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Review

Strategies for radiolabelling antibody, antibody fragments and affibodies with fluorine-18 as tracers for positron emission tomography (PET)

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This paper is dedicated to Professor Antonio Togni on the occasion of his American Chemical Society Award for Creative work in Fluorine Chemistry.

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ABSTRACT

The use of fluorine-18 as a radionuclide for positron emission tomography (PET) has become increasingly popular over the last two decades and cancer and neurology clinical centres worldwide are increasingly establishing competence in this modality for diagnostic imaging. Progress has been particularly impressive for small molecule pharmaceutical candidates and low molecular weight affinity peptides, where clearance rates of the peptides in the body are compatible with the half-life of fluorine-18 ($t_{1/2}$ = 110 min). However high molecular weight proteins present challenges as they circulate and clear much more slowly. This review focuses on the methods used to radiolabel antibodies and their derivatives with fluorine-18 as tracers for PET. The very high specificity of these biomolecules for disease indicators at the molecular level makes labelling them attractive, however antibodies can circulate for days within the blood, with slow clearance times due to their high molecular weights, and this is inconsistent with the relatively short half-life of fluorine-18. Thus, lower molecular weight fragments of antibodies present more realistic targets for labelling. This review describes the approaches and protocols which have been successfully used to radiolabel antibodies and particularly antibody fragments with fluorine-18, and highlights this challenging aspect of fluorine-18 labelling for PET imaging.

1. Introduction

Positron emission tomography (PET) is a widespread imaging modality for clinical applications such as: disease diagnosis, tumour detection and brain imaging [1]. The fluorine-18 isotope is particularly suitable for use as a positron emitter owing to its half-life ($t_{1/2}$ = 110 min) which is long enough to achieve relatively elaborate synthetic sequences from cyclotron generated fluoride-18. When compared to other commonly used radionuclides; oxygen-15 ($t_{1/2}$ = 2 min), nitrogen-13 ($t_{1/2}$ = 10 min) and carbon-11 ($t_{1/2}$ = 20 min), the longevity of fluorine-18 represents a clear advantage. Furthermore, the positron emission energy of fluorine-18 is weaker than all other first row positron emitters which optimises resolution in imaging [2,3]. On the other hand, the half-life of fluorine-18 is short enough to achieve a high signal to noise ratio on a timescale which complements the pharmacokinetic profile of pharmaceutical type molecules of interest, in contrast to more persistent heavier radionuclides [4].

Consequently, there is a mature research activity focussed on radiolabelling with the fluorine-18 isotope. To date the focus has been on low molecular weight drug like molecules and extending to bioactive peptides with molecular weights in the 1–4 kDa range. The area has been the subject of a number of recent comprehensive reviews [5–9]. With the advent of antibody engineering technologies, there has been a steady interest in exploring technology for the radiolabelling of antibodies for clinical imaging, due to their exquisite affinity to molecular markers of disease on cell surfaces. Early reports tended to focus on the labelling of whole antibodies (160 kDa) but clinical applications have been hindered by slow circulation kinetics resulting from their high molecular weights [10]. Typically, antibodies circulate in the blood for several days rendering them incompatible with fluorine-18 due to a disparate shorter half-life. As a result, efforts to radiolabel smaller antibody fragments (25–110 kDa), which circulate much more rapidly, have been pursued and with more success. These fragments are composed of various derivatives of their parent antibodies (Fig. 1) but universally incorporate an antigen-binding site to retain binding affinity. To reduce molecular weight further the radiolabelling of even smaller non-antibody, antigen-targeting molecules known as affibodies (6.5–14 kDa) has been explored and these possess additional advantages such as chemical stability [11].

The limited half-life of fluorine-18 demands that isotopic incorporation into the biomolecule is completed as fast as possible to prevent excess radioactive decay. The fluorine-18 isotope is generated in a cyclotron wherein the bombardment of oxygen-18 enriched water [$^{18}OH_2$] with high energy protons generates an aqueous solution of

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fluoride-18 in pico- to nano- molar concentrations [3]. Difficulty arises when attempting to utilise this solution for labelling because of the poor nucleophilicity of aqueous fluoride [12]. Methods generally rely on a drying step wherein water is removed and fluoride-18 is fixed as its potassium salt in an aprotic organic solvent with a phase transfer catalyst (usually kryptofix) to bind K^+. The resulting organic solution containing soluble fluoride-18 ion is then used to radiolabel a prosthetic group which can be selectively conjugated to a desired moiety on the biomolecule [13]. Recently efforts have been made to circumvent the need for drying entirely. These strategies include; exploitation of the labile B(boron)-F bond to facilitate isotopic exchange in aqueous media [14], the use of chelating ligands to trap Al(aluminium)-fluorine-18 [15], aqueous inverse electron demand Diels-Alder (IEDDA) reactions [16] and enzymatic methods [17]. Also of note are pre-targeting strategies which typically involve the injection of an antibody-streptavidin construct several days prior to administration of a low molecular weight fluorine-18 tagged biotin [18].

1.1. *Antibody and antibody fragments*

Antibodies are large proteins (immunoglobulins − Igs) which have different functions in the immune system. Immunoglobin G (IgG) is the most abundant (Fig. 1). Structurally they have two heavy (H) and two Light (L) polypeptide chains, held together through disulphide bridges. The H and L chains can be divided into variable (V) and constant (C) domains. The variation in the V_H and V_L chains determines the unique antigen-recognition site. IgG has a high molecular weight of 160 kDa, resulting in its persistence in the circulatory system for several days after injection. However, molecular weight fragments of the antibody present better candidates for radiolabelling as their uptake and clearance times are more rapid. In this review radiolabelling of several categories of engineered antibody fragments are highlighted. This inset provides an indication of the topology of the different fragment categories.

The Fc region of antibodies is not associated with antigen binding but fulfils a number of biological roles; it binds to receptors on target cells and confers effector functions which result in the destruction of the targeted cell [19,20]. The Fab region is the antigen-binding domain and is therefore integral to biotechnological applications. The region consists of two constant domains (the light domain C_L and the heavy domain C_H1) and two variable domains (the light domain V_L and the heavy domain V_H) the latter of which is responsible for the unique specificity of the antigen binding site. The antibody fragments discussed here will include; the $F(ab')_2$ homodimer fragment (~110 kDa), the Fab' fragment (~55 kDa), the Fab fragment (~50 kDa) and the Fv/scFv fragment (~25 kDa). Diabodies (~55 kDa) are non-covalent dimers of the Fv fragment ($(Fv)_2$) or the scFv fragment ($(scFv)_2$), in the case of cys-diabodies a free thiol moiety is present which leads to disulphide-stabilisation. The $(Fab')_2$ homodimer consists of both Fab regions linked by disulphides and therefore retains the entire binding region of the antibody. The Fab' fragment is derived from the homodimer by enzymatic reduction and retains a free thiol group. In contrast, the Fab fragment does not retain the thiol moiety and consists only of the four domains (C_L, C_H1, V_L and V_H) linked by disulphide interactions. In contrast Fv and scFv consist only of the two variable domains (V_L and V_H) and in scFv fragments these variable domains are linked by a hydrophilic peptide chain consisting of about 15 amino acid residues. Similarly a dsFv fragment is based on Fv but with an interchain disulphide bond [20,21]. A promising class of engineered proteins are affibodies which combine the recognition properties of antibodies with several desirable traits including small size (6.5–14 kDa) and increased stability to heat and harsh chemical conditions. Affibodies consist of 58 amino acids arranged into three helices. Of these, 13 residues may be subject to mutagenesis to determine the specificity of binding. These residues have been varied combinatorially to provide extensive libraries of affibodies each with a unique binding site on a target antigen [22,23].

Another class of Immunoglobulin G is the camelid-derived heavy-chain variant which possesses a non-binding region as found in the Fc region of conventional IgG but a distinct binding region consisting only of a single variable domain V_{HH}. The V_{HH} fragment is also known as a nanobody, reflecting its relative low molecular weight (12–15 kDa) which aids its biotechnological application. Furthermore, nanobodies possess properties such as low immunogenicity and high stability which make them particularly suitable probes [24].

1.2. *Strategies for fluorine-18 labelling of antibodies and their fragments*

Early methods for the radiolabelling of antibodies and derivatives utilised radiolabelled small molecules for conjugation (Fig. 2). The first application of fluorine-18 for antibody labelling was reported in 1987 when the alkylating agents **7** and **8** were used to radiolabel the antibody IgA (162 kDa) [25]. The synthesis of **7** (20–33% yield, 45–50 min) was achieved from S_NAr of 3,5-dinitrobenzonitrile no carrier added (n.c.a.) fluoride-18, followed by the addition of sodium methoxide. The synthesis of **8** (28–40% yield, 75 min) also began with an S_NAr utilising n.c.a. fluoride-18 with 4-nitrobenzonitrile and was elaborated to the final product by addition of MeLi and $CuBr_2$. Antibody conjugation was conducted under ambient aqueous conditions (borate buffer, pH 8.0, 47 °C, 1 h). The radiochemical yields (RCY) for the labelling of IgA were 16% for **7** and 46% for 8 (Fig. 2). Later, 8 was utilised to label the F(ab') 2 fragment of the murine BB5-G1 antibody (specific for a human parathyroid antigen) in 10% yield (from 8) [26]. The labelled fragment displayed specificity of 10:1 parathyroid-to-muscle after 4 h in nude mice models.

Later the mouse Mel-14 antibody $(Fab')_2$ fragment was radiolabelled with fluorine-18 [13,27,28]. Radiolabelling was achieved using **1**, which was synthesised by S_NAr of **10** to give **6** (Scheme 1). Subsequently, oxidation and N-hydroxysuccinamide (NHS) coupling were used to obtain **1** (100 min, RCY 25%). Conjugation was achieved under ambient aqueous conditions (pH 8.5 borate buffer, 15–20 min) in a 40–60% yield which was an improvement relative to the previously described methods involving **7** and **8**. *In vitro* binding and mouse tissue distribution studies showed similar results to those observed for the antibody fragment tagged with the ^{125}I analogue of **1**. Later refinements to the method reduced the synthesis and purification time of **1** to about 45 mins [29]. These included an increased temperature for all steps and the use of dicyclohexyl carbodiimide (DCC) as the coupling promoter.

Additional early work utilised the prosthetic group **3** [30–34]. The synthesis of **3** usually begins with an S_NAr reaction of **11** with tetrabutylammonium [^{18}F]fluoride to give **12**. Amide coupling with **12** gives **3** (Scheme 2). Conjugation to the MeI-14 $F(ab')_2$ and the TP-3 Fab fragments were achieved under analogous conditions as developed for **1**. A comparative study showed that the total synthesis time (85 min) for **3** was shorter than that (95 min) for **1**, but that the overall radiochemical yields were similar (RCY 10–12%) [30].

Attempts to improve upon **1** as a prosthetic group continued with the exploration of **9** for radiolabelling of IgG and the IL-2α dsFv fragment [35,36]. The synthesis of **9** is achieved by an S_N2 reaction of **13** with fluoride-18 (Scheme 3). This significantly reduced the preparation time (30–35 min) relative to **1**, although RCYs (10–20%) were modest due to the formation of a side product resulting from ester cleavage. Labelling of IgG (borate buffer, pH 8.5, 10–15 min) was achieved in a 50–70% yield. The overall RCY was 10% and accomplished in 65 min including purification. The labelling of the IL-2α Fv fragment (borate buffer, pH 8.5, 10 min) with **9** was achieved in 33–45% yield [36–38].

Wester et al., compared **1** and **5** as tags for radiolabelling antibody IgG [12]. Their improved synthesis of **1** was achieved in a 35% RCY in 55 min (Scheme 5). Subsequent labelling of IgG was achieved (borate buffer, pH 8.5, r.t, 15 min) in 48–60% RCY. Azeotropically dried [^{18}F] fluoride was used to convert **14** to **5** in an efficient 70% RCY in 4 min (Scheme 4). However, the labelling of IgG with **5** (15W UV lamp,