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Review

Labeling Peptides with Radioiodine: An Overview of Traditional and Emerging Techniques

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Abstract

This review focuses on the synthetic methodologies for radioiodinating peptides, a crucial process for developing effective radiopharmaceuticals used in diagnostics and therapeutics. We explore direct and indirect radioiodination methods, including mechanisms, reaction conditions, and purification strategies. The focus is on the chemical approaches that enable radioiodine incorporation into peptide structures, considering the challenges of maintaining peptide integrity and biological activity. This article is intended as a detailed resource for understanding traditional approaches and recent chemical developments in radioiodination.

Keywords: radiochemistry; PET; SPECT; radioiodination; peptides

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1. Introduction

The synthesis of radioiodinated peptides is a critical area in radiopharmaceutical chemistry, playing a fundamental role in the development of novel agents for molecular imaging and targeted therapy. Peptides exhibit remarkable specificity and affinity for biological targets, making them highly valuable in the diagnosis and treatment of various diseases, including cancer, cardiovascular disorders, and neurological conditions [1]. When combined with the unique nuclear properties of radioiodine isotopes, these radiolabeled biomolecules serve as powerful tools for positron emission tomography (PET) and single-photon emission computed tomography (SPECT) imaging, as well as radiotherapy [2–4]. Iodine-based radioisotopes (^{123}I, ^{124}I, ^{131}I) provide versatile options for both diagnostic and therapeutic applications due to their diverse half-lives and emission properties [5–9]. ^{123}I is commonly used for SPECT imaging owing to its favorable gamma emission and short half-life (13.2 h), whereas ^{124}I enables PET imaging with a relatively long half-life (4.2 days), beneficial for tracking slow biological processes, despite its higher positron energy and reduced resolution compared to ^{18}F. ^{131}I, with its beta and gamma emissions, serves as a dual-purpose isotope for imaging and radiotherapy but is less ideal for high-resolution imaging. In contrast, ^{18}F is prized for its optimal PET imaging characteristics—short half-life (109.8 min), low positron energy, and high resolution—though its short half-life can limit applications requiring extended imaging times. ^{64}Cu, offering both β^+ and β^- decay, supports PET imaging and radiotherapy, and its intermediate half-life (12.7 h)

balances logistical flexibility with imaging resolution. Compared to ^{18}F and ^{64}Cu, iodine isotopes are advantageous in settings requiring longer imaging windows, theranostic use, or easy labeling chemistry, but may be less suited for applications prioritizing high spatial resolution or short biological half-lives [10].

Among the earliest and most widespread applications of radioiodinated peptides is their use in radioimmunoassays, particularly with iodine-125 (^{125}I). This technique has been instrumental in the sensitive and specific quantification of peptide hormones and other biologically active peptides in complex biological matrices, and continues to serve as a benchmark in immunoassay development [11,12].

While radiometal-labeled peptides (e.g., ^{68}Ga-, ^{64}Cu-, ^{177}Lu-labeled) have gained prominence in nuclear medicine due to their excellent stability, favorable pharmacokinetics, and suitability for theranostic applications, radioiodinated peptides continue to offer unique advantages in specific contexts. Iodine isotopes allow for direct labeling without the need for bifunctional chelators, simplifying synthesis and avoiding potential alterations in peptide conformation or receptor affinity. Additionally, radioiodination enables high-specific-activity labeling, which is advantageous for receptor-saturable targets. However, a key limitation of iodine-labeled peptides is their susceptibility to in vivo deiodination, particularly with tyrosine-based labeling, leading to thyroid uptake and background signal [13]. In contrast, radiometal complexes, when properly chelated (e.g., via DOTA or NOTA), tend to exhibit greater in vivo stability.

Despite these advantages, the synthesis of radioiodinated peptides presents significant challenges. The primary concern is maintaining the structural integrity and biological activity of the peptide while ensuring efficient incorporation of the radioiodine. Peptide radioiodination methods can be broadly categorized into direct and indirect approaches, each offering distinct benefits and limitations. Direct radioiodination involves the electrophilic substitution of radioiodine into specific amino acid residues, such as tyrosine or histidine, within the peptide structure. While this method is relatively straightforward, it may result in the modification of residues that are critical for the peptide's biological function, potentially altering its binding properties. Indirect radioiodination, on the other hand, employs prosthetic groups that are pre- or post-labeled with radioiodine and conjugated to the peptide at sites distant from functionally important residues. Although this approach introduces a larger chemical moiety, it is often better suited for preserving the native conformation and biological activity of the peptide, while also enabling site-specific and potentially more stable labeling [1,2].

Recent advances in radioiodination methodologies have led to the development of innovative chemical strategies that enhance radiochemical yields (RCY), improve in vivo stability, and expand the range of applicable peptide substrates. These advancements include the use of novel prosthetic groups, click chemistry approaches, and optimized purification techniques that enhance the efficiency and specificity of the labeling process. Additionally, the integration of computational modeling and automation in radiolabeling procedures has further refined the synthesis of radioiodinated peptides, making them more accessible for clinical and research applications [14,15].

Given the growing demand for radiolabeled peptides in nuclear medicine, it is essential to understand the chemical principles underlying their synthesis. This review is structured to first introduce the rationale and key principles of peptide radioiodination, followed by detailed discussions of direct and indirect labeling strategies, specific examples from the recent literature and purification techniques. To better guide readers in selecting the most appropriate radioiodination approach, we have compiled a comparative summary of the main iodine-incorporating methods discussed in this review. Table 1 provides an overview of these strategies, highlighting the key features, optimal use cases, and relevant references.

Table 1. Overview of iodine radiolabeling methods with their typical applications and representative references.

Method	Key Features	Use Case	Key References
Electrophilic iodination	Mild conditions, wide substrate scope	Small molecules, tyrosine/ histidine-containing peptides	[16,17]
Isotope/halogen exchange	Simple, no precursor synthesis	Molecules with existing halogen groups	[18,19]
Nucleophilic substitution	Requires good leaving groups	Prosthetic groups	[13,20,21]
Iododestannylation	One-step, highly efficient	Aryl-containing molecules, pre-functionalized systems	[22–24]
Iododesilylation	Lower radiochemical yields than Iododestannylation	Aryl-containing molecules, pre-functionalized systems	[2,14]
Diazotisation	High reactivity of intermediate	Aryl-containing molecules, pre-functionalized systems	[25–27]

2. Direct Radioiodination Methods

2.1. Electrophilic Iodination

Direct radioiodination involves the direct incorporation of radioiodine into the peptide structure, usually through electrophilic substitution. This methodology is most feasible when the peptide contains amino acids that are susceptible to iodination, such as tyrosine and histidine [28].

The most common method for direct radioiodination of peptides involves targeting tyrosine residues [29]. Tyrosine contains a phenol group that is highly reactive towards electrophilic attack by iodine (Figure 1). The reaction typically proceeds via an electrophilic aromatic substitution mechanism. The radioiodine, usually in the form of iodide (I^-), is first oxidized to a more electrophilic species, such as I^+, using an oxidizing agent. This electrophilic iodine then attacks the ortho positions on the tyrosine's phenolic ring, leading to the formation of a carbon–iodine bond. Commonly used oxidizing agents are Chloramine-T and Iodogen. Chloramine-T can be quite harsh and might lead to unwanted side reactions, such as oxidation of cysteine residues or hydrolysis of peptide bonds [30]. This oxidizing agent is typically used in aqueous solutions, and the reaction is quenched with sodium metabisulfite. Iodogen, on the other hand, is a milder oxidizing agent and is often preferred for peptide iodination. Another advantage of using Iodogen is that this is an insoluble reagent that can be easily removed by filtration after the reaction [31], helping in reducing the exposure of the peptide to harsh conditions. The reaction is normally carried out in a heterogeneous system. The reaction of both oxidizing agents is generally performed in a buffered solution at a slightly basic pH (around 7.0–7.5) to facilitate electrophilic substitution and is typically carried out at room temperature for a short time (5–10 min) to minimize any potential degradation of the peptide [16,17]. Direct iodination of tyrosine can result in mono- or, less commonly, di-iodination—particularly in carrier-added conditions where excess iodine is present—potentially affecting the peptide's biological activity and pharmacokinetics. Additionally, oxidants such as chloramine-T and Iodogen generate reactive chlorine species that may also lead to the formation of mixed halogenated products, such as monochloro-monoiodo derivatives. Control over the degree and nature of halogenation is typically achieved by adjusting the stoichiometry of the reagents and optimizing reaction conditions. Histidine is another amino acid that can be directly radioiodinated, but it is less reactive than tyrosine. The iodination of histidine occurs at the imidazole ring via an electrophilic substitution mechanism similar to that of

tyrosine (Figure 2). The reaction is generally slower and less efficient than that of tyrosine, often resulting in lower radiochemical yields [32].

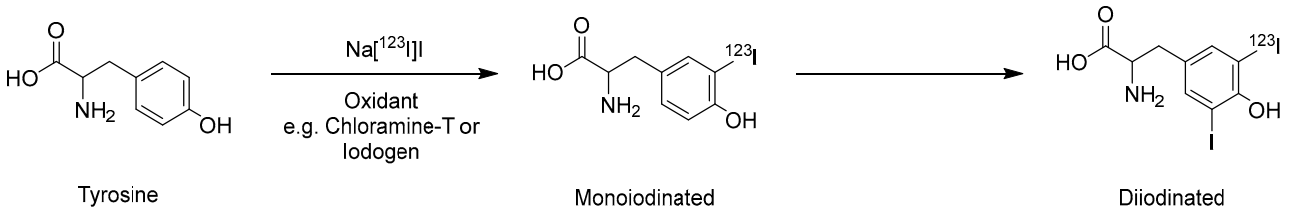

Figure 1. Direct iodination of Tyrosine residues through an SEAr reaction.

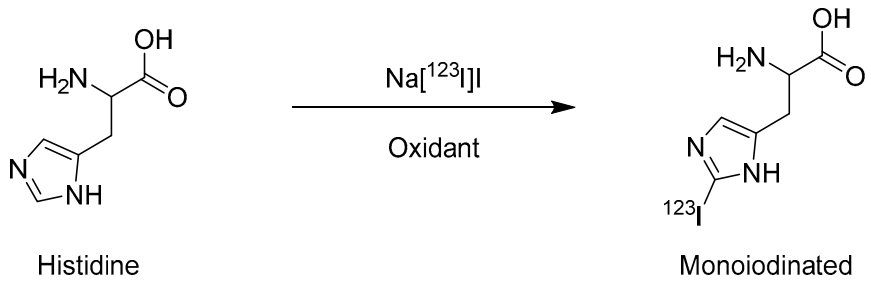

Figure 2. Direct iodination of Histidine residues through an SEAr reaction.

2.2. Isotope/Halogen Exchange of Unnatural Amino Acids Bearing Halogen Substituents

Isotopic and halogen exchange reactions represent a class of direct radioiodination methods in which a stable iodine or halogen atom within a molecule is replaced with a radioactive isotope of iodine. While traditionally applied to small molecules, this approach can also be extended to peptides that incorporate halogenated unnatural amino acids—such as iodinated phenylalanine derivatives—directly into their sequence [33]. In such cases, the peptide backbone itself can serve as the substrate for radioiodination via halogen exchange, eliminating the need for prosthetic groups. The reaction typically involves a substitution reaction where the radioiodide ion displaces the existing stable iodide (Figure 3). This method is often carried out at high temperatures in the presence of metal catalysts such as copper or nickel [18,19]. For example, the radioiodination of [^{123}I]IPEB, a metabotropic glutamate receptor subtype 5 radioligand, was achieved via bromine–iodine exchange at high temperatures in the presence of copper and tin sulfate [34]. The nickel(0)-mediated approach can also be used in isotope exchange [35]. One of the major limitations of this radioiodination method is the difficulty of separating non-radioactive and radioactive products, which can be a problem when high molar activities are needed [14].

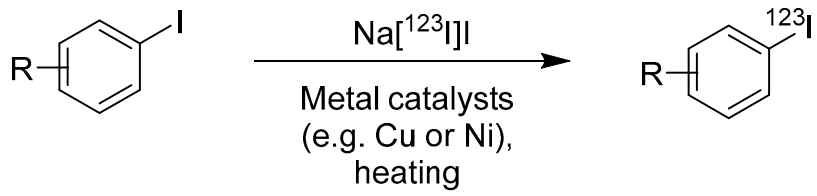

Figure 3. Standard example of direct iodination through isotopic exchange.

3. Indirect Radioiodination Methods: The Conjugation of the Prosthetic Groups

Prosthetic groups are versatile intermediates used in indirect radioiodination, allowing for the introduction of radioiodine either before [20] or after [36] their conjugation to the target peptide, thus enabling flexible and site-specific labeling while preserving the

peptide's biological activity. This approach is particularly useful when direct radioiodination is not feasible or when milder reaction conditions are necessary. Various prosthetic groups have been developed to facilitate the efficient and site-specific radioiodination of peptides such as activated esters, maleimide-, azide-, alkyne-, syndone-, or tetrazine-derivatives. These are ready to be conjugated to the peptide using several reactions such as amide bond formation, thioether formation, copper(I)-catalyzed azide-alkyne cycloaddition, strain-promoted azide-alkyne cycloaddition (SPAAC), or the inverse electron demand Diels–Alder (IEDDA) reaction.

3.1. Activated Esters and Maleimide Derivatives

In indirect radioiodination, activated esters serve as prosthetic groups to facilitate the introduction of radioiodine into macromolecules. This approach is particularly useful when direct labelling is not feasible due to harsh reaction conditions or when modifications to the molecule, such as adding tyrosine, are not possible. The use of prosthetic groups allows reactions to proceed under milder conditions, preventing macromolecules from being affected by oxidizing agents, and can lead to a radioisotope-labelled macromolecule that is stable to deiodination in vivo [13].

Activated esters like NHS (*N*-ester of hydroxysuccinimide), Tfp, pentafluorophenyl ester, and their water-soluble SO_3H-substituted derivatives are commonly employed (Figure 4) [28,37–39]. Conjugation is typically achieved by reacting the peptide with a labelled prosthetic agent in a polar solvent like DMF or acetonitrile, in the presence of a tertiary amine. For peptides soluble in water, the reaction can occur in aqueous buffer solutions with a pH of 8–9 or in aqueous–organic reaction media with acetonitrile. Unlike peptides, protein radioiodination is ideally performed in aqueous buffer solutions at a pH close to the physiological range, with conjugation efficiency depending on pH, protein concentration, and temperature.

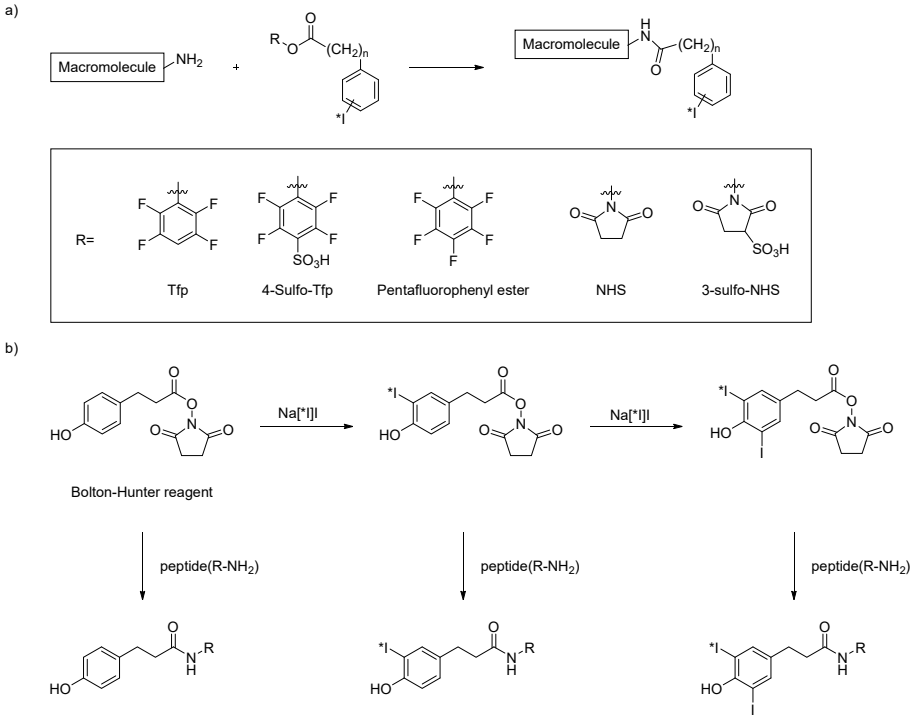

Figure 4. Indirect radioiodination using activated esters. (**a**) Conjugation of activated esters with NH_2 groups of macromolecules. (**b**) Iodination using the Bolton–Hunter method. (* means that the iodine could either be at 3 or 4 position).

The formation of peptide/amide bonds between the carboxyl fragment of the prosthetic group and the amino group of the macromolecule is a widely used approach. While Tfp esters are more stable than NHS esters at high pH, NHS esters are more commonly used. Higher protein concentrations can yield better conjugation reaction results but may decrease the molar activity of the target product.

An example is the preparation of a radioiodinated bovine serum albumin (BSA) conjugate using *N*-Tetrachlorophthaloyl (TCP) as a bifunctional linker (a chemical moiety that contains two reactive ends), yielding the ^{125}I-containing conjugate with 58–75% yield and 99.8% radiochemical purity after purification. Biodistribution studies in mice showed significantly less absorption of the conjugate in the thyroid gland compared to [^{125}I]-iodinated BSA [38].

One of the first and most widely used prosthetic groups is the Bolton–Hunter reagent (*N*-succinimidyl-3-(4-hydroxy-5-[*I]iodophenyl)propionate), which can be conjugated to the *N*-terminus peptide sequence or side chain amino groups, such as lysine [40–42]. The activated aromatic fragment of the Bolton–Hunter reagent is radioiodinated with the necessary isotope of iodine and then conjugated with a macromolecule to obtain the target compound (Figure 4). The most common prosthetic agents used for radioiodination include *N*-succinimidyl-4-[*I]iodobenzoate (PIB), *N*-succinimidyl-3-[*I]iodobenzoate (SIB), and *N*-succinimidyl-5-[*I]iodo-3-pyridinecarboxylate (SIPC) (Figure 5) [20,21,43].

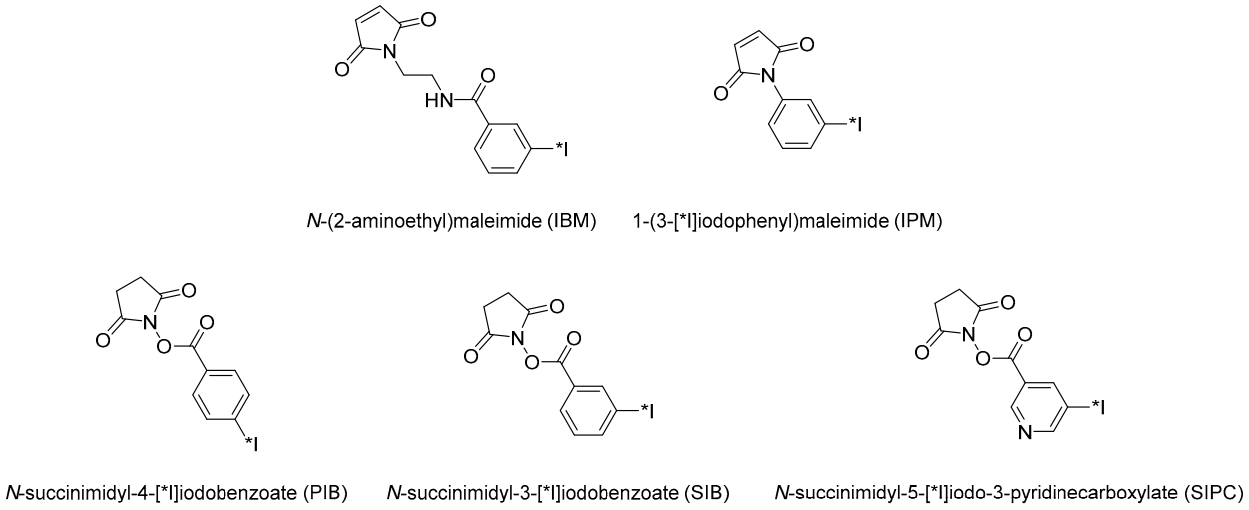

Figure 5. Typical prosthetic groups (activated esters and maleimide derivatives) used in radioiodination of macromolecules. (* represent the radioactive isotope).

Maleimide derivatives are also used as prosthetic groups for -SH fragments modification in indirect radioiodination, with conjugation reactions typically performed under physiological conditions and generally giving relatively high radiochemical yields (RCYs). An example of using a maleimide derivative is in the synthesis of a tumor-targeting radioiodine derivative of the F3 peptide, called ([^{125}I]IBMF3), which can be used as a radioligand for tumor imaging by SPECT synthesized with an overall RCY of 73% [43].

Other maleimide derivatives, such as *N*-(2-aminoethyl) maleimide (IBM) or 1-(3-[*I]iodophenyl) maleimide (IPM), have been proposed as prosthetic groups for the radioactive iodination of macromolecules (Figure 5) [2,44]. Maleimide-functionalized molecules for radioiodination can exhibit varying degrees of stability depending on factors like the specific maleimide derivative, the conjugation strategy, and the surrounding environment. While the maleimide moiety itself is relatively stable, the thioether bond formed with a sulfhydryl group can be susceptible to hydrolysis and thiol exchange reactions, potentially impacting the stability of the radioiodinated conjugate [45–47].

In a 2018 paper, S. Mushtaq et al. [48] reported the labeling of an alkylaldehyde with ^{125}I using an organotin precursor, with high radiochemical yield (72 ± 6 percent) and radiochemical purity (>99 percent) (Figure 6). Then, by exploiting the condensation reaction of aryl-diamine and alkyl-aldehyde in the presence of a Cu^{2+} catalyst under mild conditions to create a maleimide prosthetic group, the radioiodinated precursor was bound to the c[RGDfK(C)] aryl-diamine-derived peptide and also to human serum albumin.

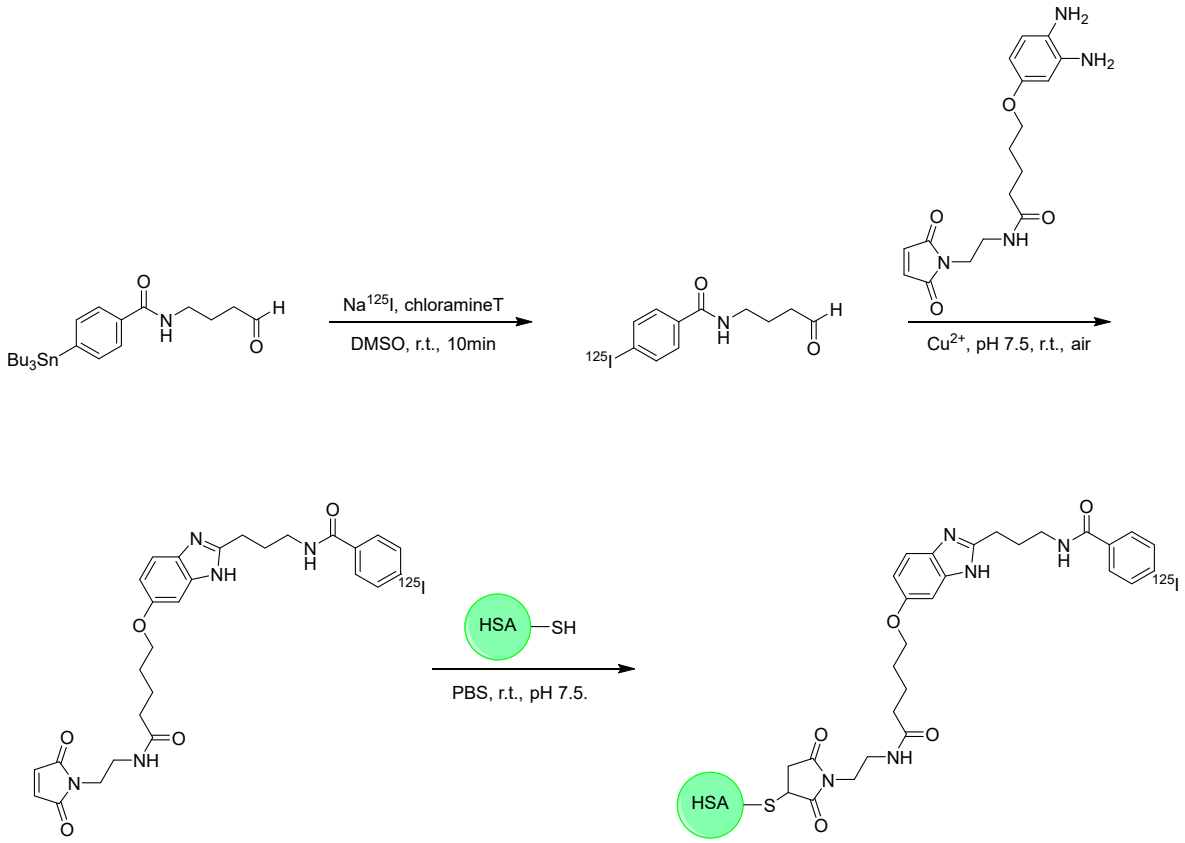

Figure 6. Labeling/maleimide conjugation with ^{125}I using an organotin precursor.

3.2. Cycloadditions

Le Saux et al. [49] explored the use of Strain-Promoted Sydnone-Alkyne Cycloaddition (SPSAC) for radioiodination, offering a novel approach to radiopharmaceutical development. The research focuses on sydnones, which are easily synthesized and chemically stable, as a means to introduce radiohalogens into biomolecules. As already mentioned, direct electrophilic substitution, has limitations, including the requirement for accessible tyrosine residues and potential instability due to dehalogenation. To overcome these limitations, the use of prosthetic groups such as the proposed sydnones has become common. The researchers compared various bioorthogonal systems and focused on SPSAC due to its high reactivity and suitability for low reactant concentrations, which is ideal for radiotracer development. They designed and synthesized a series of iodinated sydnones with different substituents at the N_3 and C_4 positions to modulate reactivity (Figure 7). The synthesis of bifunctional precursors involved creating *N*-arylsydnones with a carboxylic acid function. Boronic acid precursors and iodinated references were obtained through amide bond formation.

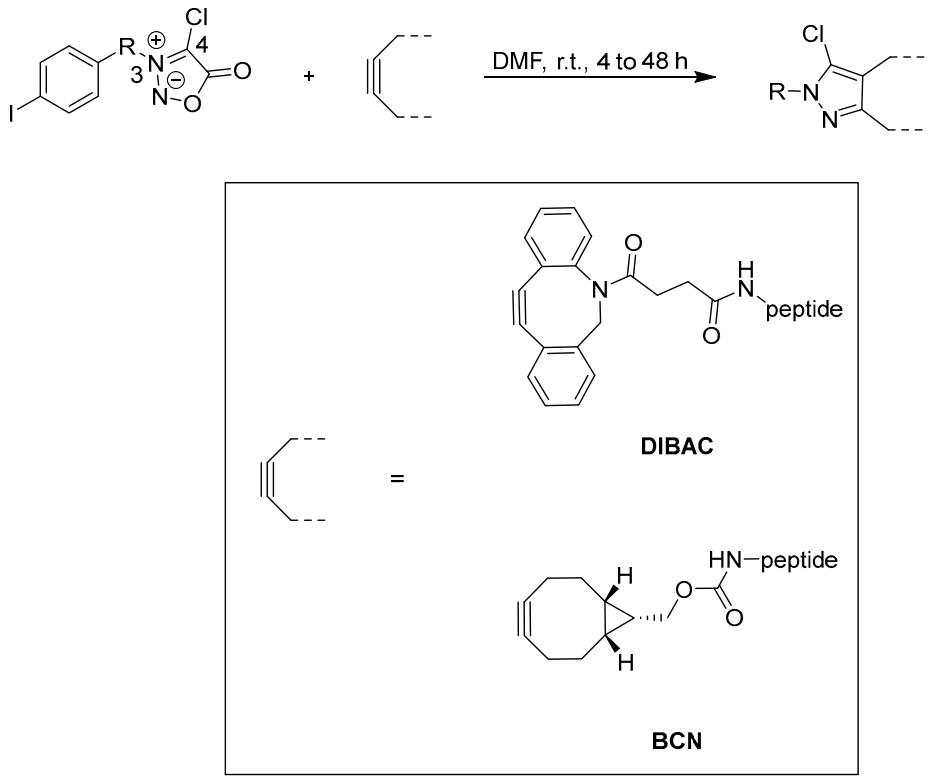

Figure 7. Radioiodination through the Strain-Promoted Sydnone-Alkyne Cycloaddition (SPSAC) approach. N_3 and C_4 positions are highlighted.

Kinetic studies were performed using model peptides with dibenzoazacyclooctyne (DIBAC) or bicyclononyne (BCN) clickable handles to evaluate the impact of N_3 and C_4 substituents on sydnones reactivity. The study found that *N*-alkylsydnones showed the slowest reaction kinetics, while *N*-arylchlorosydnones exhibited higher reaction rates with the BCN peptide. The high reaction rate of cycloaddition with BCN proved nearly 11 times higher with the chlorosydnone compared to azide.

Radiochemistry experiments involved obtaining radioiodinated sydnones and benzyl azide via the iododeboronation reaction. The radioiodinated prosthetic groups were then evaluated in cycloaddition reactions with model peptides, showing similar trends to the non-radioactive evaluations. The *N*-arylchlorosydnone reported in Figure 7 was identified as the most reactive, achieving high radiochemical yields with both DIBAC and BCN peptides.

The study concludes that SPSAC is a promising approach for radiopharmaceutical design, offering a modular system to tune reactivity and functionality for optimal bioconjugation. The optimized system, particularly with BCN, outperformed previously reported SPAAC reactions. The correlation between non-radioactive and radioactive kinetics assays suggests that non-radioactive kinetics assay is a robust predictor of radioactive conditions. The methods developed are also of interest for labeling with astatine-211, a potential alpha emitter for cancer therapy, because astatine exhibits similar reactivity to iodine.

Albu et al. developed a convenient method to prepare radioiodinated tetrazines so that a bioorthogonal reverse electron demand Diels–Alder reaction could be used to label biomolecules with iodine-125 [50]. Tetrazine was prepared by employing a high-throughput oxidative destannylation reaction that simultaneously oxidized the precursor dihydrotetrazine, as reported in Figure 8. Later, their utility was demonstrated through antibody and hormone labeling experiments.

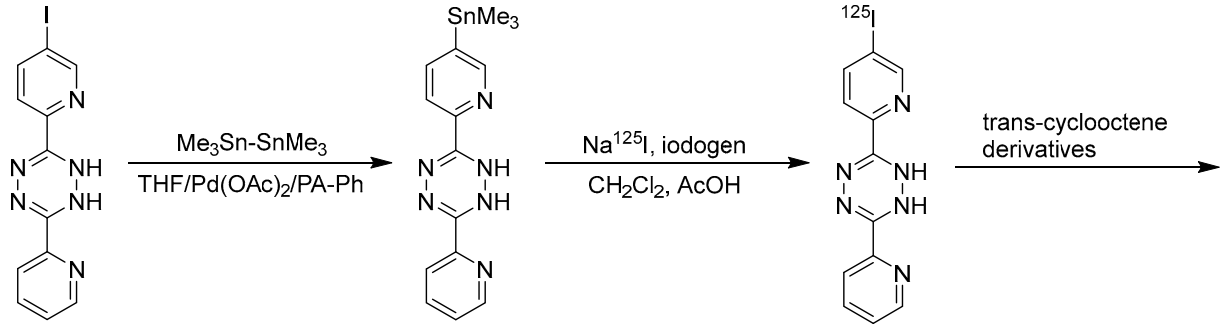

Figure 8. Method to prepare radioiodinated tetrazines.

Similarly, M.H. Choi and coworkers present a rapid and highly efficient method for radioactive iodine labeling of biomolecules conjugated with trans-cyclootene group using the reverse electron-demand Diels–Alder reaction [51]. The tetrazine structure radioiodination reaction (Figure 9) was conducted always using a stannylated precursor to obtain an ^{125}I-labeled product with high radiochemical yield (65 ± 8%) and radiochemical purity (>99%). Subsequently, the radiolabeled tetrazin derivative was used to radiolabel two biomolecules, transcyclotene derivatives, such as cRGD peptide and human serum albumin.

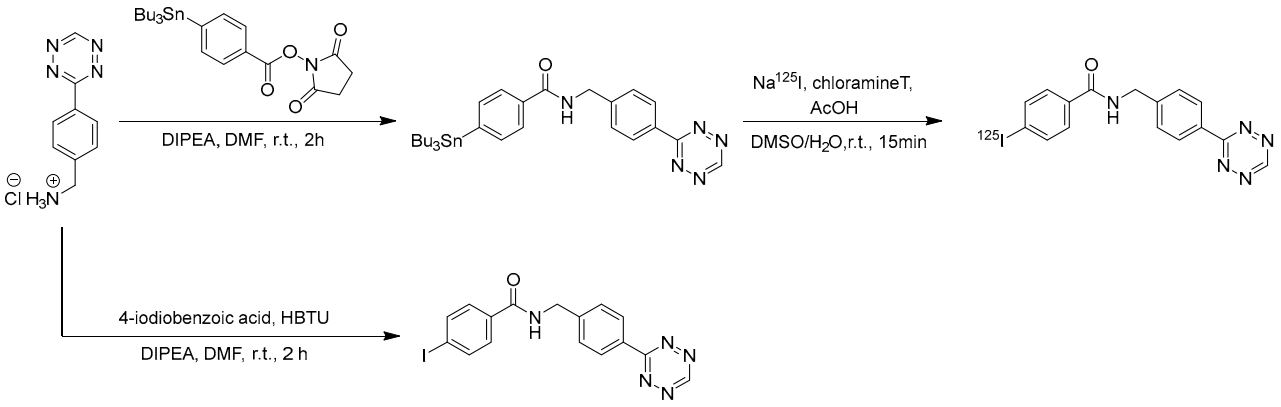

Figure 9. Stannylated precursor to obtain an ^{125}I-labeled product.

4. Indirect Radioiodination Methods: The Labelling of the Prosthetic Groups

4.1. Iododestannylation

Iododestannylation is a frequently used method for introducing radioiodine, involving the reaction of a stannane precursor with an in situ generated iodinated reagent derived from NaI and an oxidant. The transformation results in radiolabeled derivatives through an ipso S_EAr reaction (Figure 10) [22–24].

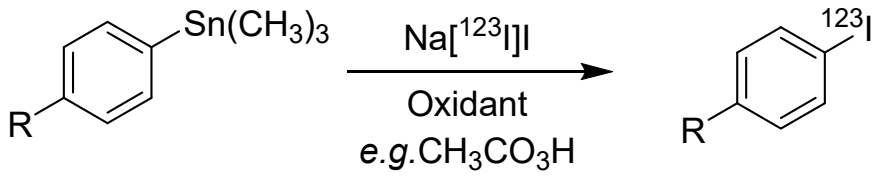

Figure 10. Iododestannylation through an S_EAr reaction.

The reaction starts with a stannane precursor and uses an in situ generated iodinated reagent from NaI and an oxidant. The transformation proceeds smoothly and selectively via an ipso S_EAr reaction, which leads to the radiolabeled derivatives. Aryltrialkylstannanes

are typically synthesized from halogenated precursors through metalation or palladium-mediated reactions. The main advantage of iododestannylation is that it proceeds under mild conditions, and even deactivated aromatic compounds can enter these reactions, yielding iodine derivatives stable in vivo. Stannylation and destannylation reactions usually give high product yields, even when small amounts of starting trialkyltin are used. Unfortunately, the major drawback is the potential contamination of the radiotracer with organotin residues, which can hinder its use in clinical settings. Also, aryltrialkylstannanes can have stability and toxicity issues.

Because contamination with organotin compounds is a significant concern in radioiodination protocols, several strategies have been developed to minimize residual organotin levels in the final product. One such approach involves the use of fluorine-rich organostannanes to enable solid-phase extraction and efficient removal of tin residues [52]. More recent solutions include the use of fluoride-assisted solid-phase extraction or reactions with stannylated derivatives in ionic liquids, followed by filtration through SiO_2 [53]. Notably, Valliant et al. [52] demonstrated that radioiododestannylation using fluorine-rich organostannanes not only facilitates effective purification but also delivers high radiochemical yields. For example, [^{125}I]SIB was synthesized using this approach and isolated with a 67% radiochemical yield (RCY) and 100% radiochemical conversion (RCC), underscoring the utility of this method for preparing small-molecule radiotracers such as [^{125}I]AGI-5198.

4.2. Iododesilylation

Iododesilylation is another method for radioiodination that uses silanes as precursors for labelling molecules (Figure 11). Compared to iododestannylation, iododesilylation generally results in lower RCYs because the carbon–silicon bond is more stable. It typically involves the reaction of a silylated precursor with an electrophilic source of iodine, often in acidic media [2,14]. The reaction is generally conducted in acidic media such as trifluoroacetic acid (TFA). To promote the formation of the reactive radioiodine species in situ, reagents such as *N*-chlorosuccinimide (NCS) and trifluoroperacetic acid can be employed [14]. [^{131}I]MIBG was labelled in 85–90% RCY starting from the corresponding aryltrimethylsilane in TFA, using trifluoroperacetic acid to generate the reactive ^{131}I-species in situ [54]. [^{123}I]iodometomidate (IMTO), a high-affinity ligand of adrenal steroidogenic enzymes, has been radioiodinated from a polymethacrylamide-supported precursor [55]. Interestingly, solid-phase organic chemistry can be used for this reaction, enabling rapid purification of reaction products through simple filtration. In the radioiododesilylation of iodometomidate (IMTO), after the reaction, TFA was neutralized with a polymer-supported amine. The purification step of [^{123}I]IMTO was then performed through elution on a Sep-Pak cartridge, while the unreacted polymethacrylamide-supported precursor could not be eluted. The radiotracer was obtained in 85% RCY and 94% RCP.

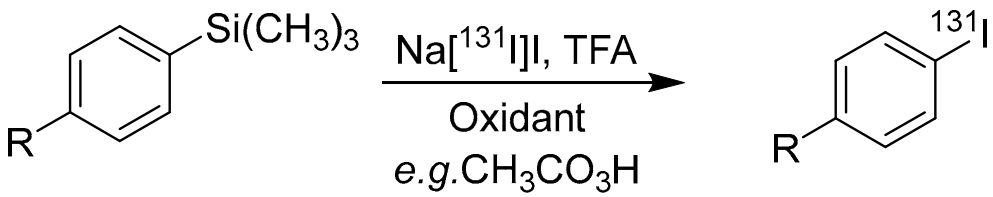

Figure 11. Iododesilylation through an S_EAr reaction.

In summary, while iododesilylation may not always provide the high RCYs seen in iododestannylation, it remains a useful method, particularly when combined with techniques like solid-phase chemistry to aid in purification.

4.3. Diazotisation

Diazonium salts are employed in nucleophilic aromatic radioiodination reactions and can be used to radioiodinate aryl amines (Figure 12) [25]. Diazonium salts readily undergo nucleophilic aromatic radioiodination. A challenge with using diazonium salts is their instability. To circumvent this, storage-stable triazenes can be used as synthetic equivalents. However, this approach has limitations, including the need for modified precursors, using a large excess of reagents, and the potential for side reactions [25–27]. Diazotisation is typically performed using sodium nitrite at low temperatures in an aqueous solution of hydrochloric or sulfuric acid. The resulting diazonium salt solution is then treated with radioactive sodium iodide. A significant disadvantage of the diazotization method is the high reactivity of intermediate aryl cations or radicals, which can lead to the formation of by-products. An efficient methodology for radioiodinating aryl amines involves using a polymeric reagent to generate nitrite anions. This allows diazotization and the subsequent Sandmeyer reaction to occur under mild conditions and at low concentrations of diazonium salts. This method has been successfully applied to prepare SPECT diagnostics, including [^{125}I]Iomazenil, [^{125}I]CNS1261, and [^{125}I]IBOX, with RCYs ranging from 47% to 75% [25]. Triazene derivatives are less reactive than diazonium salts, which can limit their use in radiolabeling applications (Figure 12).

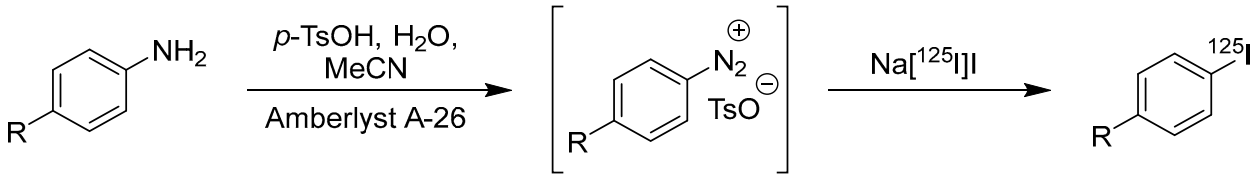

Figure 12. Standard example of radioiodination through diazonium salts.

In summary, while diazonium salts offer a direct route for nucleophilic aromatic radioiodination, their instability and the potential for side reactions have led to the development of alternative strategies, such as using triazenes or polymeric reagents, to achieve milder and more controlled reaction conditions.

4.4. Iodonium Salts

Iodonium salts are highly reactive electrophilic reagents widely employed in radioiodination reactions due to their excellent leaving group ability and compatibility with mild conditions. Their use enables efficient labeling of aromatic compounds with radioiodine isotopes, facilitating the synthesis of radiotracers for imaging and therapeutic applications [2]. L. Navarro's study provides several new alternatives to conventional and suboptimal approaches currently in use for radioiodination and astatination of biomolecules (Figure 13) [56]. The study proposes the use of ^{125}I- and ^{211}At-labeled azide and tetrazine prosthetic groups for bioorthogonal conjugation, designed and tested in a comparative study of five bioorthogonal systems. Radiolabeling, in particular, was performed from precursors of ^{125}I ariliodonium salts. All five bioconjugation reactions conducted on a clickable model peptide produced quantitative yields in times ranging from less than a minute to several hours, depending on the system used. Finally, one of the bioorthogonal systems was used for tagging an IgG.

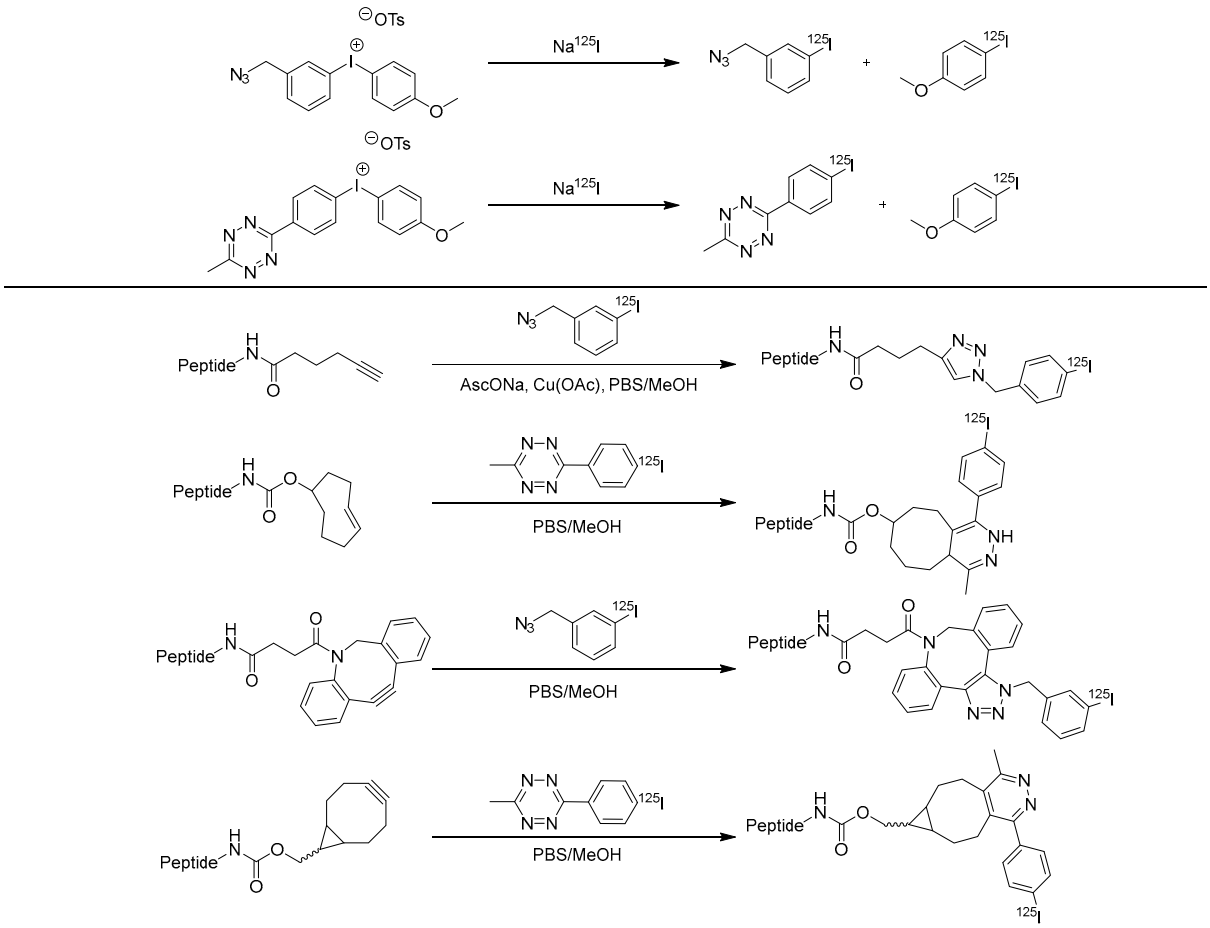

Figure 13. ^{125}I-labeled azide and tetrazine prosthetic groups for bioorthogonal conjugation.

A comparative study of the radiohalogenation of arylhalogen salts with ^{125}I and ^{211}At was performed by F. Guérard and colleagues [57]. Initial experiments were performed on a model compound, which showed the higher reactivity of astatide compared with iodide. From the kinetic studies, a significant difference in the activation energy (Ea = 23.5 and 17.1 kcal mol^{-1} with ^{125}I and ^{211}At, respectively) was inferred along with computational studies. The good to excellent regioselectivity of the halogenation and the high yields obtained with variously substituted arylodonium salts indicate that this class of compounds is a promising alternative to the stannan chemistry currently used for radiohalogen labeling of tracers in nuclear medicine. The same group describes the development of an alternative approach to arylstannan chemistry for radiolabeling antibodies with radioiodine or astatine, based on precursors of arylodium salts [58]. Bifunctional aryl salts were designed and tested for the synthesis of ^{125}I^{-} and ^{211}At^{-}labeled prosthetic groups for bioconjugation (Figure 14). The nature of the electron-rich aryl group was varied and its impact on the regioselectivity of radiohalogenation was evaluated. Under optimized conditions, both radioiodination and astatination were performed very efficiently under mild conditions (radiochemical yields > 85%). The ionic nature of the precursors was exploited to develop an efficient purification approach: the HPLC step, usually required in conventional approaches to optimize removal of toxic organotin precursors and side products, was replaced by filtration through a silica cartridge with significantly reduced loss of radiolabeled product. The radioiodinated and astatinated prosthetic groups were then efficiently conjugated to an anti-CD138 monoclonal antibody (conjugation yield of 75–80%), exploiting the *N*-hydroxysuccinimidyl ester group.

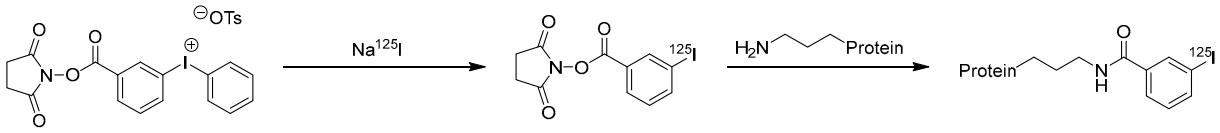

Figure 14. Radioiodination of proteins with arylodium salts.

4.5. Boronic Acids

Kondo et al. [59] explored the impact of water on the efficiency of copper-mediated radioiododeboronation, a method used to directly radiolabel small molecules and peptides with radioiodine. The study seeks to optimize reaction conditions for various peptides, especially considering the solubility of peptides in water-containing solvents. The central goal was to improve the design of boronic peptide precursors and radiolabeling protocols for reproducible direct radiolabeling of diverse peptides.

The study uses copper-mediated nucleophilic reactions with a boronic precursor, a promising method for labelling an aromatic ring with radioiodine. This method occurs in open air at room temperature without complex materials and can be adapted for direct radiolabeling of peptide mimetics, solving issues of classical direct radiolabeling such as byproduct formation. Small molecules were firstly used as models before synthesizing ^{125}I-labelled octreotate derivatives ([^{125}I] *m*/*p*-IBTA).

Key findings and observations were the following. Methanol (MeOH) and ethanol (EtOH) are optimal solvents, yielding high radiochemical conversions (RCCs > 95%). Acetonitrile (MeCN), *N*, *N*-dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) resulted in significantly lower RCCs. Adding MeOH to aprotic polar solvents improved RCCs, but adding H_2O did not. Increasing water content in MeOH solvent decreases RCCs. This decrease may be due to poor solubility of boronic precursors or target compounds. RCCs can be improved by increasing the amount of the copper catalyst $Cu(py)_4(OTf)_2$ and extending the labelling reaction time. In H_2O/MeOH solvents, substrates with electron-donating groups have higher RCCs than those with electron-withdrawing groups. This aligns with previous research on Chan–Evans–Lam (CEL) coupling reactions [60–62], where electron-rich arylboronic acids lead to faster catalyst turnover.

In the proposed mechanism, the reaction involves the formation of complexes, with the presence of MeOH promoting complex formation required for transmetalation. Water can interfere with this process, reducing the formation of the necessary complex and lowering RCCs.

Direct radiolabeling of peptides was then performed using copper-mediated radioiododeboronation. The synthesis of [^{125}I] *m*/*p*-IBTA required a larger amount of copper catalyst to exceed 97% RCC (Figure 15).

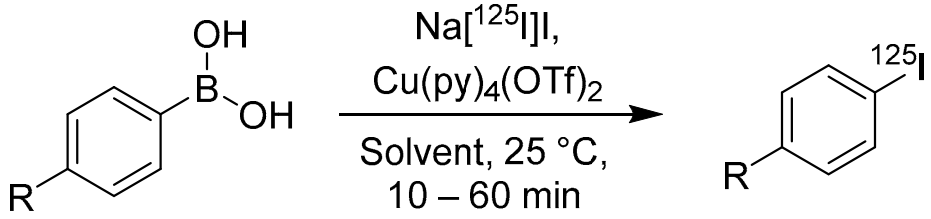

Figure 15. Standard example of copper-mediated radioiododeboronation.

The study concludes that high RCCs can be achieved using alcohol solvents, but water content should be minimized. Adjusting the amount of copper catalyst and reaction time can improve RCCs in water-containing solvents. The amount of copper catalyst used was lower than the levels of concern defined in the draft guidance ICH Q3D [63]. Inductively

coupled plasma mass spectrometry (ICP-MS) analysis showed that $Cu(py)_4(OTf)_2$ can be effectively removed, with the labelled material containing substantially less copper residue than any level of concern.

5. Purification and Other Considerations for Radioiodination

After radioiodination, purification is necessary to remove unreacted radioiodine, precursors, and byproducts. The most common method for purifying radioiodinated peptides is HPLC. HPLC allows for high separation efficiency and can separate the radiolabeled product from impurities based on their physicochemical properties [64]. It is easy to monitor with UV and gamma detection and allows for high separation efficiency. Another useful method for separating radioiodinated peptides from unreacted iodide, especially when the peptide's structure is well-defined and there is only one labeling site, is Gel Permeation Chromatography (GPC)—examples include Sephadex G-50, G-25 or LH-20 [65,66]. Solid-Phase Extraction (SPE) with C18 cartridges can also be used to remove unreacted radioiodide, but it has limitations such as low separation efficiency [67]. SPE is generally useful for single separations based on evident lipophilicity differences. Finally, solid-state synthesis combined with simple filtration can be used to isolate radioiodinated products. For example, filtration through SiO_2 was used to separate [^{125}I]SIB [58]. Several other factors must be considered for effective radioiodination such as the following: (1) Radiochemical Yield (RCY): RCY is the percentage of the radioactive material that is incorporated into the desired product. Optimizing reaction conditions is crucial to achieve high RCY [68]. (2) Radiochemical Purity (RCP): RCP is a measure of the proportion of the total radioactivity that is associated with the desired product. High RCP is essential for accurate imaging and effective therapy [69]. (3) Molar Activity: Molar activity refers to the amount of radioactivity per mole of the radiolabeled compound. High molar activity is desired for better imaging contrast and to minimize the amount of administered substance. (4) Stability: The stability of the radiolabeled peptide is crucial for maintaining its integrity and biological activity during in vivo studies and clinical applications. (5) Scale of the Reaction: Radioiodination is typically carried out in micromolar amounts, and reoptimization is needed to adapt the technique to larger amounts. (6) Metabolic Stability: Understanding the metabolic pathways of the radiolabeled drug is crucial to prevent possible deiodination processes [13]. For clinical applications, especially where radiotracers are intended for intravenous administration, purification must comply with stringent pharmaceutical standards, including radiochemical purity, sterility, and apyrogenicity [70]. Rapid purification is essential due to the short half-lives of iodine isotopes such as ^{123}I and ^{124}I. Solid-phase extraction (SPE) is one of the most commonly employed techniques, using cartridges such as C18, alumina, or ion exchange columns to remove unreacted iodine and by-products; this method can be performed in-line and is highly amenable to automation under good manufacturing practice GMP conditions [71]. Semi-preparative HPLC offers high resolution and is often used in combination with fraction collection and sterile filtration (e.g., through 0.22 μm filters) for the purification of I-labeled antibodies and larger biomolecules. Quenching agents and scavengers (e.g., sodium thiosulfate or sodium metabisulfite) are sometimes used during work-up to neutralize residual oxidants. The choice of purification strategy is guided by the nature of the radiotracer, its formulation route, and the need for rapid turnaround in clinical settings [72,73].

6. Modern Examples of Biological Applications of Radioiodinated Peptides

Building on the radioiodination methodologies discussed above, we next highlight several recent applications starting with a recent one in the field of systemic amyloidosis imaging and therapy. Systemic amyloidosis is a progressive disorder marked by the extra-

cellular deposition of amyloid fibrils in organs and tissues, leading to organ dysfunction. Current treatments primarily focus on reducing the production of amyloid precursor proteins. However, a curative approach likely necessitates the removal of existing amyloid deposits. A recent study describes the development and characterization of a prototypic pan-amyloid binding peptide–antibody fusion molecule, murine Igp5 (mIgp5), designed to enhance macrophage uptake of amyloid [74]. Radiolabeling was fundamental for achieving the results. The mIgp5 molecule is a murine IgG1-IgG2a hybrid immunoglobulin with a pan amyloid-reactive peptide, p5, genetically fused to the *N*-terminal of the immunoglobulin light chain. The p5 peptide is a synthetic polybasic l-amino acid reagent known to bind amyloid fibrils and associated heparan sulfate glycosaminoglycans through electrostatic interactions. The researchers radiolabeled mIgp5, along with m11-1F4 and peptide p5, with iodine-125 (^{125}I). This was achieved using chloramine T as an oxidant.

The radioiodinated products served several critical purposes in the study. Firstly, they were used in in vitro binding studies, specifically a suspension-phase pulldown assay, to assess differences in solution phase reactivity with amyloid-like fibrils and patient extracts. This also functioned as a quality control step to ensure the radiolabeling process did not compromise the binding capabilities of the molecules.

Secondly, and perhaps most significantly for in vivo investigations, ^{125}I-mIgp5 was used in biodistribution measurements in a murine model of systemic AA amyloidosis and in healthy wild-type control mice. This involved small animal SPECT/CT imaging to visualize the distribution of radioactivity over time. Measurement of tissue radioactivity in harvested organs was also studied, expressed as percent injected dose per gram of tissue (%ID/g), to quantify the uptake and retention of ^{125}I-mIgp5 in different organs. Finally, microautoradiography (ARG) of tissue sections was used to determine the microdistribution of ^{125}I-mIgp5 at a cellular level and to assess its co-localization with amyloid deposits identified by Congo red staining.

These in vivo studies demonstrated that ^{125}I-mIgp5 bound rapidly and specifically to amyloid deposits in organs such as the liver and spleen of mice with AA amyloidosis, with sustained retention for up to 72 h. Importantly, there was minimal non-specific uptake in healthy tissues. In contrast, the control molecule ^{125}I-mIgp5G, with a mutated peptide sequence, showed no significant retention in amyloid-laden organs, highlighting the specificity of mIgp5 for amyloid. The findings from the autoradiography precisely correlated the presence of the radioactive tracer with the amyloid deposits, further confirming the in vivo amyloid-targeting ability of mIgp5.

Radioiodination of the stapled peptide VIP116 was successfully achieved using a dehalogenation-resistant radioiodination prosthetic agent SIB [75]. The conjugation of the [*I]SIB prosthetic agent was specifically directed to a lysine residue present on the VIP116 peptide. This lysine residue is connected via an aminohexanoic spacer at the *N*-terminus of the peptide. The synthesis process involved two main steps, similar to the preparation of the non-radioactive analogue: (1) Synthesis of SIB (the radioiodinated [*I]SIB itself was first synthesized). This was accomplished using a tributyltin precursor and *N*-chlorosuccinimide as the oxidizing reagent with a yield of 75.9 ± 7.8%.

The conjugation of the synthesized [*I]SIB to 50 µg of VIP116 was carried out by incubating the labelling mixture at room temperature for 20 min. The conjugation yield obtained was 46.2 ± 8.0% relative to the starting [^{125}I]SIB activity. Following the conjugation reaction, the radioiodinated peptide, [*I]SIB-VIP116, was purified. Reversed-phase high-performance liquid chromatography HPLC (RP-HPLC) was utilized for this purification step. Subsequently, the purified [*I]SIB-VIP116 underwent solid-phase extraction (Empore™ C18) to remove residual acetonitrile from the formulation. The purified product was stored in an elution mixture comprising ethanol and PBS in a 60:40 ratio at 4 °C. The synthesized

[*I]SIB-VIP116 exhibited high stability when stored at 4 °C in its elution mixture, remaining 97.7% intact at day 7. In vitro studies in human serum at 37 °C also confirmed high stability, with 98.2% intact at 1 h and only decreasing slightly to 96.0% intact after 72 h of incubation. The final labeled peptide was measured to have a lipophilicity (logD) of 1.45 ± 0.05 (n = 4), suggesting a generally lipophilic character. It is speculated that the SIB conjugation may have contributed to this lipophilicity.

Prostate-specific membrane antigen (PSMA) imaging probes are crucial for prostate cancer diagnosis and therapy [76,77]. Two boronic precursors were synthesized by Kondo et al. [78] for the radiosynthesis of the ^{125}I-labeled PSMA probe, namely [^{125}I]mIB-PS: a carboxylic acid-unprotected precursor and a carboxylic acid-protected precursor. The protected precursor was synthesized from a *t*Bu-protected peptidomimetic urea precursor and 3-boronobenzoic acid, followed by purification (yield: 60.2%). The unprotected precursor was obtained by treating the protected one with trifluoroacetic acid (yield: 73.0%).

The copper-mediated radioiodination was performed using these precursors (Figure 16).

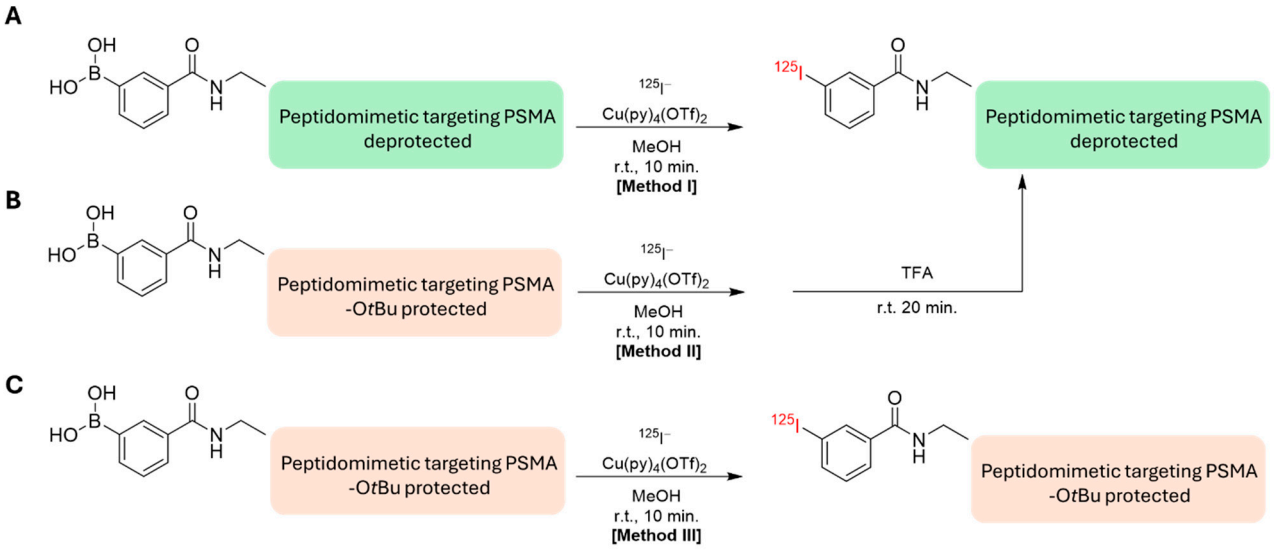

Figure 16. Radiosynthesis of [^{125}I]mIB-PS via boronic precursors using the three methodologies described (Method I–III) by Kondo et al. [78]. (**A**) Method I; (**B**) Method II; (**C**) Method III.

Method I (unprotected precursor): the molecule reacted with Cu(py)$_4$(OTf)$_2$ and [^{125}I]NaI in methanol at room temperature for 10 min, followed by HPLC purification. The radiochemical yield for [^{125}I]mIB-PS was 77.1% ± 6.0%.

Method II (protected precursor): the molecule reacted with the copper catalyst and [^{125}I]NaI at room temperature for 10 min. After solvent removal, TFA was added to perform continuous deprotection of *t*Bu groups at room temperature for 20 min, without intermediate purification. The product was then purified by HPLC to yield [^{125}I]mIB-PS. This method resulted in a significantly higher RCY of 93.8% ± 2.6%.

Method III (protected precursor, no deprotection): using the protected molecule under the same conditions as Method II but omitting the TFA deprotection step yielded the protected product, [^{125}I]mIB-PS(tBu), with an even higher RCY of 98.0 ± 1.6%.

The study confirmed that the copper-mediated radioiodination successfully produced [^{125}I]mIB-PS under mild conditions (room temperature, 10 min reaction time). Using the carboxylic acid-protected boronic precursor in Method II provided a higher RCY for the final product compared to the unprotected precursor (Method I). The continuous deprotection step in Method II also streamlined the synthesis process.

The synthesis of the radioiodinated SARS-CoV-2 receptor binding domain (RBD) probe involved labeling the RBD protein with the positron-emitting medical nuclide, ^{124}I [79]. The radioiodination was achieved using an *N*-bromosuccinimide(NBS)-mediated method.

Successful synthesis yielded ^{124}I-RBD with a radiochemical yield of 83.9% ± 4.6%. Radio-TLC analysis demonstrated a radiochemical purity of over 99% for the purified ^{124}I-RBD. Stability studies showed that ^{124}I-RBD maintained a radiochemical purity of over 99% after incubation in saline or 5% human serum albumin at room temperature for 120 h (5 days), indicating high in vitro stability. Quality control parameters, including appearance, volume, pH, radiochemical purity, ethanol content, endotoxins, sterility, and specific activity, were within the specified limits.

This radioiodination technique produced a stable ^{124}I-labeled RBD probe suitable for further biological evaluation.

Cyclic RGD peptides (cRGDs) are recognized ligands for integrins [80]. The radioiodination technique employed for the synthesis of the radiolabeled bicyclic RGD peptide derivatives in the study of Kondo et al. [81] involved site-specific radioiodination using [^{125}I]SIB. This approach was selected based on the design of the peptide precursors, which lacked other reactive functional groups except for specific ε-amino groups at the *N*-terminal Lys in bcRGDpal and bcRGDiba, and the amino group of the terminal β-alanine in bcRGDazide, thus directing the radioiodination to these sites.

For the synthesis of the single-unit probes, specifically [^{125}I]bcRGDpal and [^{125}I]bcRGDiba, the corresponding peptide precursor (200 μg) was dissolved in a mixture of borate buffer (0.1 M, pH 8.5) and acetonitrile. This solution was then combined with [^{125}I]SIB (10–20 MBq) dissolved in acetonitrile. The reaction mixture was incubated at 40 °C for 1 h. Following the reaction, the desired radiolabeled peptides were purified using reversed-phase HPLC. This purification step successfully separated the radiolabeled products from any unreacted precursor, ensuring they were obtained in a non-carrier-added form. The RCY from [^{125}I]SIB was 59% for [^{125}I]bcRGDpal and 70% for [^{125}I]bcRGDiba. Both probes demonstrated radiochemical purities exceeding 99% after HPLC purification.

The synthesis of the dimeric probe, [^{125}I]bcRGDdimer, involved a multi-step process. First, the radiolabeled precursor [^{125}I]bcRGDazide was prepared using the same procedure as for [^{125}I]bcRGDpal and [^{125}I]bcRGDiba, utilizing [^{125}I]SIB. The RCY for [^{125}I]bcRGDazide from [^{125}I]SIB was 40%, with an RCP > 99%. Subsequently, [^{125}I]bcRGDdimer was synthesized via a click chemistry reaction. The reaction mixture contained equimolar amounts of [^{125}I]bcRGDazide and the non-radiolabeled bcRGDalkyne precursor, along with $CuSO_4 \cdot 5H_2O$ (50 eq.) and ascorbic acid (100 eq.), dissolved in dry DMF. This mixture was stirred for 1 h at room temperature. Similar to the single-unit probes, the dimeric product was purified by RP-HPLC. The RCY for [^{125}I]bcRGDdimer from [^{125}I]bcRGDazide was 61%. The overall yield for [^{125}I]bcRGDdimer from [^{125}I]SIB was lower (24%). Despite the lower overall yield, the RCP of [^{125}I]bcRGDdimer also exceeded 99% after purification.

7. Conclusions

The field of radioiodinated peptide synthesis continues to evolve, driven by the need for more efficient, selective, and stable radiolabeling techniques. The choice between direct and indirect radioiodination methods depends on multiple factors, including the peptide's structure, functional groups available for labeling, and the intended application. While direct iodination remains a widely used approach due to its simplicity, indirect methods provide greater flexibility and control over labeling site specificity, thereby preserving peptide functionality and improving in vivo stability.

Advancements in radioiodination chemistry have led to the development of novel prosthetic groups, click chemistry techniques, and improved purification strategies, all of

which contribute to the enhanced efficiency of radiolabeling. Additionally, recent innovations in computational modeling and high-throughput screening have facilitated the optimization of reaction conditions, reducing the time and resources required for peptide radiolabeling. These technological improvements not only expand the scope of radiopharmaceutical applications but also enhance the translational potential of radioiodinated peptides from bench to bedside. Another key frontier is the development of dual-modality imaging agents, where radioiodinated peptides are conjugated with fluorescent or MRI-active probes for complementary diagnostic insights [82,83]. Another promising direction in radionuclide theranostics lies in the development of double-labeled peptide-based probes ensuring consistent pharmacokinetics across both diagnostic and therapeutic applications. By leveraging the strengths of dual-labeling strategies, we can achieve higher imaging resolution with PET tracers such as gallium-68 while simultaneously enabling effective targeted radionuclide therapy with isotopes like lutetium-177, actinium-225 or iodine-131 [80,84–86].

Despite these significant advancements, challenges remain in the field of radioiodination. One major hurdle is the potential for deiodination in vivo, which can lead to reduced imaging contrast and off-target effects [13]. Strategies to address this issue include the use of metabolically stable prosthetic groups and the incorporation of iodine isotopes into structurally protected positions within the peptide. Additionally, further research is needed to refine purification protocols to achieve high radiochemical purity while minimizing peptide degradation.

Looking ahead, the continued exploration of novel chemical methodologies, coupled with advancements in radiopharmaceutical development, will pave the way for more effective and versatile radioiodinated peptides. The integration of artificial intelligence and machine learning in radiochemistry is expected to further optimize labeling strategies, enabling the rapid identification of the most suitable conditions for peptide radioiodination [87,88]. Moreover, the development of dual-labeled peptides that combine radioiodine with other imaging or therapeutic isotopes presents an exciting avenue for multimodal diagnostics and theranostics [84].

In conclusion, radioiodinated peptides remain at the forefront of molecular imaging and targeted therapy, offering invaluable tools for the diagnosis and treatment of various diseases. The continuous refinement of radiolabeling strategies will not only improve the efficacy of these radiopharmaceuticals but also expand their clinical applicability. By addressing current challenges and leveraging cutting-edge technologies, the field of peptide radioiodination is poised for significant breakthroughs that will further advance the landscape of nuclear medicine.

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