Synthesis of carbon-14–labelled peptides

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Carbon-14 (14C)–labelled active pharmaceutical ingredients (APIs) and investigational medicinal products (IMPs) are required for phase 0/I to phase III mass balance and micro-dosing clinical trials. In some cases, this may involve the synthesis of 14C-labelled peptides, and the analysis can be performed by accelerated mass spectrometry (AMS). The 14C-peptide is typically prepared by the solid-phase peptide synthesis (SPPS) approach using custom-made glassware for the key coupling steps. Further modification of the purified 14C-peptide can then be performed.

KEYWORDS
accelerated mass spectrometry, biomolecules, carbon-14 peptides, solid-phase peptide synthesis

1 | INTRODUCTION

The application of biomolecules in drug delivery systems is a major growth area within the pharmaceutical and biotechnology sectors. An important aspect of these drug delivery systems is active pharmaceutical ingredients (APIs) based on peptides. Currently, therapeutic peptides are being developed as potential new treatments in oncology.1 A critical component of the development of any drug is an assessment of its ADME (absorption, distribution, metabolism, and excretion) profile, most commonly implemented by using a 14C-labelled version of the parent drug.2

In order to achieve the peptide labelling, there are a number of other radioisotopes that can be used, such as tritium and iodine-125.3 Labelling with iodine-125 normally involves incorporation of iodine-125 into tyrosine or histidine residues within the peptide or protein.4 However, the benefit of using 14C for ADME programmes is that the label is placed within the core of the drug. This approach reduces the risk of “wash out” or need to use a modified structure. One limitation of 14C labelling is with the low maximum specific activity of 62.4 mCi/mmol (theoretical maximum) compared with tritium that is 28.6 Ci/mmol. Practice, the maximum specific activity of 14C-labelled compounds is between 50 and 60 mCi/mmol. This limitation regarding 14C-labelled compounds becomes significant as the molecular weight of the molecule increases. As with tritium, the specific activity can be increased by incorporating several 14C-labelled amino acids into the peptide sequence. The specific activity can be adjusted by diluting with unlabelled material. Also, this limitation of using 14C can be overcome through the use of accelerated mass spectrometry (AMS) during metabolite studies.5

The methodology to the synthesis of a 14C-labelled peptide is shown in Figure 1.

Stage 1. Involves the synthesis of the peptide using amino acid residues as far as the step prior to the introduction of the 14C-labelled amino acid. This typically involves augmentation of the amino acids, by solid-phase peptide synthesis (SPPS), within a peptide synthesiser.

Stage 2. Incorporation of the 14C-amino acid residue. The most cost-effective strategy is to insert the 14C-labelled amino acid at the terminal of the peptide. However, in most cases, the unlabelled amino acids are generally added after the 14C-labelled amino acid. The most common amino acids to target for introduction of the 14C-label are glycine, alanine, and valine. These amino acids can be incorporated into the peptide with specific activities up to a maximum of 50 to 60 mCi/mol per 14C-label. In addition, the specific activity of
the peptide can be further increased by incorporating several $^{14}$C-amino acids. The coupling of the $^{14}$C-labelled amino acid to the resin-bound peptide is performed using custom-made glassware designed to maximise coupling efficiency without damaging the resin support.

Stage 3: Cleavage of the crude $^{14}$C-peptide from the resin support, followed by a purification method, such as reversed phase chromatography (RP-HPLC). This stage enables a range of analytical methods to confirm identity, radiochemical purity, and stability.

Stage 4: Further functionalisation of the $^{14}$C-peptide. For example, by PEGylation, BIOTINylation, or conjugation to other biomolecules.

The manufacture of peptides labelled with carbon-14 provides a number of challenges, as demonstrated by the following two case studies: the first involving the preparation of a $^{14}$C-labelled BIOTINylated 84-mer (Figure 2) and the second incorporating two disulfide bridges in a 16-mer peptide. A subsequent sequence of selective oxidative folding generates the desired secondary structure in the 16-mer peptide (Figure 3).

### 2 | CASE STUDY 1: A BIOTINYLATED CARBON-14 84-MER PEPTIDE

During the first case study, we required 8 mg of a 84-mer $^{14}$C-peptide BIOTINylated on the C-terminus with a specific activity of >300 mCi/mmol, illustrated in Figure 2. The molecular weight was around 10 kDa, and for the product to be of any use for the planned ADME studies, the specific activity had to be greater than 300 mCi/mmol. BIOTINylation occurs first by the deprotection of the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (ivDde) protecting group using 3% hydrazine monohydrate in DMF, followed by coupling with BIOTIN-NHS. The $^{14}$C-peptide-BIOTIN was cleaved from the resin under acidic conditions, followed by purification and lyophilisation. This gave $^{14}$C-peptide-
BIOTIN with a radiochemical purity (HPLC) > 98 area %, chemical purity (HPLC) > 98 area %, and specific activity > 300 mCi/mol. The product was formulated in an aqueous solution containing 1% ethanol at a radiochemical concentration of 20 nM/mL. BIOTINylation was rapid, specific, and unlikely to perturb the natural function of the molecule because of the small size of BIOTIN.

3 | CASE STUDY 2: A 16-MER PEPTIDE CONTAINING TWO DISULFIDE BRIDGES

This case study demonstrated a selective oxidative folding of a 16-mer 14C-peptide, achieved by generating two disulfide bridges between the cysteine residues (Cys₄ and Cys₁₂; Cys⁷ and Cys₁₅), as shown in Figure 3. The linear peptide was prepared by incorporating the 14C-label on the 6th amino acid from the C-terminus of leucine. The linear peptide was then selectively folded by the removal of the protecting groups, followed by oxidation with iodine, to form the two disulfide bridges between the four cysteine amino acids. Purification and isolation gave 1.5 mCi of 14C-leucine-labelled peptide (16-mer) with two disulfide bridges and a chemical and radiochemical purity ≥ 95 area %.

4 | DISCUSSION

Peptides can be labelled with radioactive 14C to track the passage of the biomolecule through a biological system. The manufacture of these 14C-peptides is custom made to the clients’ requirements, with regard to the position of the labelled amino acid. The requested labelled amino acid may have a single 14C-label or multiple 14C-labels. Consequently, the simplest 14C-amino acid is glycine, which can be labelled at either carbon atom giving a maximum specific activity of 120 mCi/mol. The best approach is to identify a stable biological position in the peptide and then insert a 14C-labelled amino acid into the peptide. All unlabelled amino acids can be coupled together using an automated peptide synthesiser. Once the unlabelled amino acid sequence has been assembled, work can begin on the 14C part of the peptide.

This part usually requires a different approach and involves the manual synthesis of the 14C-peptide portion. The application of the glass peptide bubbler (Figure 4)
facilitates the coupling of the protected $^{14}$C-amino acid residue onto the peptide sequence, followed by a deprotection step, to generate the $^{14}$C-peptide.

The glass peptide bubbler consisted of a sintered glass vessel, fitted with a three-way tap. The resin containing the unlabelled peptide was added into the reaction vessel and capped at the top of the vessel with a rubber septum. The system was purged with nitrogen for at least 5 minutes. The operation of the system involves adding solvents from the top of the vessel, ensuring that any resin on the sides is rinsed down into the resin bed. The resin bed is agitated by manipulating the tap that controls the flow of nitrogen to the reaction vessel. The solvents and reagents are then removed via the sinter, under vacuum. The $^{14}$C-labelled amino acid, containing the coupling “cocktail,” was added to the reaction vessel via a syringe and allow to agitate under nitrogen. The resin was washed with a solvent, and the $^{14}$C-peptide was deprotected from the resin.

A major disadvantage of peptides as therapeutic agents is their short biological lifetime in the human body. This can result in degradation of the peptide, and subsequently, these metabolites have the potential to initiate an immune response.

In order to reduce these problems, the $^{14}$C-peptide can undergo a modification such as PEGylation. This process involves a covalent modification using polyethylene glycol (PEG) and increasing the therapeutic performance, such as improving inadequate water solubility, lowering toxicity, or enhancing a poor pharmacokinetic profile. The $^{14}$C-peptide is modified by coupling the amino acid sequence to PEG to produce a PEGylated $^{14}$C-peptide and therefore has a different pharmacological profile compared with the parent peptide: Having the attached PEG group will alter the mass of the peptide and change both peptide solubility and stability profile.

The PEGylation of $^{14}$C-peptides can be difficult. However, these problems can be circumvented by using a synergy approach involving both the peptide and radiolabelling groups. These PEG covalent modifications require a reactive functional group at the terminal of the $^{14}$C-peptide. The simplest method to PEGylate $^{14}$C-peptides—which have a primary amine linker—is to use a PEG compound that contains an NHS ester group at one end.

An alternative approach is to use BIOTINylated lysine to couple the $^{14}$C-peptide. The use of linkers can be used to couple the BIOTIN molecule to the $^{14}$C-peptide. The aim of this chemical linker is to minimise the BIOTIN interfering with the therapeutically active conformation of the peptide. A common linker used is 6-aminohexanoic acid because it produces a regular peptide amide, with both BIOTIN and peptide.

Also, BIOTIN (vitamin H) can be coupled to a $^{14}$C-peptide sequence using a solid-phase approach. The BIOTINylation to the $^{14}$C-peptide takes place at the thiol (SH) or amino (NH$_2$) sites. An example of this is a $^{14}$C-peptide bound to a solid support (polystyrene resin) containing a side chain lysine residue. This lysine residue is available to react with BIOTIN reagents and, on cleavage from the solid support, generates the BIOTINylated $^{14}$C-peptide.

5 | CONCLUSION

APIs based on peptides are a growing area of drug delivery systems, particularly as new treatments in oncology. Radiolabelled versions of the parent drug are required for ADME studies. $^{14}$C is often the preferred labelling isotope because of the lowered risk of “wash out” or the need to use a modified structure. The limitations of a relatively low specific activity, traditionally overcome with the incorporation of more than one label, have now been overcome with the advent of accelerated mass spectrometry (AMS).

The short biological lifetime in the human body of peptides as therapeutic agents is overcome by PEGylation and BIOTINylation strategies. However, these techniques have posed significant challenges to the preparation of $^{14}$C-peptides. A synergistic collaboration between both the peptide and radiolabelling groups has been developed to provide successful solutions to the delivery of complex, bespoke $^{14}$C-peptides for customers.

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REFERENCES

