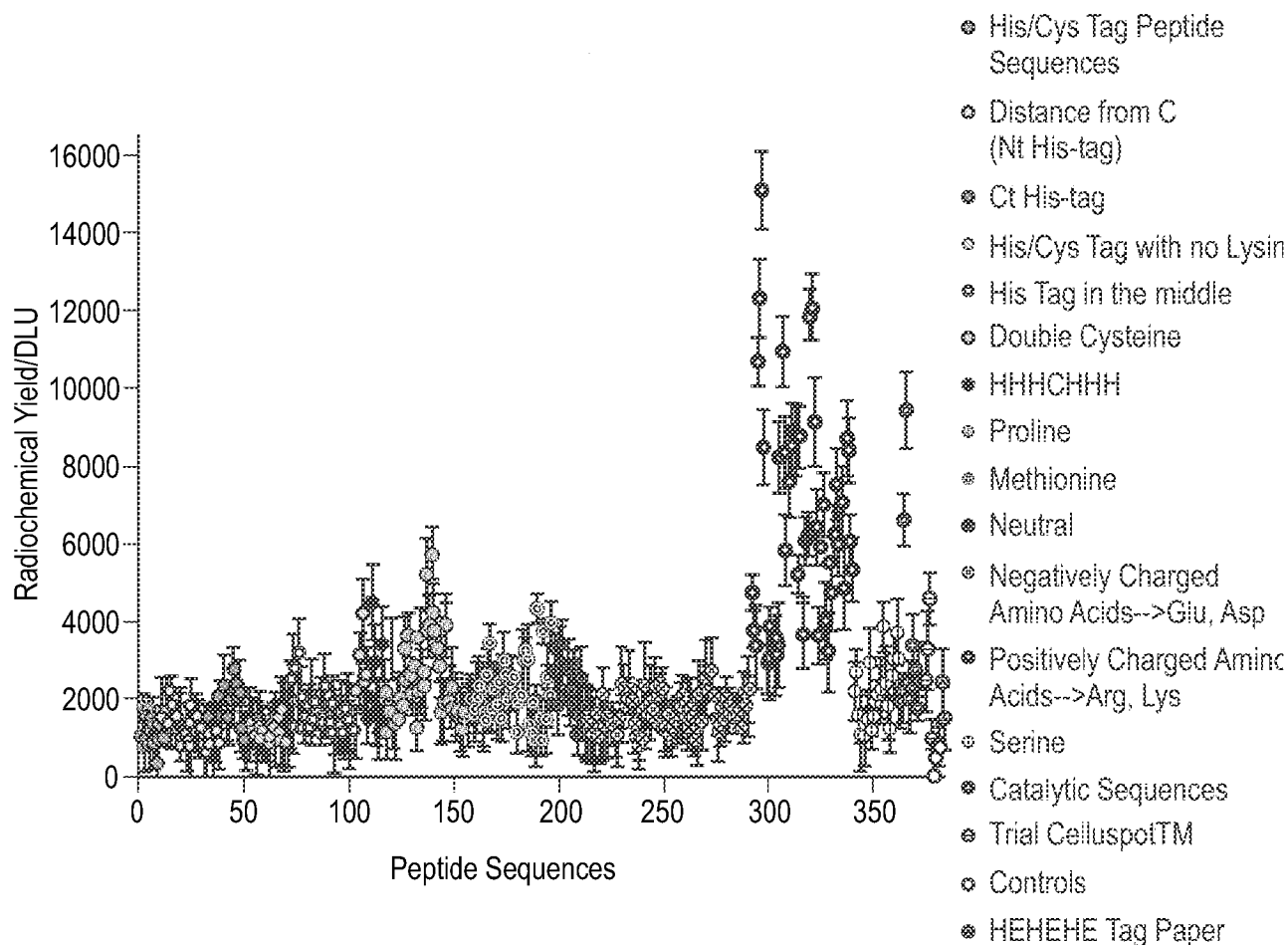


Fig. 4

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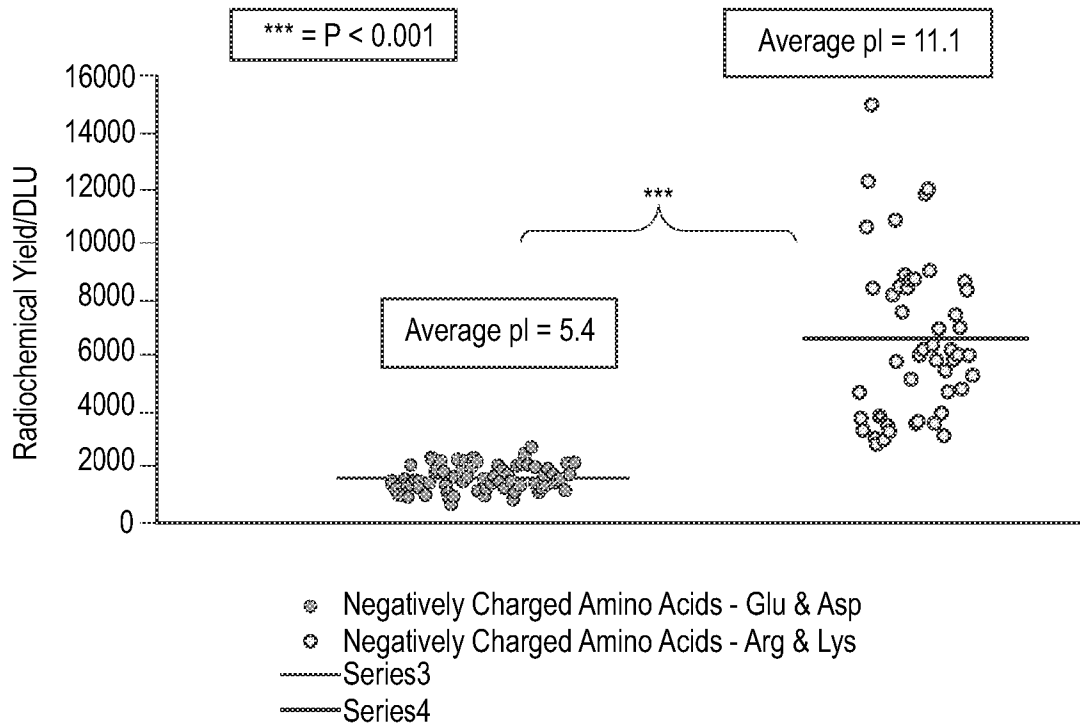


Fig. 5

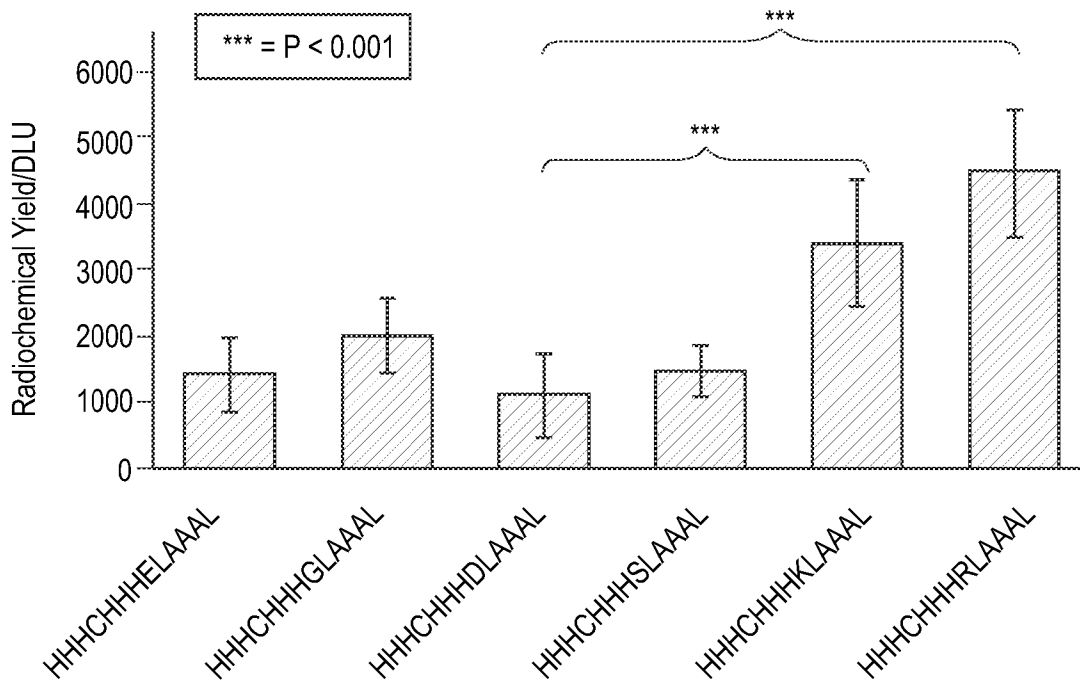


Fig. 6

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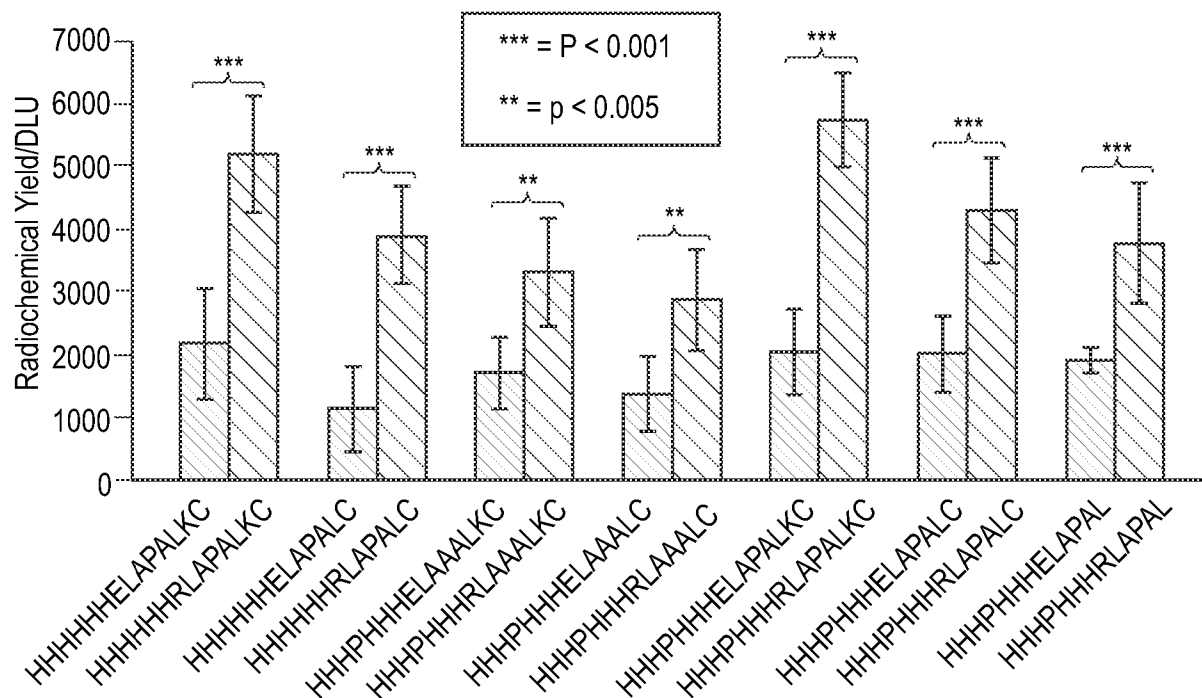


Fig. 7

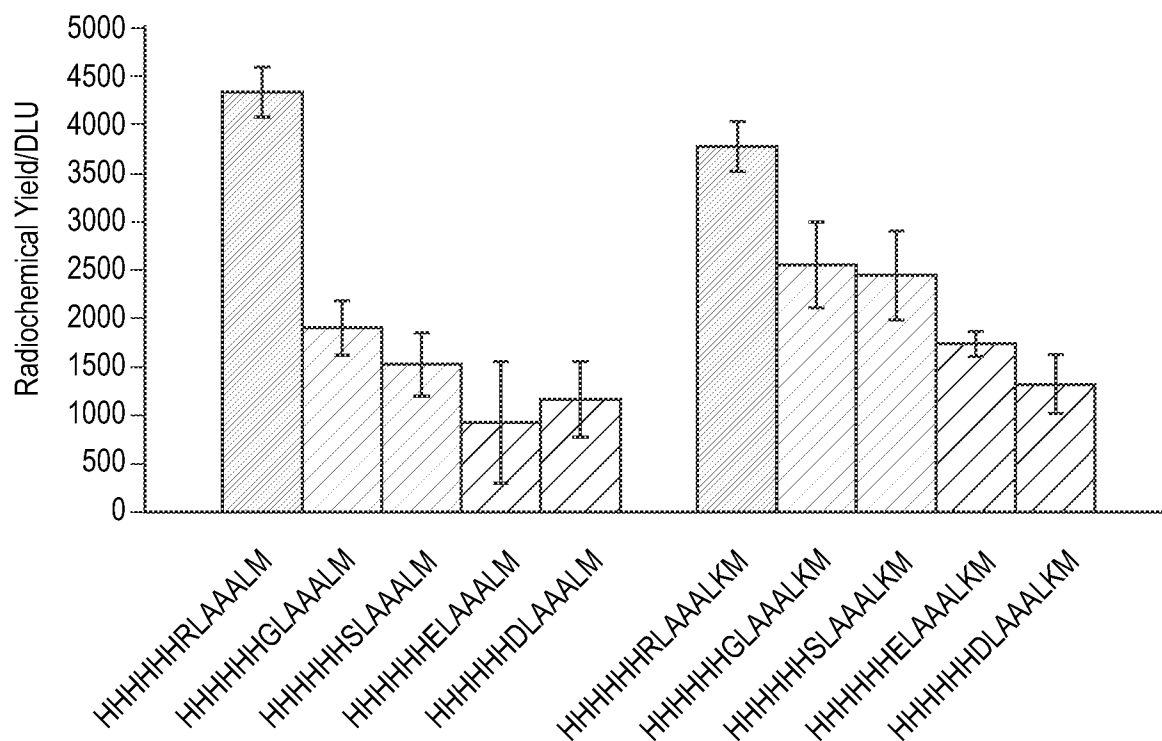


Fig. 8

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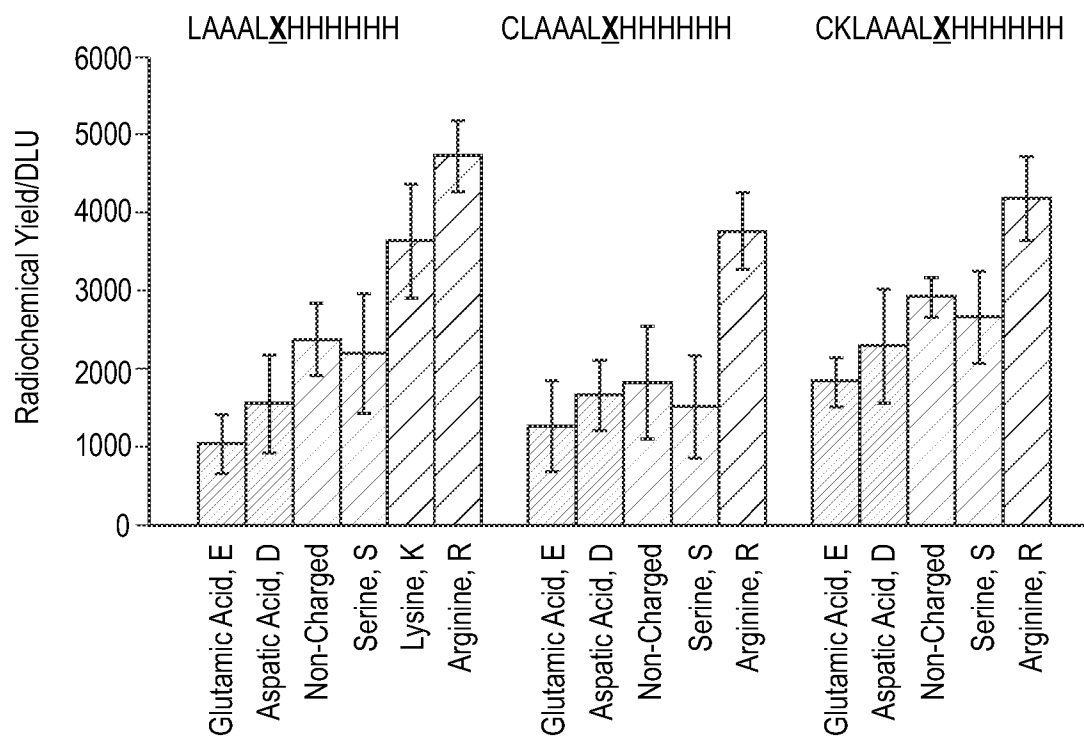


Fig. 9

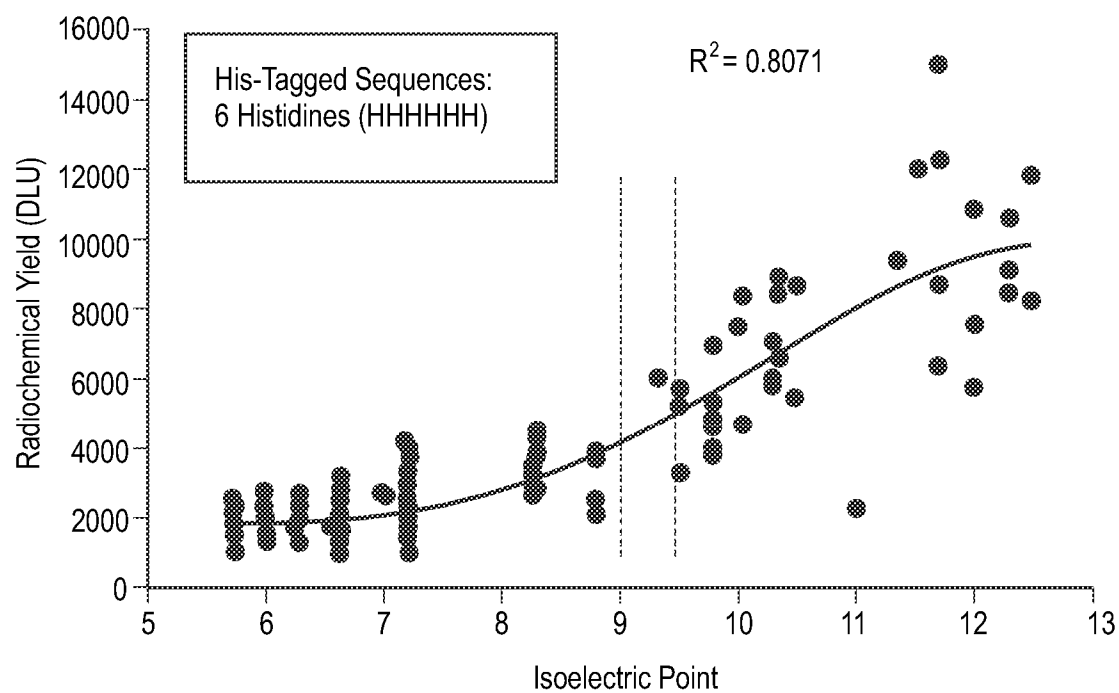


Fig. 10

6/23

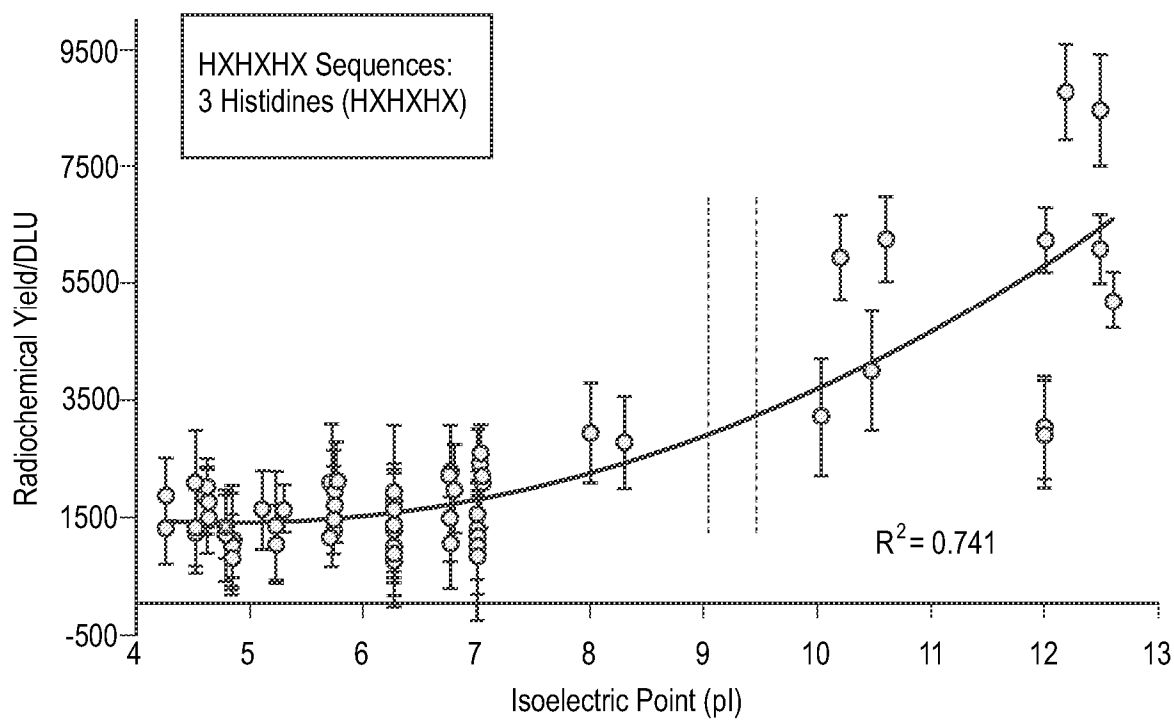


Fig. 11

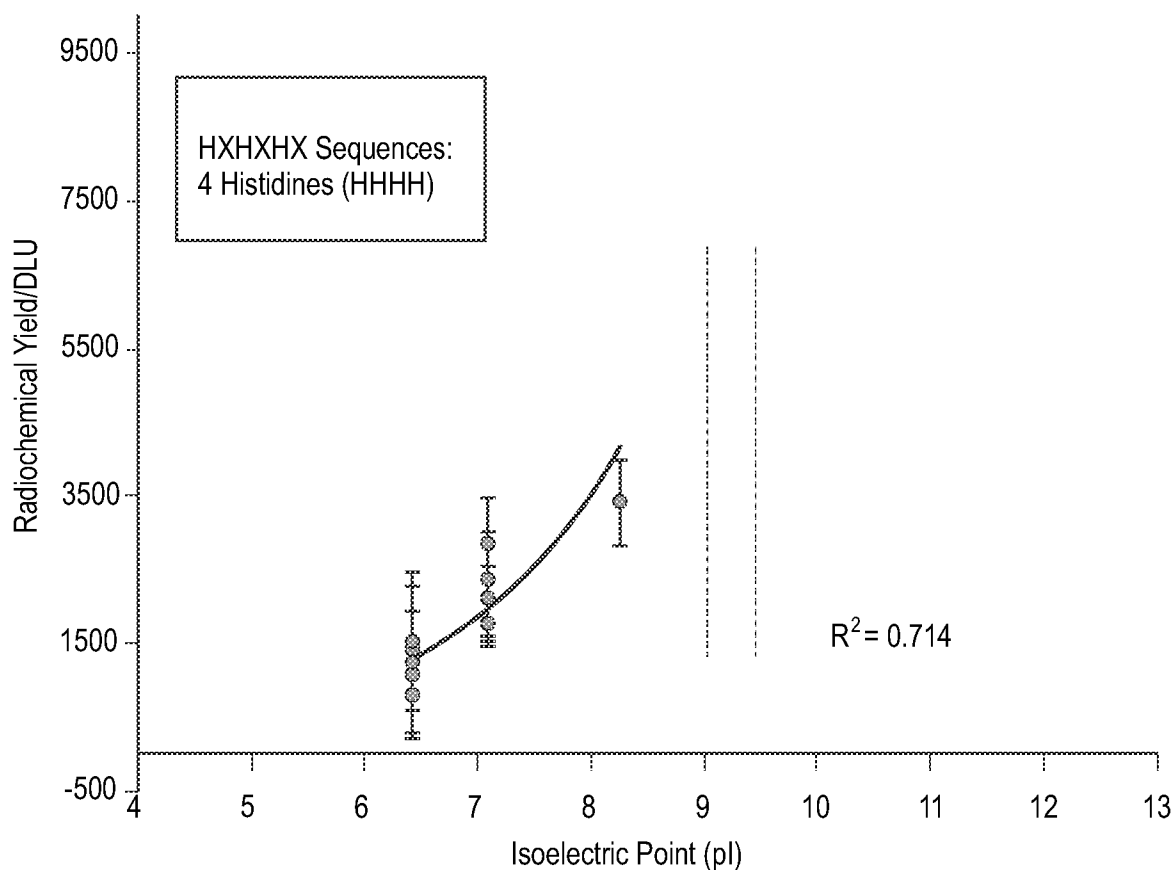


Fig. 12

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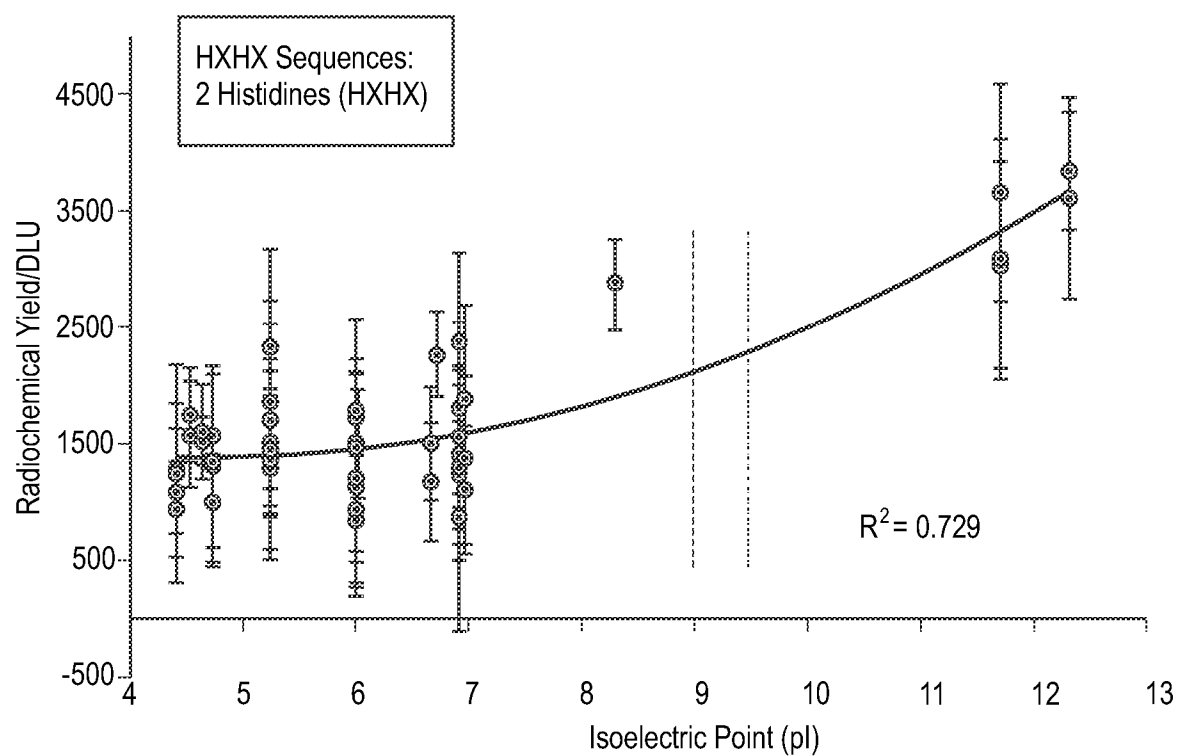


Fig. 13

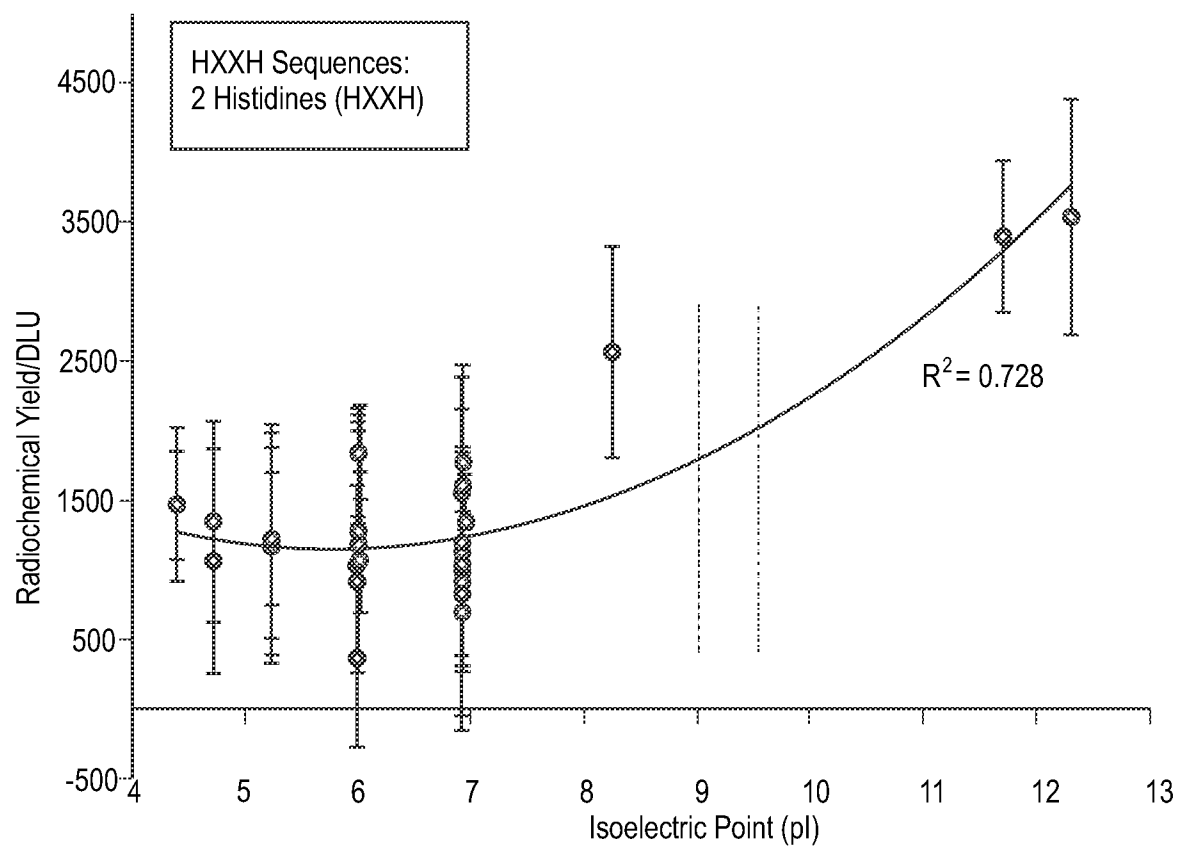


Fig. 14

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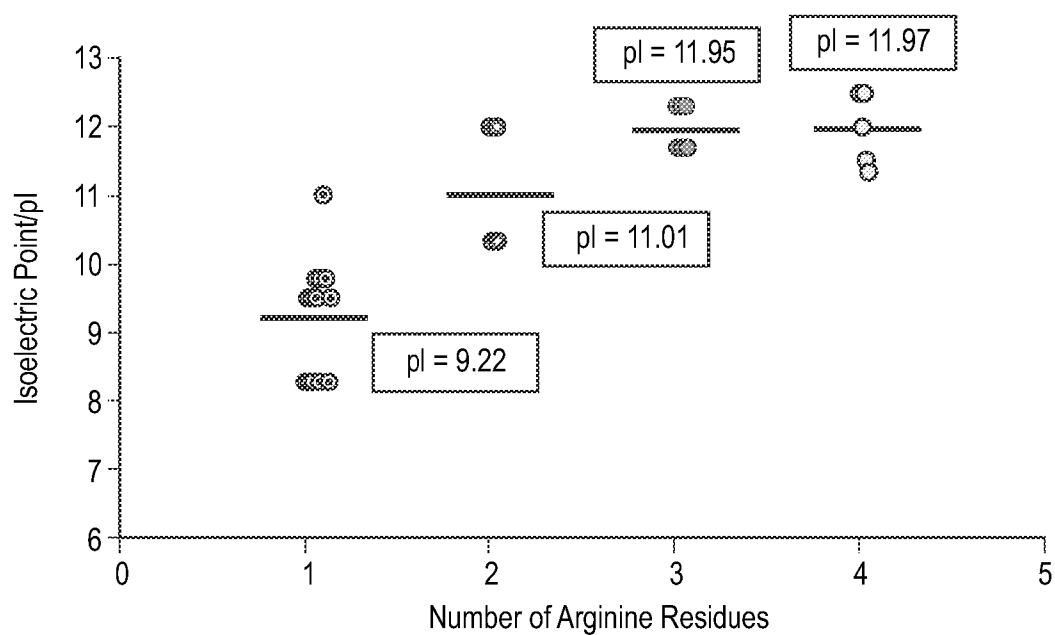
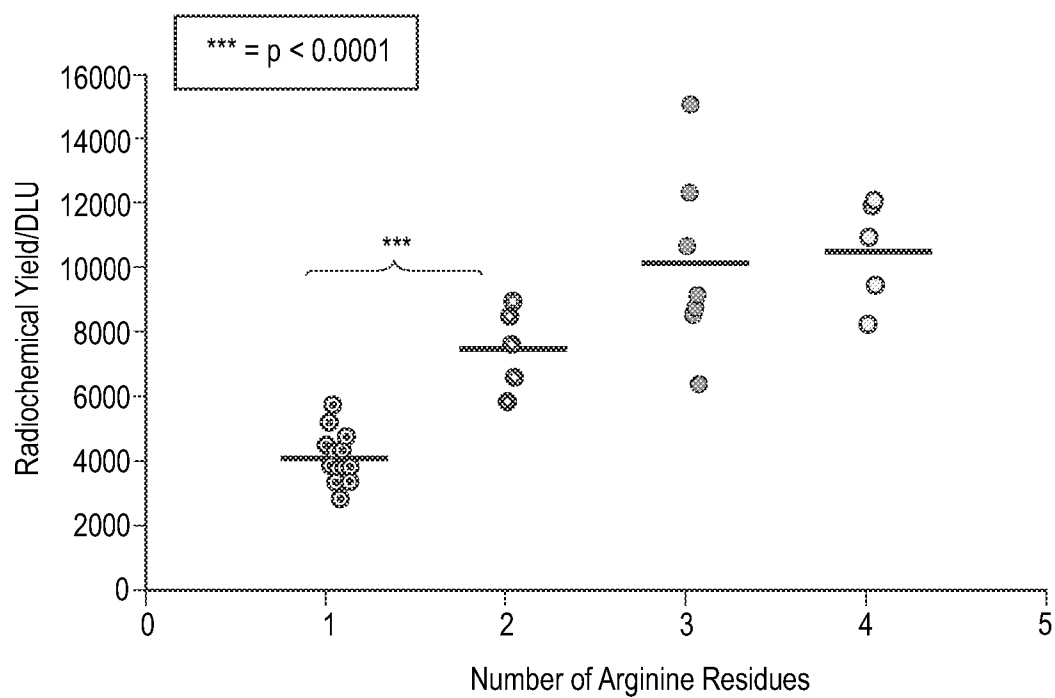


Fig. 15

9/23

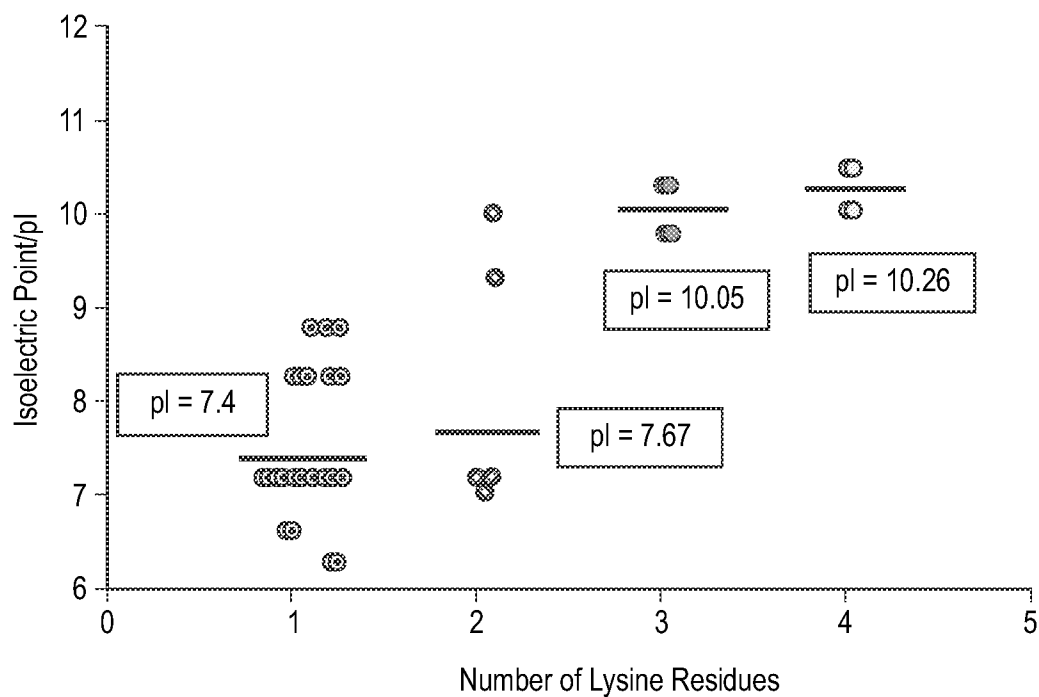
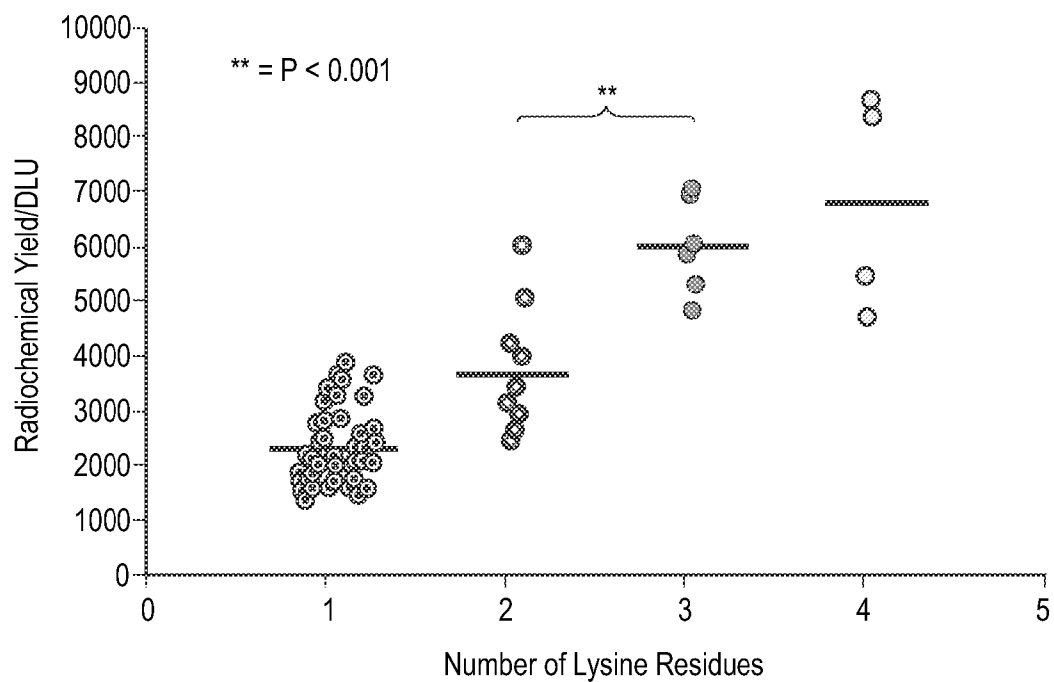


Fig. 16

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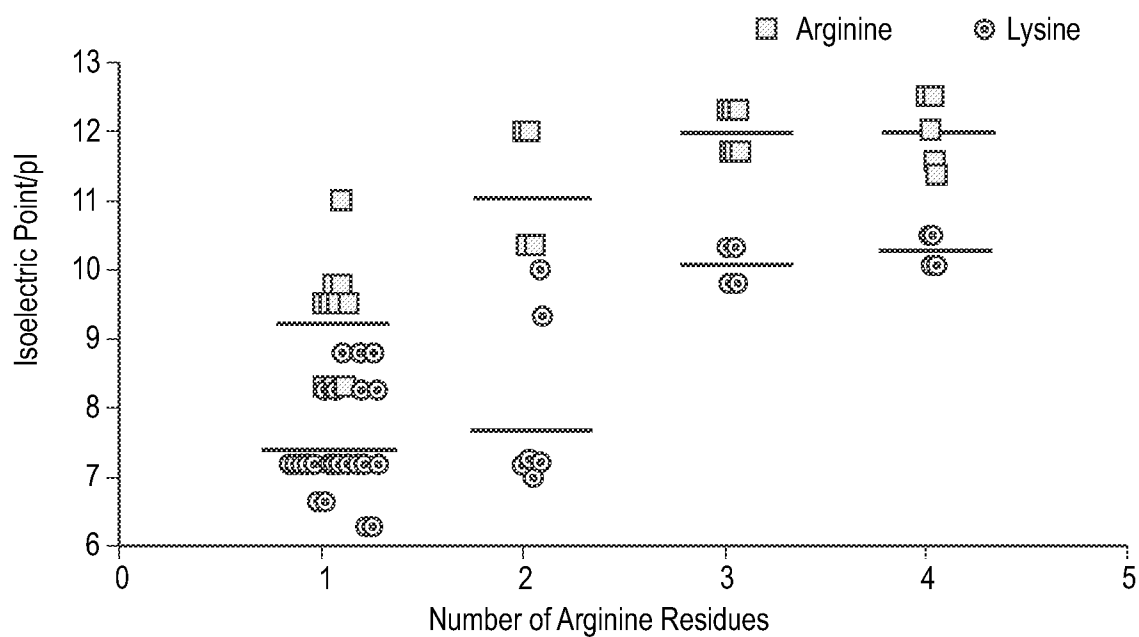
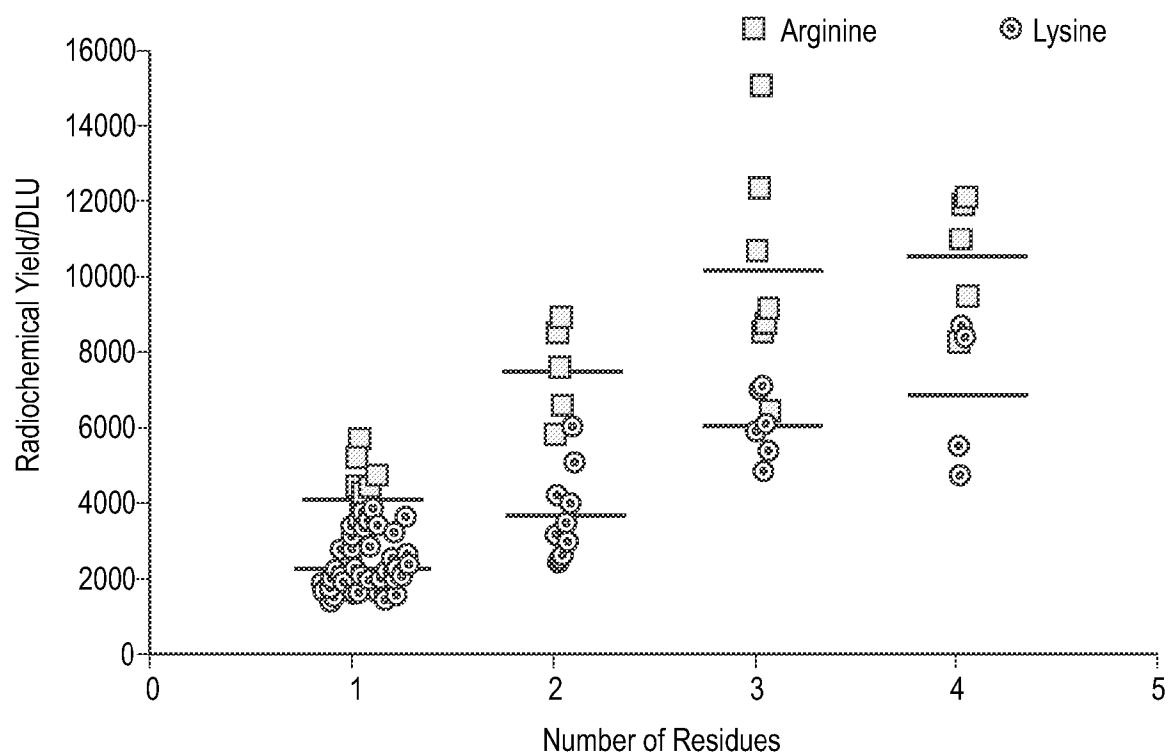


Fig. 17

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● HHHHHHXXRRR ● HHHHHHXRXR ⊙ HHHHHHXRXR ⊙ HHHHHHXRXRXR
 ⊙ HHHHHHRXRXR ⊙ HHHHHHRRXXRR ⊙ RHRHRHXXX ● RHRHXXXXX
 ⊙ RHRRHXXXXX ⊙ XRRHHHHHRRX ⊙ XRRHHHRHHHRRX ○ RXRXHHHHHHXRXR

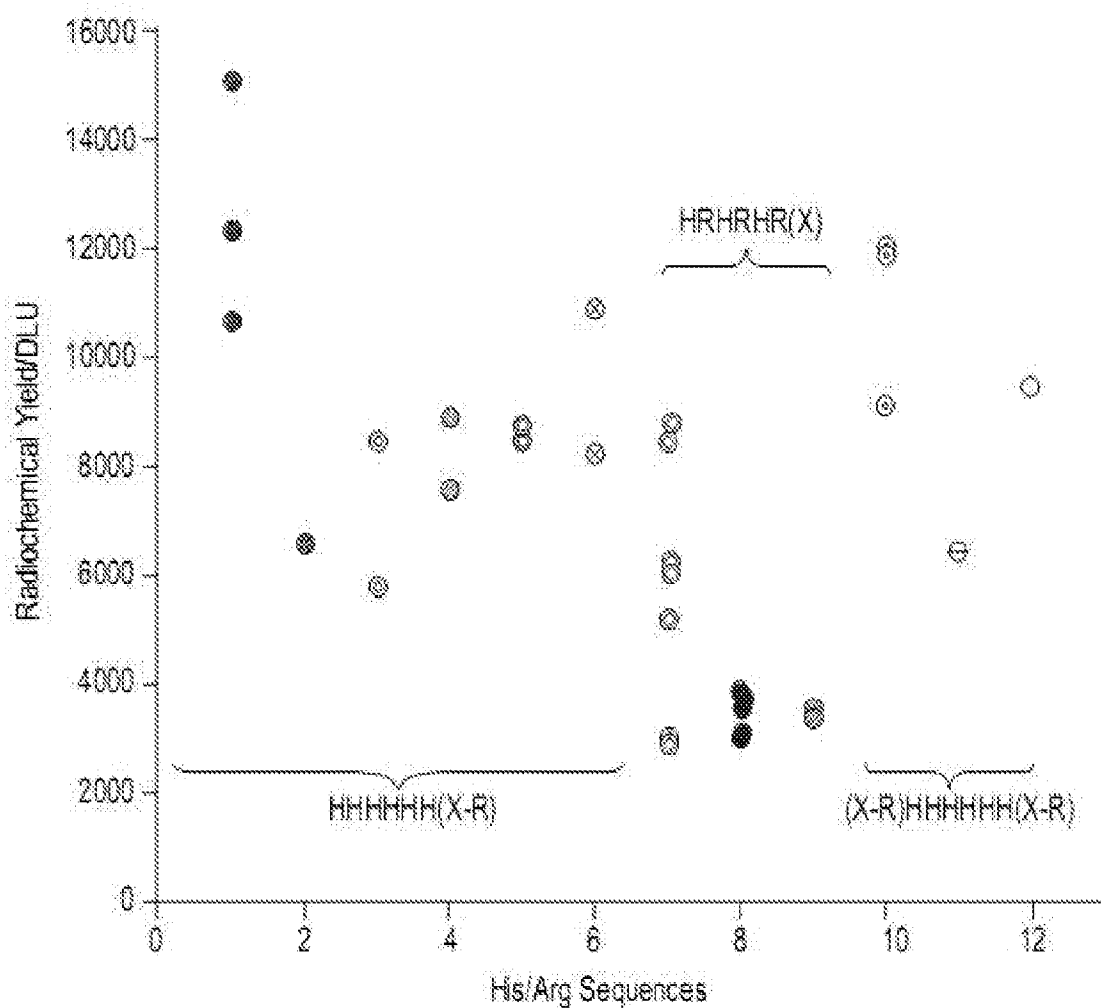


Fig. 18

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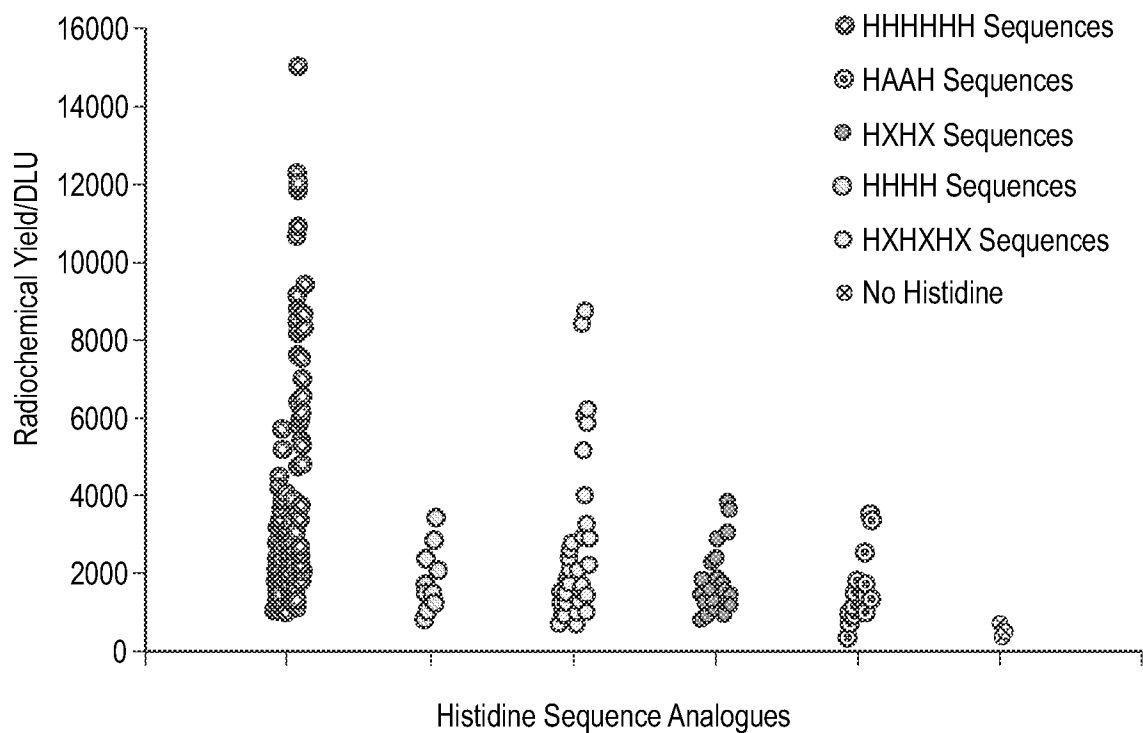
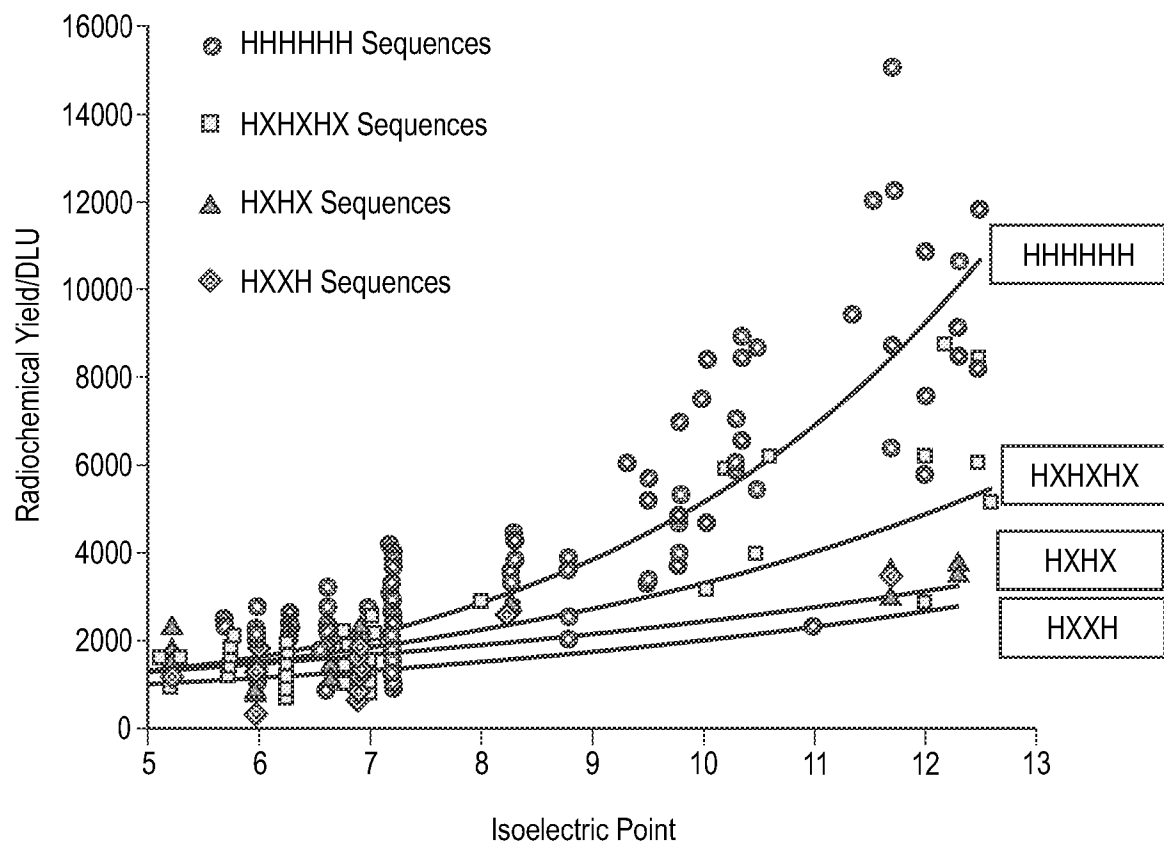


Fig. 19

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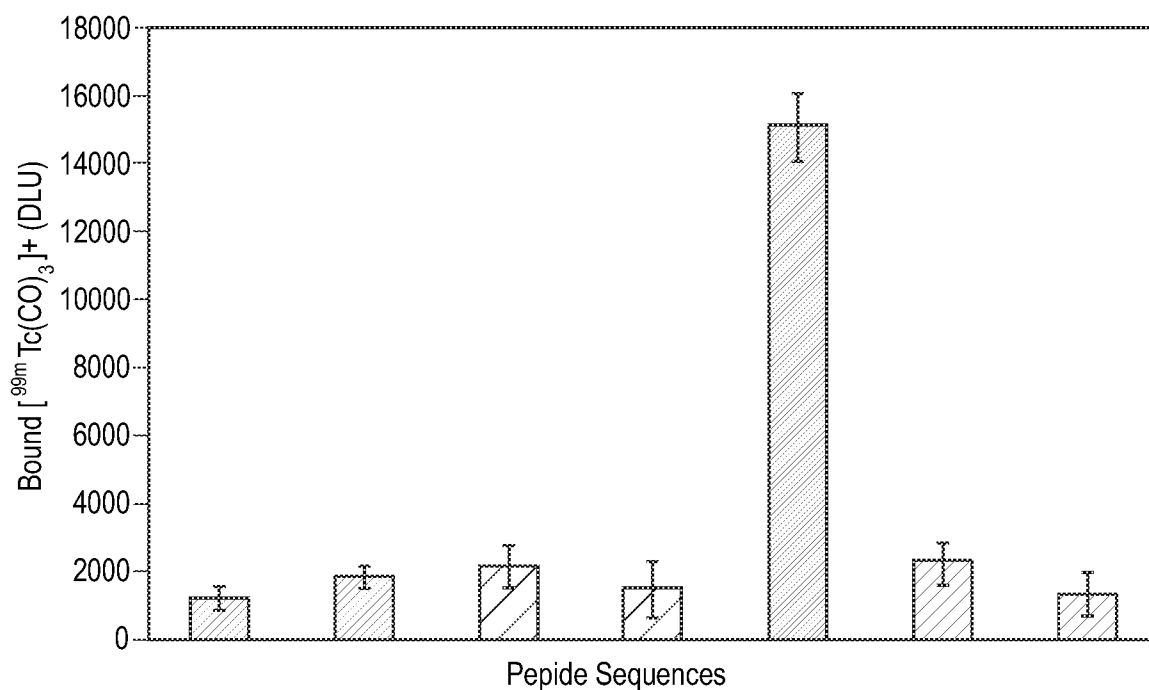


Fig. 20

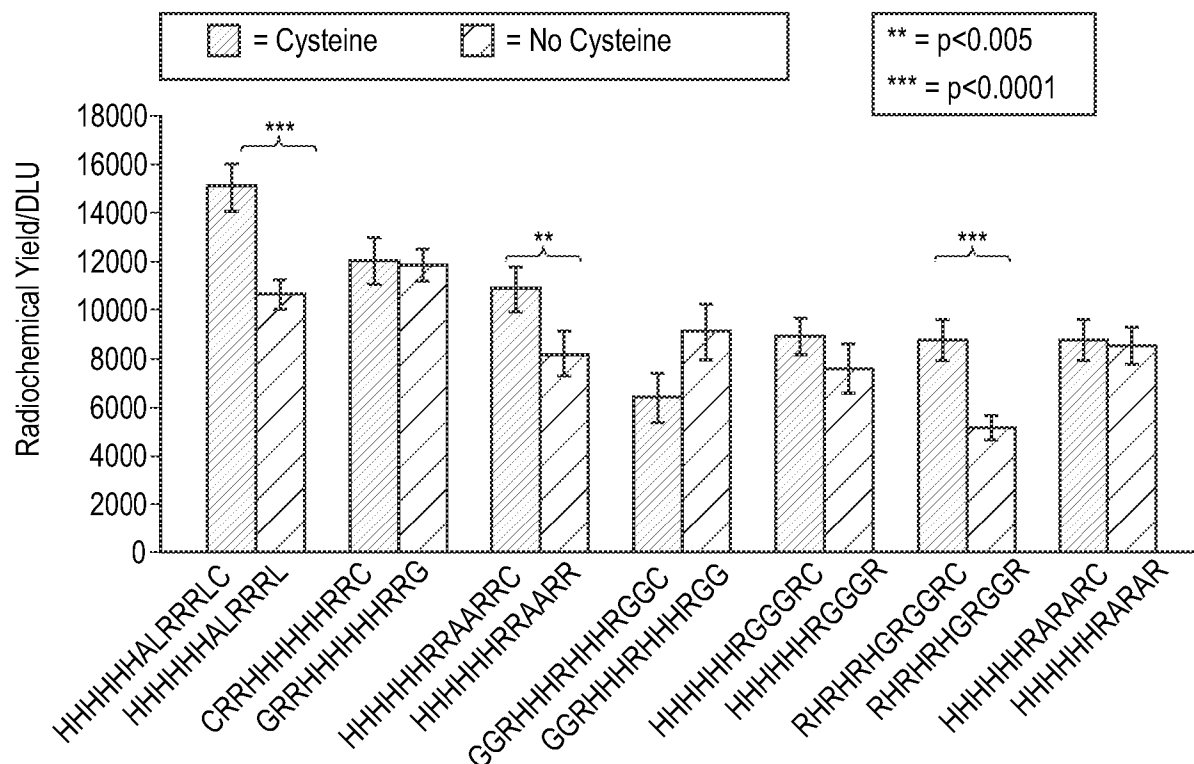


Fig. 21

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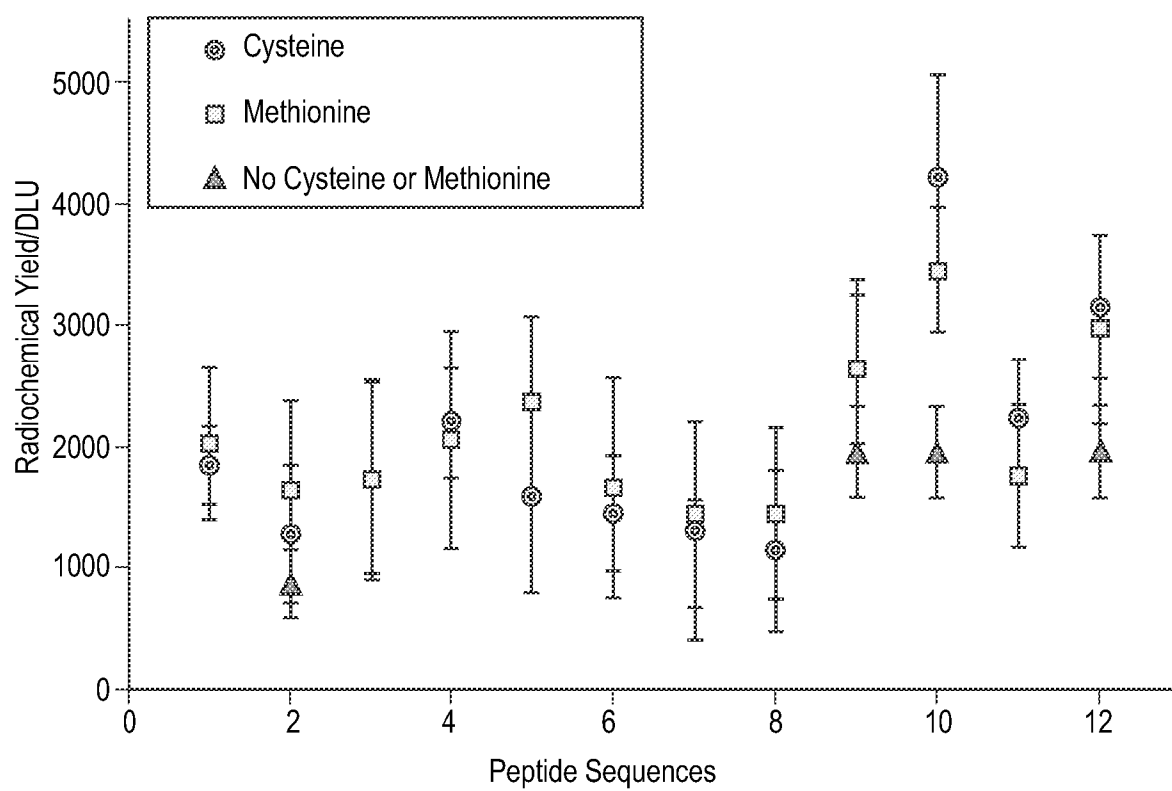


Fig. 22

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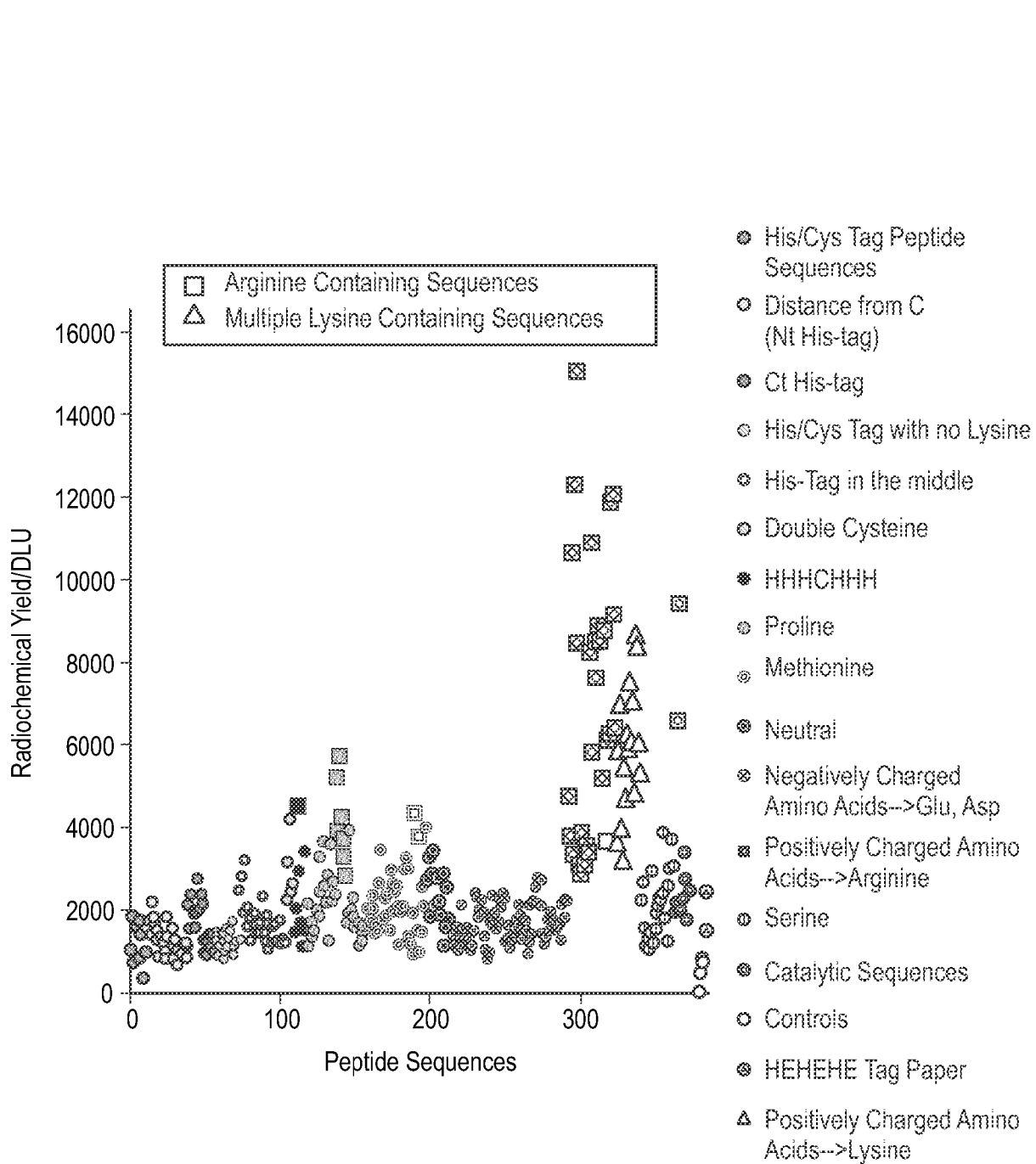


Fig. 23

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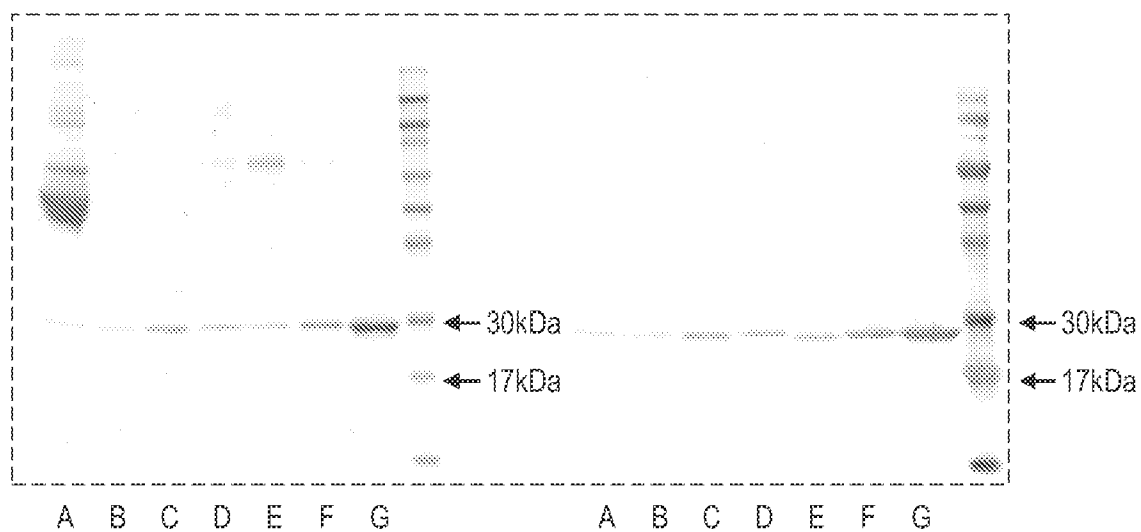


Fig. 24a

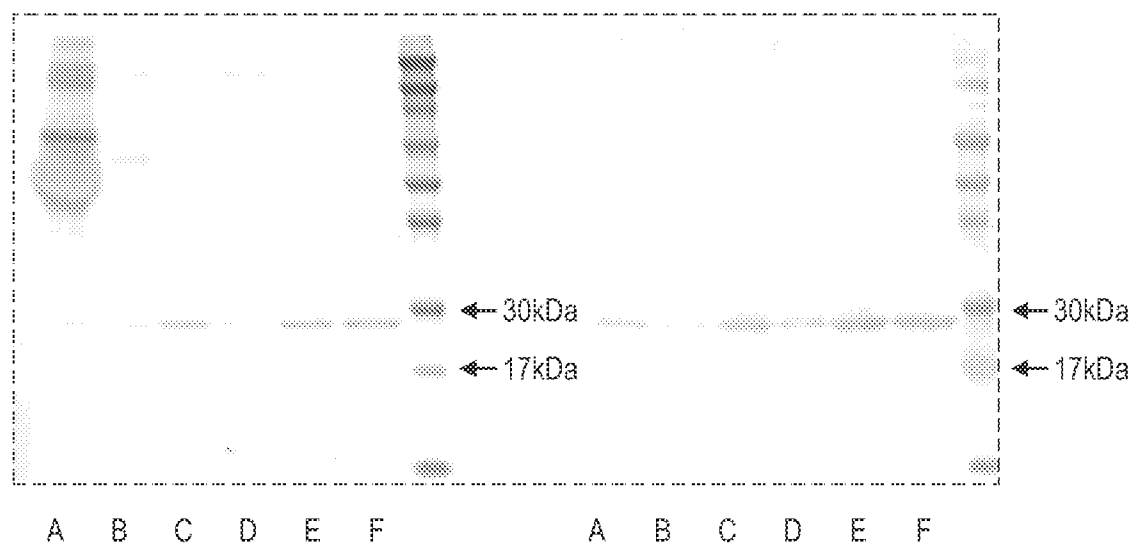


Fig. 24b

17/23

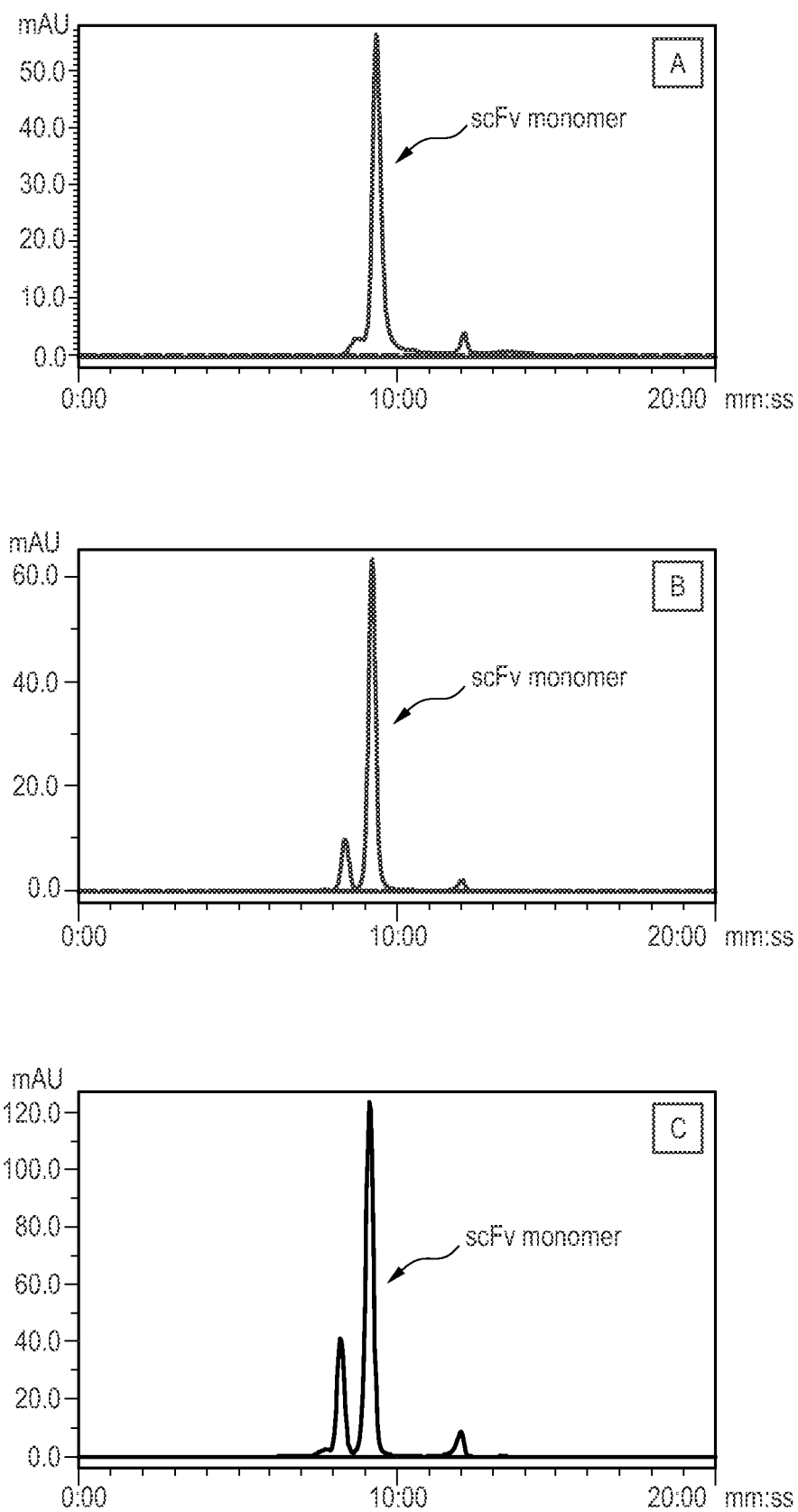


Fig. 25

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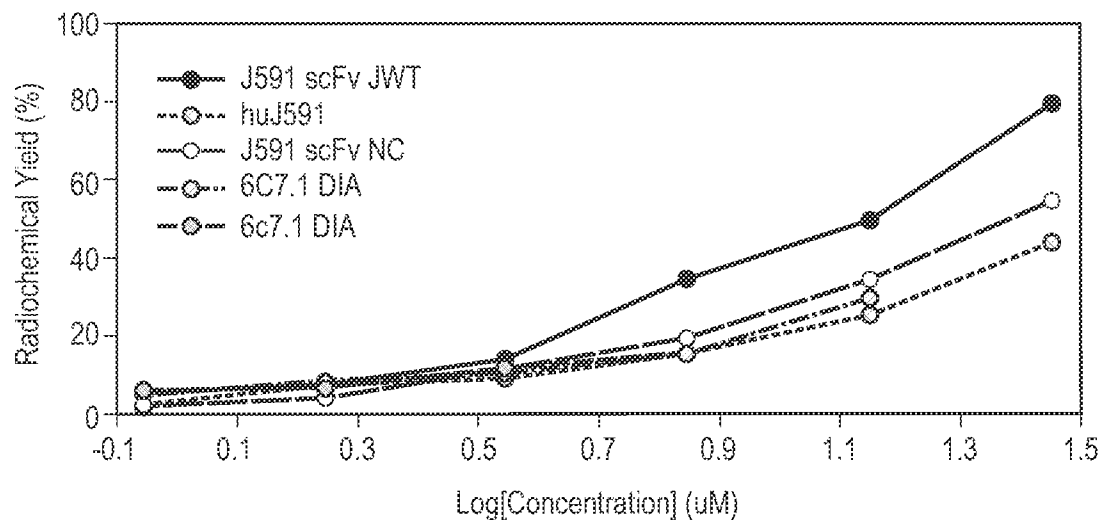


Fig. 26a

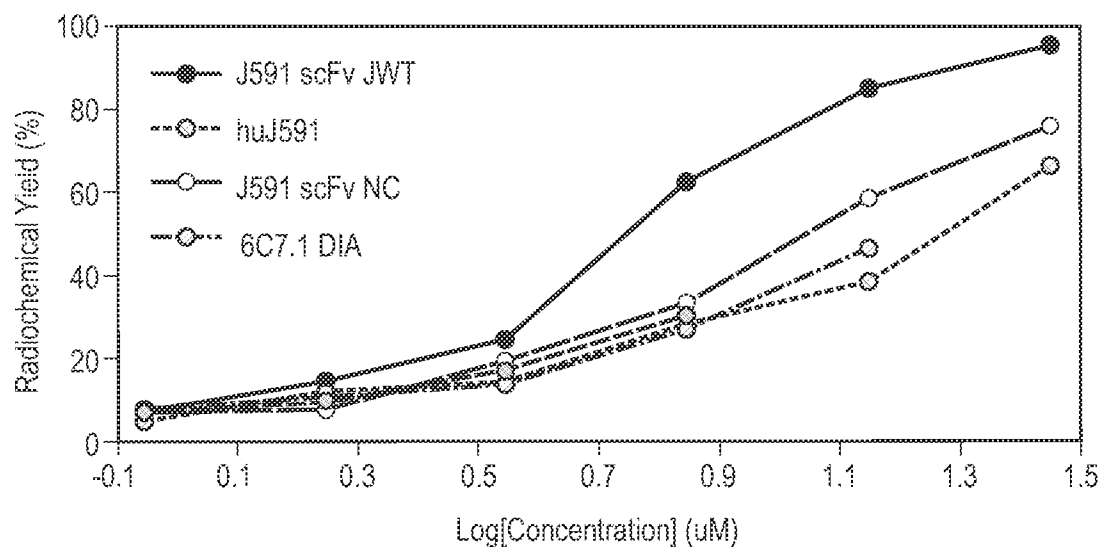


Fig. 26b

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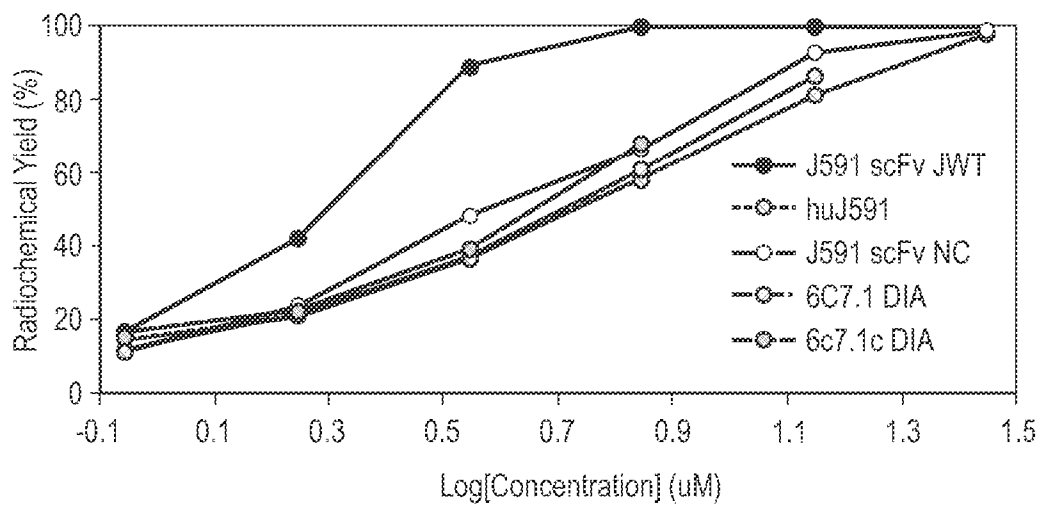


Fig. 26c

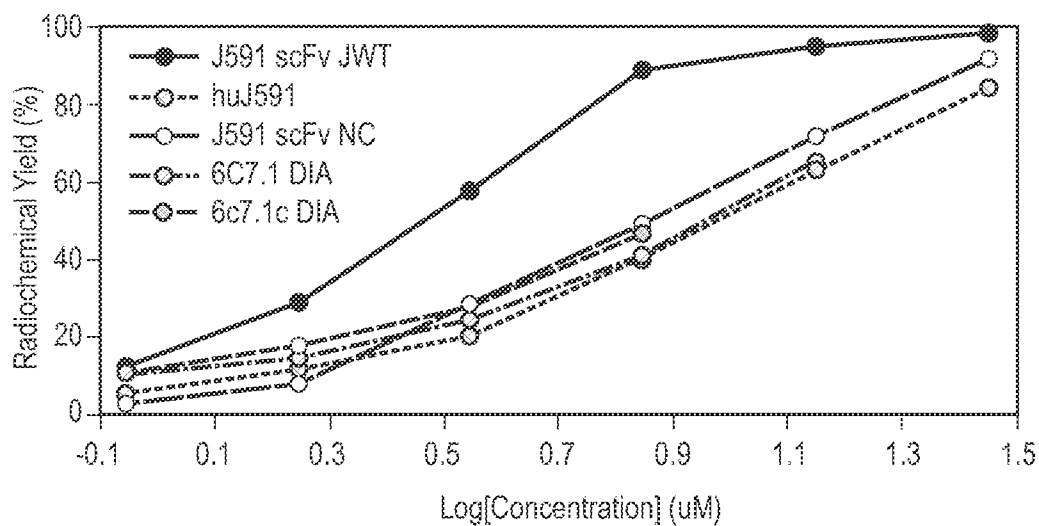


Fig. 26d

20/23

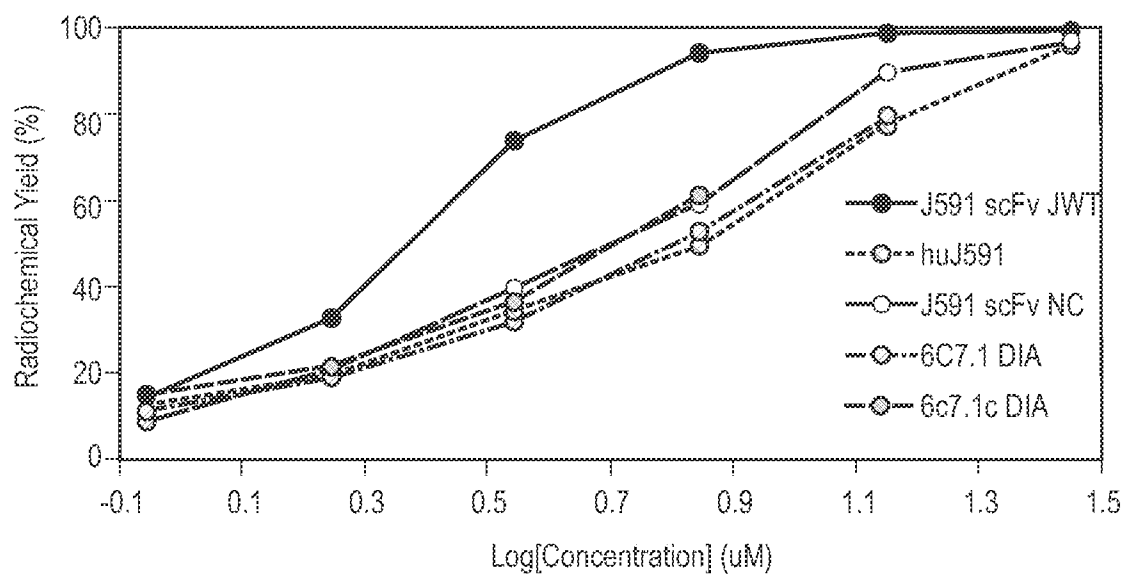


Fig. 26e

21/23

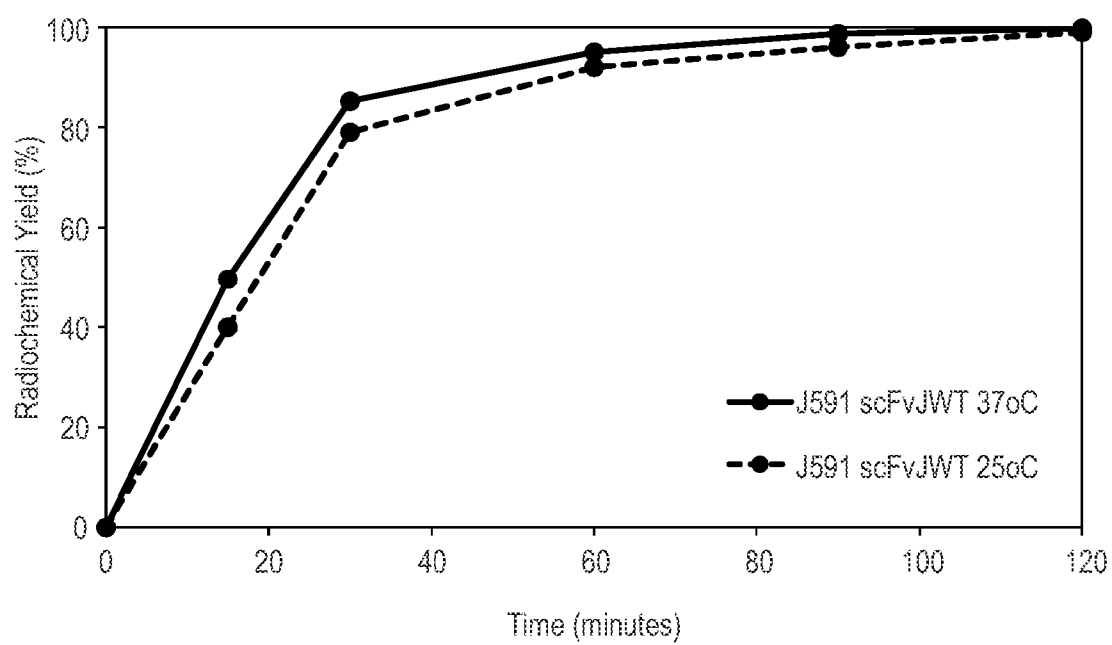


Fig. 27

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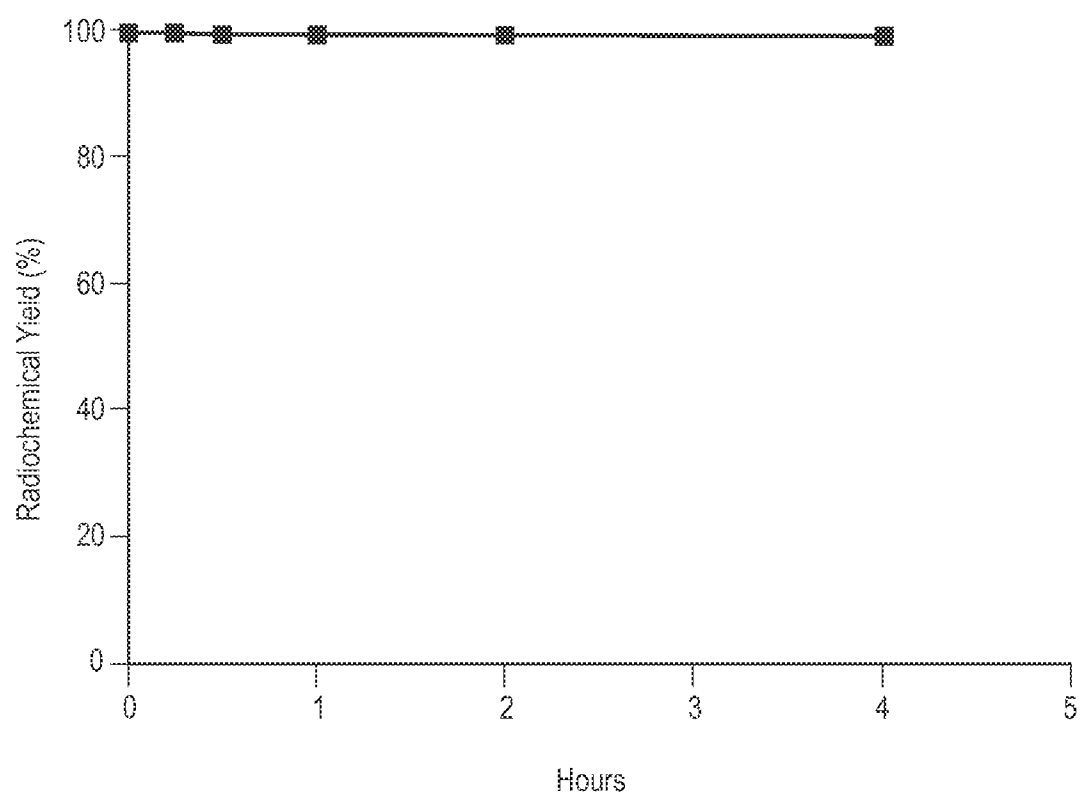


Fig. 28

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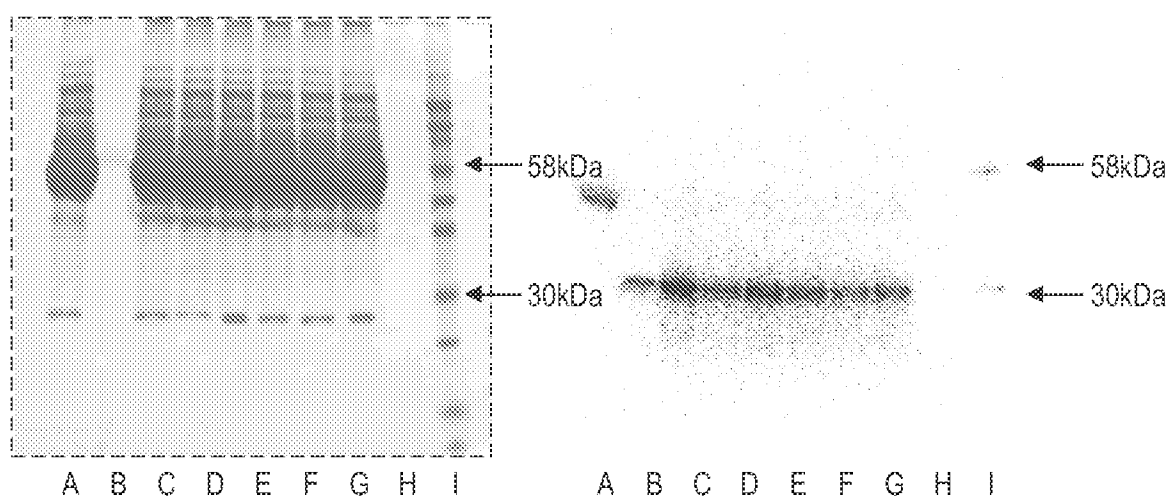


Fig. 29

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2013/052191

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K51/10

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of Box C.



See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

4 November 2013

Date of mailing of the international search report

19/11/2013

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International application No

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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