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Fluorinated Tags to Study Protein Conformation and Interactions Using ^{19}F NMR

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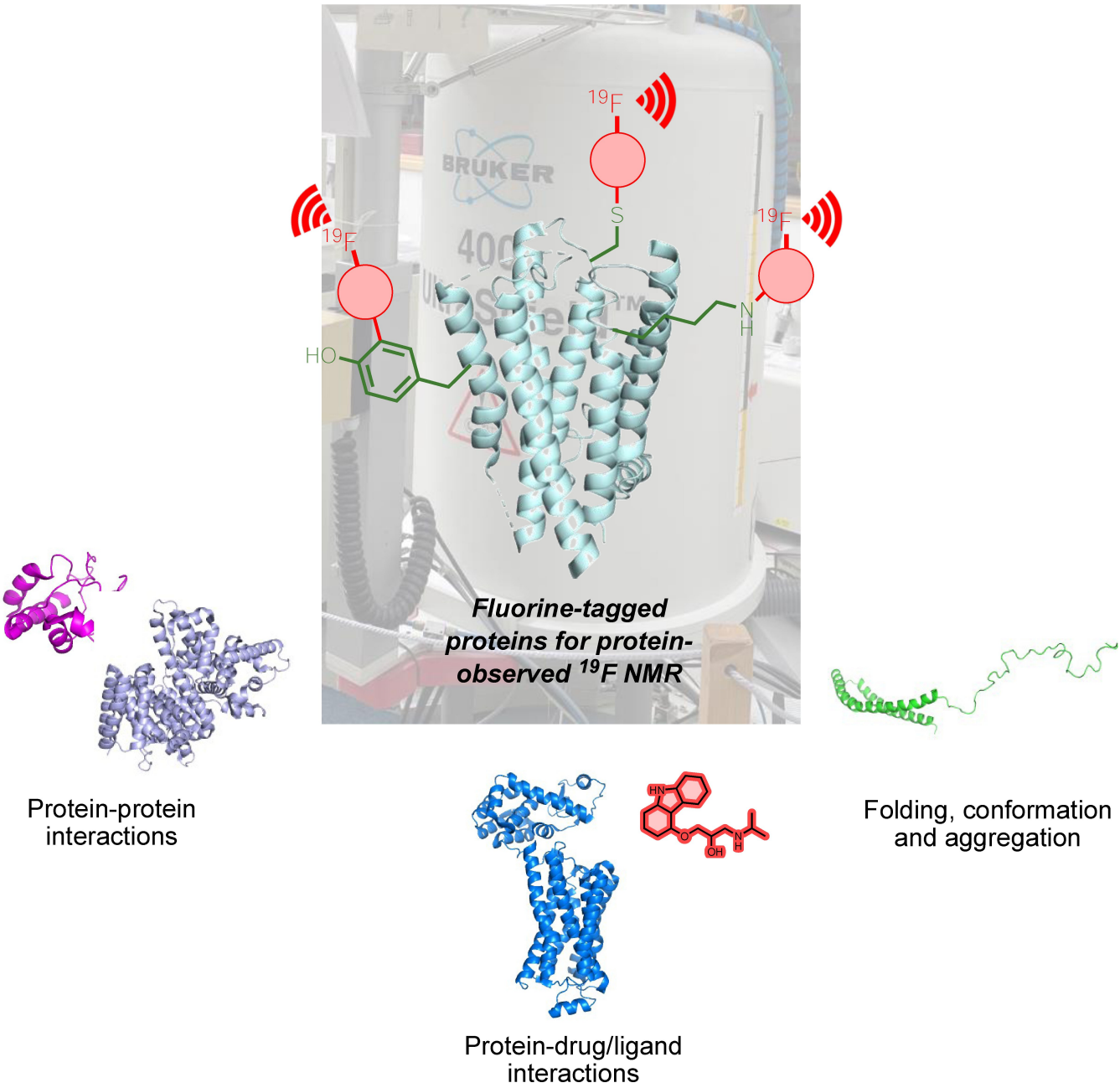

The incorporation of fluorine atoms into a biomacromolecule provides a background-free and environmentally sensitive reporter of structure, conformation and interactions using ^{19}F NMR. There are several methods to introduce the ^{19}F reporter – either by synthetic incorporation *via* solid phase peptide synthesis; by suppressing the incorporation or biosynthesis of a natural amino acid and supplementing the growth media with a fluorinated counterpart during protein expression; and by genetic code expansion to add new amino acids to the amino acid alphabet. This review aims to discuss progress in the field of introducing fluorinated handles into biomolecules for NMR studies by post-translational bioconjugation or 'fluorine-tagging'. We will discuss the range of chemical tagging 'warheads' that have been used, explore the applications of fluorine tags, discuss ways to enhance reporter sensitivity and how the signal to noise ratios can be boosted. Finally, we consider some key challenges of the field and offer some ideas for future directions.

1. Introduction

^{19}F NMR has been widely used for the study of protein folding, conformation, and protein-ligand interactions, as will be discussed in this review. The motivations to employ ^{19}F NMR for biological studies are numerous:[1,2] fluorine is scarcely found in nature in the form of organic fluorine, therefore, the study of fluorinated proteins, peptides and small molecules in biological samples using ^{19}F NMR typically exhibits no background signals. The spin $^1/_2$ ^{19}F nucleus also overcomes the inherent challenges of analyzing biological samples using ^{1}H NMR, namely the abundance of overlapping proton resonances and large water signals complicating the analysis; whilst proton-decoupling of ^{19}F NMR spectra further simplifies the spectra, producing single resonances for each species or conformer. Therefore, it is often possible to use simple 1D ^{19}F NMR spectra to detect changes of protein structure and conformation.[3–8] ^{19}F NMR exhibits a wide chemical shift window (~300 ppm) and high sensitivity to its local chemical environment. Combined with its 100% natural abundance and similarly high sensitivity to ^{1}H (83% of ^{1}H), ^{19}F NMR affords a particularly useful tool with which to detect and quantify (using appropriate relaxation delay times in the acquisition) different closely related species e.g., conformers and metabolites, as well as having applications in fragment-based ligand binding studies,[9–11] and for in-cell NMR experiments.[12–15]

Perhaps the most widely used way to 'tag' proteins to study them has been through the use of fluorescent labels, allowing the visualisation of spatial location of molecules of interest using fluorescence microscopy.[16] It is also possible to incorporate a number of different fluorescent labels into a system under study to determine e.g. protein conformation, co-localisation or proximity studies using FRET pairs.[17] Whilst the importance of fluorescent labels is undoubted; background fluorescence, photobleaching, spectral overlap and the size of the fluorescent label affecting the behavior of the system can be notable limitations to their use.[16] On the other hand, fluorine atoms or fluorinated groups are relatively small and mostly non-perturbing and are stable in aqueous conditions. A wide range of different fluorinated building blocks are also available commercially, which permits the incorporation of multiple ^{19}F reporters with a range of distinct chemical shifts, allowing simultaneous study of multiple species at atomic resolution.

Techniques such as X-ray crystallography and cryo-electron microscopy have allowed researchers to observe the molecular structures of proteins and their complexes in fine detail, however, these images typically represent only a static averaged snapshot of the protein structural ensemble. Proteins are dynamic species that can adopt a variety of conformations relating to their function. In the simplest of cases, some proteins switch between a series of 'active' and 'inactive' conformations, whereas in intrinsically disordered proteins, there exists a multitude of different conformations and oligomers. The sensitivity of ^{19}F NMR to different conformations is, therefore, well suited to investigating protein behavior. For example, the incorporation of trifluoromethyl methionine into amyloid-β residues 1–40 *via* solid phase peptide synthesis (Figure 1 A), followed by real-time ^{19}F NMR analysis allowed the identification of at least six transient oligomeric intermediates formed during the lag phase of fibrilization, which is difficult to detect by X-ray or fluorescence-based techniques.[18] ^{19}F NMR was also used to study the membrane interactions of α-synuclein, an intrinsically-disordered protein associated with Parkinson's disease by biosynthetically substituting the four native tyrosine residues using either 3-fluoro-L-tyrosine (3FY) or trifluoromethyl-L-phenylalanine (tfmF).[19] The resulting ^{19}F NMR spectra exhibited 4 distinct resonances, which were used to determine which parts of the protein were bound to membranes by observing signal broadening. The same approach showed that ^{19}F-labeled proteins were observable in cells at concentrations of 50–100 μM.[20] Notably, small (~10 kDa) proteins such as α-synuclein containing 3-fluoro-tyrosine were observable in *E. coli* cells, but for larger proteins the ^{19}F signals were undetectable. However, the incorporation of trifluoromethyl-L-phenylalanine (three times more fluorine atoms) allowed larger proteins (up to 100 kDa) to be observed in cells.

^{19}F NMR has also found use in studying protein-ligand interactions, for example, in probing the interactions of lectin from *Ralstonia solanacearum* with carbohydrates, by replacing binding-site tryptophans with various fluorinated tryptophan analogues.[21] The same *protein-observed* ^{19}F NMR approach can

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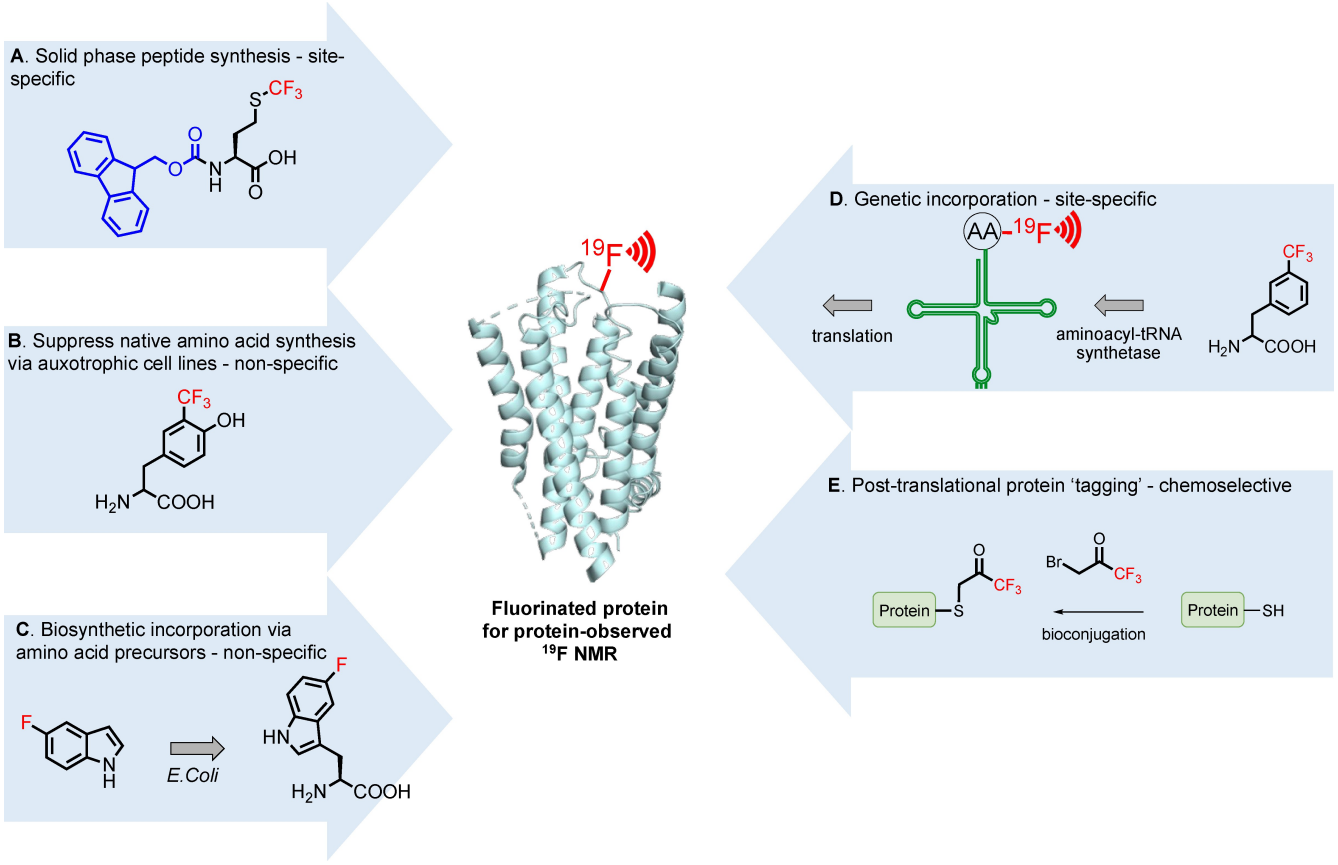

Figure 1. Approaches used to install ^{19}F NMR reporters into proteins.

also be used to understand *where* a ligand binds, for example, the dual-labelling of the KIX domain of CREB Binding Protein (CBP/p300) with 4-fluorophenylalanine and 3-fluorotyrosine led to the identification of a previously unknown small-molecule ligand binding site.[22] Indeed, this methodology has been particularly useful in fragment-based drug discovery campaigns, in which a fluorine-labelled protein is incubated with small molecule compound libraries to identify binders often by perturbation of the fluorine resonances[10,23] depending on the binding affinities and rates of exchange between bound and unbound states. Wherein, a strong binding interaction results in slow exchange and generally the observation of separate signals for the bound and unbound forms; weak binding affords a single signal at a chemical shift representing the weighted-average of the bound and unbound states, and intermediate strength interactions afford broad signals due to intermediate rates of exchange.[23] There are also several other NMR experiments used to detect binding interactions, such as nuclear Overhauser effect, saturation transfer difference, and T_2-relaxation spectroscopy. Therefore, protein-observed ^{19}F NMR has proved to be fruitful for identifying small molecule binders.[10,24–28] However, it is also possible to use similar ligand-observed ^{19}F NMR experiments to identify binders from fluorinated fragment libraries.[29] Indeed, one group has even

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developed a dedicated fluorinated compound fragment library of 115 fluorinated sp^3-rich compounds of diverse shape for ligand-observed ^{19}F NMR.[30]

Whilst we will discuss the benefits of protein tagging subsequently, in some cases where the protein contains several conjugatable residues e.g. GPCRs containing multiple cysteines, tagging may be less suitable than genetic incorporation. Wang *et al.* reported the genetic incorporation of *meta*-trifluoromethyl-phenylalanine into cannabinoid 1 (CB1) receptors expressed in insect cells.[31] Whilst considerably different active and inactive conformational states of CB12 have been observed in the crystal state, the ^{19}F NMR analysis was able to clearly report differences in chemical shift and line shape at two sites upon agonist and antagonist binding, showing that these two sites are sensitive to ligand-induced conformational changes. Given the environmental sensitivity of this technique, the authors also found that an allosteric modulator and agonists synergistically stabilise a previously unreported pre-active state.

The above is merely illustrative of some of the important examples of using ^{19}F NMR to study protein conformations and interactions. There are many other applications of ^{19}F NMR in biology, as we refer the reader to some excellent recent reviews on this topic.[2,32,33] For this review, we focus on the applications of protein 'fluorine-tagging' to install the ^{19}F NMR reporter *via* bioconjugation.

2. Discussion

2.1. Incorporating ^{19}F NMR Reporters into Proteins

As alluded to in the introduction, fluorine has been readily incorporated into peptides as a reporter for ^{19}F NMR analysis using solid phase peptide synthesis and installing synthetic fluorine-containing amino acids (Figure 1 A), however, notwithstanding native chemical ligation approaches, this is fundamentally limited to relatively small biomolecules <100 amino acids. The first functionally active fluorinated proteins e.g., aldolase and glyceraldehyde-3-phosphate dehydrogenase produced by biosynthetic means were prepared and isolated in 1961 by feeding rabbits with fluorinated tyrosine and phenylalanine.[34] One way to more efficiently incorporate fluorinated amino acids into a protein is to suppress the biosynthesis of the non-fluorinated counterpart e.g., by using auxotrophic cells (Figure 1 B). In 1974 fluorine-labelled alkaline phosphatase was isolated from *E. coli* after supplementation of cell culture media with 3-fluorotyrosine, and was used to study its

motional properties by analysis of ^1H–^{19}F dipolar relaxation.[35,36] This approach can suffer from non-specific incorporation at several sites, the non-canonical amino acid not being recognised by the host translational machinery and can afford a number of fluorinated proteins due to non-selective incorporation.[8] Furthermore, the supplementation of culture media with fluorinated amino acids can cause bacterial growth inhibition, which presents a further challenge.[38] However, recently it was even shown that 5-fluoroindole could be used as a precursor to replace native tryptophan residues with 5-fluorotryptophan *via* a

standard *E. coli* expression system (Figure 1 C).[39] This was used to replace all five native tryptophans in HIV-1 capsid proteins so that each fluorinated residue exhibited distinct ^{19}F NMR resonances due to each experiencing different local environments and allowed the study of tubular capsid assembly by fast magic angle spinning (MAS) ^{19}F NMR.[40]

Modern molecular biology techniques allow a wider pool of fluorinated amino acids to be site-selectively incorporated into recombinant proteins in place of a canonical equivalent using orthogonal aminoacyl-tRNA synthetase/tRNA pairs (Figure 1 D).[41–43] However, this is of course limited to studying recombinant proteins. In some cases, incorporation of the non-canonical amino acid can give variable yields and incomplete protein labelling. This leads to heterogeneous products if incorporating multiple different ^{19}F labels, which complicates the NMR analysis.[44] Finally, the range of fluorinated amino acids that can be accepted by current expression systems is still relatively limited.[33]

Chemical approaches that post-translationally incorporate fluorinated reporters for ^{19}F NMR *via* bioconjugation or 'tagging' enable straightforward multi-labelling with very high efficiency and allows the design of a significantly more diverse set of tools with different chemical shifts and environmental responsiveness. Moreover, tagging of native human proteins in e.g., patient derived samples, may provide a paradigm shift, in for example, disease diagnosis. To this end, tagging can be achieved by fluoroalkylation of proteins using fluorinating reagents such as Togni's reagent,[45] however, more selective modification is achieved by targeting reactive, thiol and hydroxy groups under mild and non-denaturing conditions as discussed below. Thus far ^{19}F-tagging proteins has mostly relied on conjugating tags to naturally occurring solvent-exposed amino acid side chains, and in some cases, through introducing non-native nucleophilic residues through mutagenesis.

2.2. Current Fluorine-Tagging Approaches and Applications

Bioconjugation with fluorinated reporters, or 'fluorine-tagging', conceptually offers the opportunity for post-translational modification of proteins with a sensitive NMR reporter tag. One main advantage of this approach is that, unlike recombinant expression or protein total synthesis methods, fluorine can be incorporated into native proteins or even patient samples. Site-specific tagging, with fluorine, gives chemical biologists the ability to focus on local level conformation changes amongst the complex biological milieu. Cysteine is the most targeted and convenient residue for modification of proteins due to its high nucleophilicity, which can afford selectivity, and its relative sparsity in native proteins, accounting for ~2.3% of residues in the human proteome.[46] Unsurprisingly, around 36% of covalent warheads target cysteine,[47] and cysteine-reactive warhead groups include haloacetamides, acrylamides, and maleimides (Figure 2).

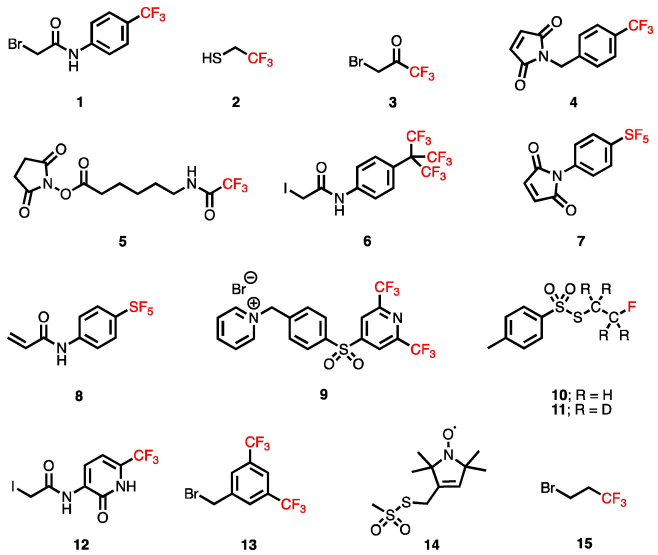

Figure 2. Examples of covalently reactive fluorine tags for protein ^{19}F NMR.

Fluorine-tagging of proteins has mostly focused on studying dynamic changes to individual protein structures, as well as the intermolecular association of proteins. Manglik and co-workers employed 2-bromo-N-(4-(trifluoromethyl)phenyl)acetamide (**1**) to probe the dynamics of the β_2-adrenergic receptor.[48] Labelling of an endogenous cysteine residue (Cys265) was easily achieved with 100% efficiency in HEPES buffer (pH 7.5), by initially employing TCEP (tris(2-carboxyethyl)phosphine) as a disulfide reducing agent, before the introduction of the fluorine tag and cooling overnight (Figure 3). The single fluorinated modification was able to differentiate distinct conformational states by exhibiting individual chemical shifts in a one-dimensional ^{19}F NMR spectrum (Figure 3). The peak area of each signal directly reflected the relative conformational population, whilst the line widths represented conformational heterogeneity and exchange rate between each state. This is a remarkable amount of information from one subtle chemical modification. Horst *et al.* also studied the β_2-adrenergic receptor but looked to probe the temperature dependence of the receptor conformation by adding 2,2,2-triflouroethanethiol (**2**) to the purification buffer to separately tag two different cysteine-residues (Cys265 and Cys327) *via* disulfide formation.[49] This allowed the determination of thermodynamic parameters that describe the equilibria between the active and inactive state using ^{19}F NMR saturation transfer experiments.

Hellmich and co-workers employed 3-bromo-1,1,1-trifluoroacetone **3** to tag *all three* native cysteine residues of a proteorhodopsin transmembrane pump protein with 100% efficiency (by Ellman's test).[50] They demonstrated that each tagged cysteine residue exhibited a distinct chemical shift by ^{19}F NMR, with different integral intensities (Figure 4). Having three unique fluorine environments dispersed throughout the protein structure enabled the monitoring of its dynamic nature. For comparison, cysteine labelling with fluorophores would not be able to identify changes at individual residues in this way.

Edwards and co-workers demonstrated that multiple *different* fluorine tagged species can be studied simultaneously as long as the tags exhibit different chemical shifts.[51] Independent labelling of two oppositely charged proteins, bovine serum albumin and cytochrome C using tag **3** (200% efficiency i.e. two tags conjugated) in tandem with 1-(4-(trifluoromethyl)benzyl)-1H-pyrrole-2,5-dione (**4**) (160–200% efficiency), respectively, they probed whether the proteins formed heterodimers. The unique chemical shifts of tags **3** and **4** afforded distinct NMR signals at different chemical shifts (Figure 5). The study showed that whilst the proteins self-aggregation was influenced by the presence of each other, there was no formation of heterodimers even at high concentrations. They went on to use this approach study a pair of much larger monoclonal antibodies, tagging one with **3** and a

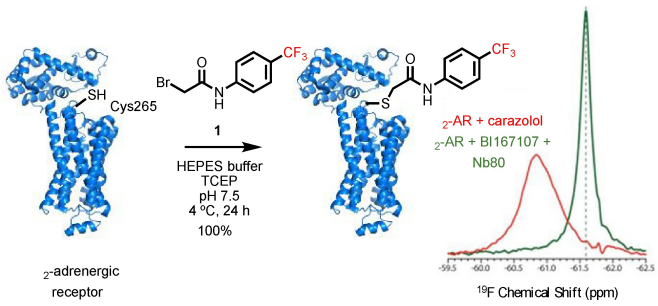

Figure 3. Reaction conditions for tagging β_2-adrenergic receptor with bromo-acetamide **1**. The ^{19}F NMR spectrum showed the fluorine-tags were sensitive to different ligand binding based on structural perturbations of the protein. Figure adapted with permission from reference [48] (copyright of Elsevier).

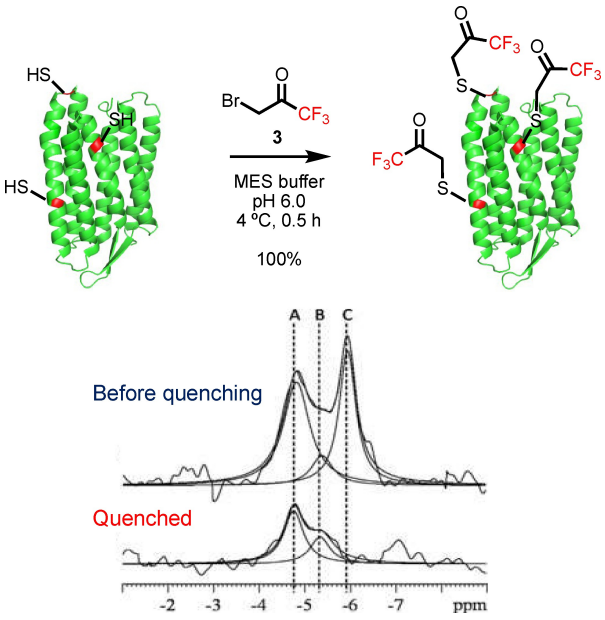

Figure 4. Reaction conditions for tagging with **3** that were employed by Hellmich and co-workers and the predicted locations of the three labelled cysteine residues (labelled red) are shown in a proteorhodopsin homology model. Below: the resulting MAS ^{19}F NMR spectrum before and after quenching of signal C by addition of a paramagnetic quencher $MnSO_4$. This data shows that signal C is the most solvent exposed. Figure adapted with permission from reference [50] (copyright of Wiley).

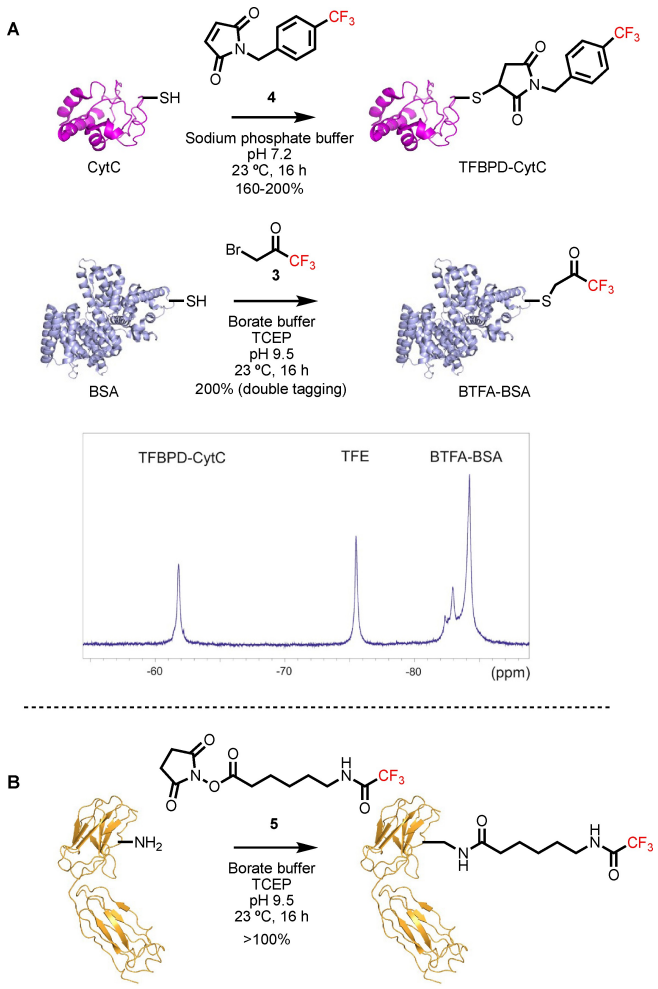

Figure 5. (**A**) Reaction conditions for labelling of a pair of proteins with different fluorinated protein tags **3** and **4**, and the resulting ^{19}F NMR spectrum showing that the signals do not overlap – a difference in chemical shift of ~20 ppm. (**B**) Conditions for antibody labelling at lysine by **5**. Figure adapted with permission from reference [51] (copyright of ACS).

second with *N*-(ε-trifluoroacetylcaproyloxy) succinimide ester (**5**), which reacts with lysine and N-terminal amines, and therefore, does not require disulfide reduction. One advantage of the lysine tag **5** is the milder reaction conditions (pH 7.2, room temperature) compared most others previously investigated tags (pH >8.0 required for **3**). One of the antibodies was known to self-associate at higher concentrations, which was clearly visible by the broad complex cluster of signals produced in the ^{19}F NMR, whilst the non-aggregating antibody retained a single sharp signal – surprising for such a large protein (~144 kDa). This neatly demonstrated the ability to study multiple processes occurring within the same solution.

So far, we have focused mainly on observing the changes in chemical shift arising due to protein conformational changes. However, there are other important parameters that can be obtained from ^{19}F NMR analysis of fluorine tagged proteins. For example, Edwards *et al.* demonstrated that three differentially tagged proteins (tagged with **3**, **4** and **5**) diffused at different rates when dissolved in serum when compared with phosphate buffer,[52] which has important implications for the relevance of studying protein behaviour in model buffer systems. Moreover, they were able to estimate the molecular radius of the proteins based on the diffusion coefficient (D_L) measurements obtained from ^{19}F diffusion ordered spectroscopy (DOSY) experiments. Extending this approach provides a useful tool to study protein aggregation. Additionally, the transverse relaxation rates (R_2) for proteins could be measured, reflecting the relative rates of tumbling in solution, which is affected by solvent viscosity, aggregation and by transient interactions. Again, in each case, the relaxation rates were found to be higher in serum compared with buffer.

In a biological sample, proteins often exist in complex and crowded mixtures, which can promote interactions, clustering, aggregation into a solid-like state and condensation into a liquid-like state.[53] Large proteins e.g., monoclonal antibodies and protein assemblies can become 'invisible' to NMR (or in a 'dark state') due to increased rates of transverse relaxation, which leads to broadened signals. This makes their analysis using conventional NMR methods challenging. Remarkably, an NMR technique termed dark state exchange saturation transfer (DEST) has been applied to pairs of orthogonally ^{19}F-tagged large (145 kDa) monoclonal antibodies (tagged using **4** and **5**) to study the reversible formation of small and large protein assemblies at high concentrations up to 400 mg/mL (Figure 6).[54] Given their large size and their propensity to form protein clusters, there is a significant dark state population that cannot be observed directly. However, selective radiofrequency saturation of the dark state population was transferable to the smaller visible monomers and small oligomeric species, attenuating their signals, due to the reversible nature of the protein interactions. This allowed the populations of clusters to be quantified, and the effects of temperature and concentration on the apparent radius of the large protein clusters to be studied.

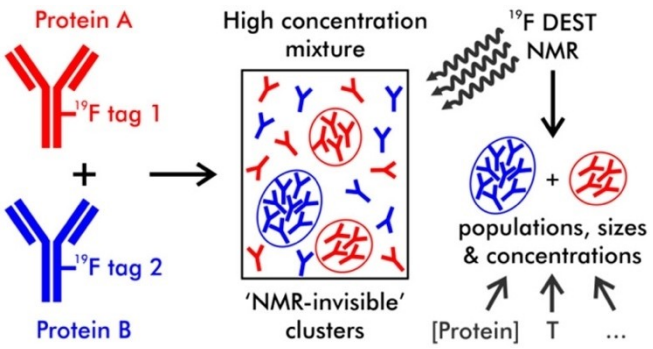

Figure 6. Analysis of invisible large protein clusters using ^{19}F dark state exchange saturation transfer (DEST) NMR. Figure reproduced with permission from reference [54] (copyright of ACS).

2.3. Enhancing Signal-to-Noise Ratio and Chemical Shift Dispersion

Poor signal to noise ratio can make analysis challenging for even trifluoromethyl-containing tagged-proteins. An example of an extremely sensitive ^{19}F NMR tag, albeit sterically large, is 4-perfluoro-*tert*-butyl-phenyliodoacetamide **6**, used by Kalbitzer *et al.* to label the cysteine residues of the protein actin.[55] The nine fluorine atoms of the tag are chemically equivalent affording a strong single resonance by ^{19}F NMR. This allowed the remarkable detection of the protein at a concentration of 40 μM with a single scan at 470 MHz. It is worth noting that the effect on the biophysical properties of the protein must be considered when selecting a tag and the level of fluorination (discussed later). Another highly fluorinated and sensitive NMR tag was developed by Hiscocks *et al.* who combined an $-SF_5$ group with maleimide **7** and acrylamide **8** warheads, respectively.[56] The highly fluorinated tags were envisaged to be useful for ^{19}F MRI or PET applications but would also provide a strong ^{19}F NMR signal. However, it is worth noting that rather than giving one 5F singlet, the SF_5 group produces a pair of ^{19}F signals – a 4F doublet and a 1F quintet due to the axial and equatorial geometries of the fluorine atoms. This may be considered a disadvantage due to making the resulting spectrum more complex than afforded by e.g. $-CF_3$. They chose the small, cancer-targeting, cyclic peptide c-RGD as a model for bioconjugation initially through a cysteine residue (Figure 7 A). Each tag afforded quantitative conversion to the labelled peptide.

An interesting feature of maleimide **7** is the potential for hydrolysis of the succinimide ring after conjugation in basic conditions (> pH 7.5). LCMS analysis showed that this occurred instantaneous upon conjugation to c-RGD and was likely driven by the strong electron withdrawing effect of the $-SF_5$ group. It is worth noting that the ring-opening of maleimides is a potentially desirable feature to make the conjugation irreversible.[57] The acrylamide warhead in general has very favourable reactivity towards thiols and is commonly employed selectively for cysteine modification (pH 7.5–pH 9.0).[58] However, acrylamide **8** was employed for the modification of a lysine residue in more strongly basic conditions (pH 9.0–10.0) (Figure 7 B), but suffered from poor solubility in a mixture of phosphate buffer and acetonitrile. However, neither of these tags have been reported for use in protein labelling at the time of writing.

Chai and co-workers developed the sensitive hexafluorinated tag **9**, for in cell ^{19}F NMR. This contains a pyridinium salt for improved water-solubility whilst the heteroaromatic sulfone is highly reactive but chemoselective for thiol nucleophiles.[13] After delivering the tagged protein into human HeLa cells by electroporation, the six equivalent fluorine atoms enabled ^{19}F NMR detection of labelled proteins (6–40.5 kD) at near-physiological concentrations as low as ~1 μM (Figure 8). A cysteine- mutant of streptococcal protein G (GB1) was labelled in < 12 hours in phosphate buffer (pH 8.0) with > 95% labelling efficiency, and the C–S linkage that formed was stable to thioether exchange in the presence of glutathione. In terms of NMR sensitivity, the tag could distinguish conformational transitions of the protein as spectral shifts, including those induced by dimerization and increasing Ca^{2+} ions. This tag was also shown to be far more sensitive to its environment in comparison to 2,2,2-triflouroethanethiol (**2**) and 3-bromo-1,1,1-trifluoroacetone (**3**). Subsequently, Zhai and co-workers also used **9** to probe the activation mechanisms of β-arrestins, a family of G-protein-coupled receptors with multiple binding partners.[59] Here they used ^{19}F NMR to monitor the structural changes associated with its activation by several phosphopeptides and observed a complex conformational energy landscape for activation and the subtle modulation during signalling. For more information, a more comprehensive discussion of the recent developments in the field of in-cell ^{19}F NMR has recently been published elsewhere.[12]

The chemical shift dispersion of a monofluoroethyl thiol fluorine tag **10** was shown to be dramatically enhanced by per-deuteration of the ethyl group (**11**).[61] Chemical shift anisotropy is also reported to be approximately two times lower in the monofluoroethyl than in the trifluoroethyl group.[61] When **11**

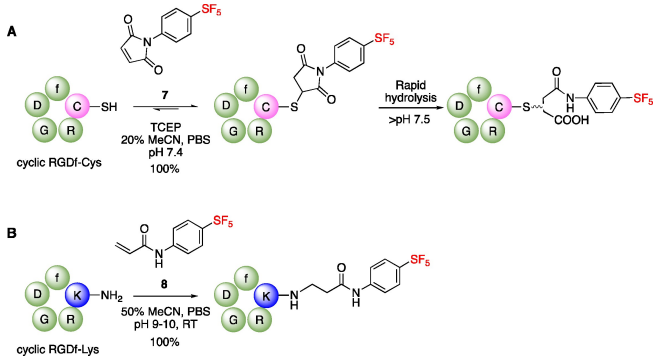

Figure 7. The reaction of highly-fluorinated SF_5 protein tags with model cRGD peptides.[56]

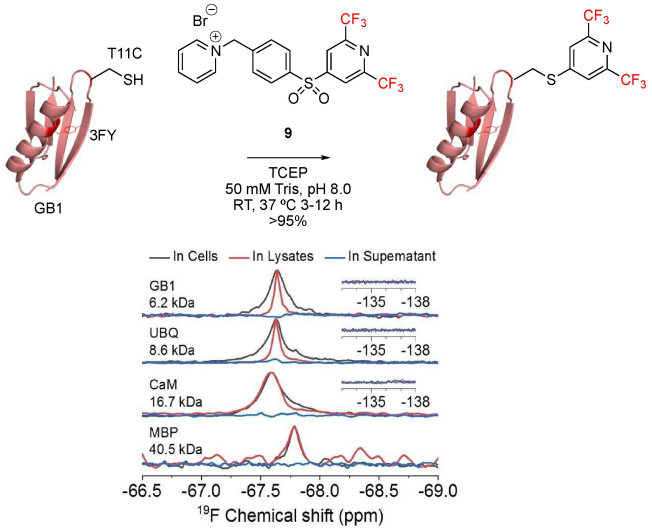

Figure 8. Reaction conditions for tagging with **9** and comparison of the difference of intracellular ^{19}F NMR sensitivity of proteins tagged with **9** (~−67.7 ppm) compared with 3-fluorotyrosine (inset: ~−136.5 ppm). Figure adapted with permission from reference [13] (copyright of Wiley).

was used to label a 134 kDa membrane transporter protein (incubation at 4 °C for 2 h or room temperature for 1 h in HEPES buffer at pH 7.4), this afforded improved chemical shift dispersion and narrower line shape for higher-resolution detection of previously unresolved separate conformers. Moreover, the use of ^{19}F NMR in combination with cryo-electron microscopy imaging of the protein, was found to be advantageous for proteins that adopt more than two conformational states.

The iodoacetamide **12** was recently reported as a protein label with amplified ^{19}F NMR chemical shift sensitivity.[62] The amplification is proposed to arise through the rapid exchange of trifluoromethylated pyridone tautomers (lactam and lactim, Figure 9) that depend on the polarity of the solvent environment, rendering the tag and the ^{19}F NMR signal, more sensitive to this environmental change compared to 2-bromo-N-(4-(trifluoromethyl)phenyl)acetamide **1**. Using **12**, human serum albumin (HSA) was tagged near to a ligand binding site at Cys34 (>97% labelling efficiency), and subsequently the effect of binding of ligands, such as ATP, cholesterol and $ZnCl_2$ on protein conformation were studied. Upon ligand binding, a protein conformational change altered the solvent exposure of the tag, which altered the weighted average population of pyridone tautomer, and was evident in the ^{19}F NMR spectrum. A comparison against HSA tagged with the phenylacetamide **1**

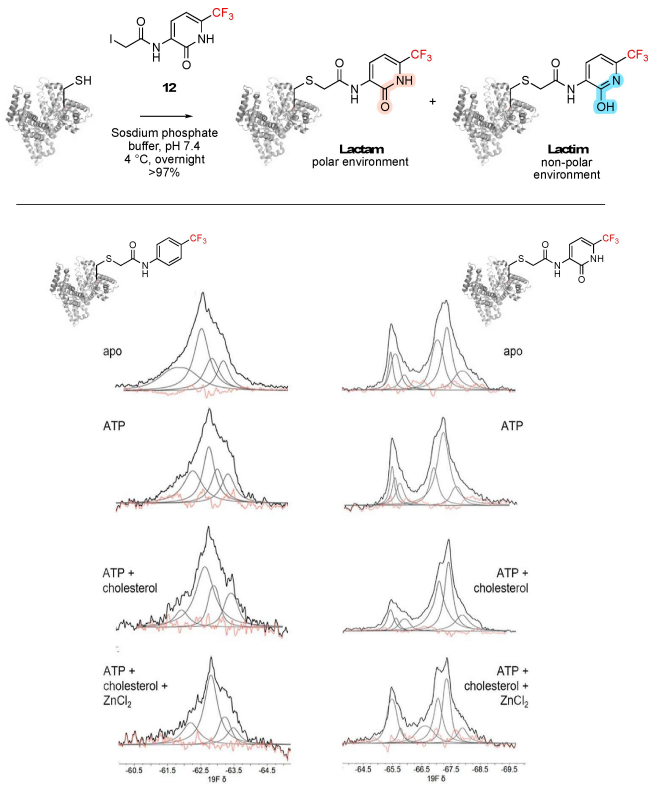

Figure 9. Reaction conditions for fluorine tagging with **12**. The effect of tautomerisation of the pyridone tag provides extra environmental sensitivity to solvent polarity and solvent exposure. The comparison of tagging of HSA with **1** or **12**, with the solvent exposure associated with binding and conformational changes apparent, in the more dispersed nature of the spectrum for the latter. Figure adapted with permission from reference [62] (copyright of ACS).

(which does not undergo tautomerism) showed considerably less sensitivity to the solvent environment with signal overlap in the resultant spectrum (Figure 9). This, therefore, suggests that tautomeric NMR tags such as **12** could afford exceptional benefits in drug fragment binding assays.

2.4. Combining ^{19}F NMR Tags with Paramagnetic Spin Tags

Some recent studies have incorporated sensitive 3,5-bis(trifluoromethyl)benzyl (**13**) and 3,5-bis(trifluoromethyl)phenyl tags into proteins. The six equivalent fluorine atoms provide a strong NMR signal, with less of the potential structural perturbations of for example 4-perfluoro-*tert*-butyl-phenyliodoacetamide (**6**). Some studies have also shown that aryl-CF_3 tags benefit from improved chemical shift sensitivity compared with alkyl-CF_3 groups.[63] Somlyay and co-workers used a combination of a ^{19}F NMR tag and a paramagnetic spin label to study the heterodimerization of the Myc-Max oncogenic transcription factor in the presence of E-box DNA and BRCA1.[64] A cysteine residue of the intrinsically disordered region of Myc was labelled with 3,5-bis(trifluoromethyl)benzyl bromide **13** (six-fold excess of **13** in 5% DMSO/95% sodium phosphate buffer, pH 7 with incubation at RT), and correspondingly, a series of positions within Max was labelled with 1-oxyl-2,2,5,5-tetra-methyl-Δ3-pyrroline-3-methyl)methanethio-sulfonate (MTSL, **14**) for paramagnetic relaxation enhancement NMR studies. The paramagnetic spin label was introduced to enhance the longitudinal and transverse relaxation rates of neighbouring residues, which caused signal attenuation for nearby ^{19}F nuclei. ^{19}F signal perturbations gave an insight into the association of the two proteins and the structural changes that govern this. Recently, a related approach incorporated a trifluoromethylphenylalanine and a high-spin Gd(III) paramagnetic tag as a 'molecular ruler' for long range ^{19}F electron–nuclear double resonance (ENDOR) distance measurements between the spin tag and a fluorinated reporter,[65] which is effective up to a distance of 25 Å,[66] as an alternative to more traditional EPR-based distance measurements in biomolecules.

Luchette *et al.* leveraged the paramagnetism of membrane-dissolved O_2, and its resultant chemical shift perturbation of ^{19}F labels to study the secondary structure and position of diacylglycerol kinase residues within an artificial lipid membrane.[67] Endogenous and mutated cysteine residues within the transmembrane 1 domain of the intact homotrimer were tagged by thioalkylaton of with 3-bromo-1,1,1-trifluoropropane (**15**). Under high pressure (100 atm), the dissolved O_2 influenced only the fluorine nuclei that were in contact with the membrane and the solubility gradient of O_2 allowed the authors to probe the depth of residues within the membrane. This allowed dynamic structural information about the protein to be garnered.

2.5. Tagging Non-Cysteine Residues

Whilst cysteine is predominantly targeted for protein bioconjugation, lysine and N-terminal amino groups can also be targeted for indirect fluorination. Larda and co-workers introduced fluorinated indoles **16** in a site-specific and non-denaturing modification of hen egg white lysozyme (HEWL) (Figure 10A).[68] Activation of the amine was achieved in the presence of formaldehyde to form an iminium ion *in situ*, which acts as an electrophile for the incoming indole. Key features of this method are the strong preference for primary amines and the conservation of the amine charge in physiological conditions after modification. The incorporation of the fluorine provides the obvious ^{19}F NMR handle, whilst the reaction itself, with differing rates of conjugation for different amines, has the potential for probing surface topology.

The phenol sidechain of tyrosine can also be targeted for bioconjugation by undergoing three component Mannich-type reactions in a similar process to that above. Tyrosine-targeting is attractive due to its typically low abundance, whilst surface exposed tyrosine residues are even less common. Vitali *et al.* targeted tyrosine residues of two proteins, HEWL and streptococcal protein G (GB1).[69] The reaction proceeds via the *in situ* formation of an imine from 4-fluoroaniline **17** and formaldehyde. The imine electrophile is then sequestered during electrophilic substitution at the carbon *ortho* to the hydroxyl group of the tyrosine sidechain (Figure 10B). It was noted by the authors that the reaction relies on a slightly acidic pH (6.5) to prevent unwanted side-products such as reaction at tryptophan. The mass spectrometry and NMR data showed that only a single GB1 residue was tagged with a yield of ~33% as the ring open product. For HEWL the data also indicated only one of three native tyrosine residues had been tagged but with tags existing as both the open and ring-closed products. Overall, for HEWL the reaction efficiency was significantly lower than for GB1, which the authors suggested may be due to the presence of fewer surface exposed tyrosine residues.

Other sidechains such as glutamine can also be indirectly fluorinated, as demonstrated by Hattori, who performed the enzymatic modification of glutamine carboxamides using transglutaminase catalysis to introduce 2,2,2-trifluoroethylamine **18** as an ^{19}F NMR handle (Figure 10C).[70] Using this approach, surface glutamine residues were labelled in model proteins FKBP12 and GF14b. NMR experiments provided insight into the protein-protein interactions of FKBP12 with another protein FK506, whilst for GF14b, they explored the interaction of the protein with a hormone and a transcription factor. They observed that the first interaction was a fast exchange whilst the second was at a slower rate.

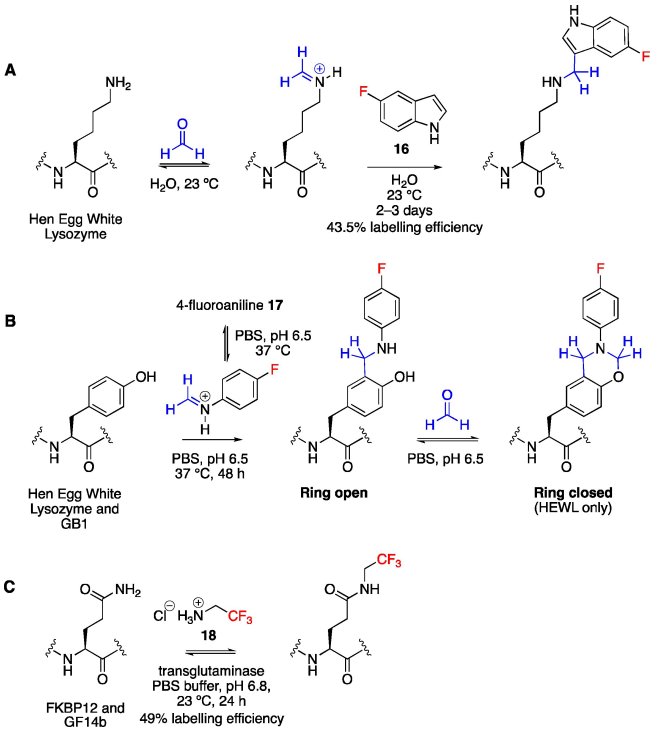

Figure 10. Fluorine tagging at non-cysteine residues. (**A**) The introduction of fluorinated indoles e.g. **16**, as ^{19}F NMR tags, by conjugation at lysine residues.[68] (**B**) Reaction conditions for tyrosine tagging *via* a three component Mannich-type reaction with fluoroanilines e.g., **17**.[69] (**C**) Reaction conditions for the enzymatic modification of glutamine with fluorinated amines e.g. **18** to via transglutaminase catalysis.[70]

2.6. Covalently-Reactive ^{18}F Tags from Radiochemistry

Some alternative warhead types have been employed in fluorinated protein tags for indirect ^{18}F radiochemical labelling and may serve as alternative chemistries for ^{19}F tagging (Figure 11). The ^{18}F radioisotope, with a half-life of around 109.7 minutes, is incorporated for the purpose of tracing biomolecules through positron emission tomography.[71] Radiolabelling of N-terminal amines can be achieved using 4-[^{18}F]-fluorobenzaldehyde **19**,[72,73] whilst [^{18}F]-fluoropropylsulfonyl chloride **20** can react with both primary and secondary amines.[74] Similarly, the activated ester 6-[^{18}F]-fluoronicotinic acid tetrafluorophenyl ester **21**, is reported to react efficiently with exposed amines on proteins.[75] Click-type reactions involving copper-catalysed azide-alkyne cycloadditions using phenylazide **22** and alkyne **23** have also been utilised for ^{18}F-radiolabelling.[76] However, this method requires the incorporation of the corresponding non-standard amino acid. These examples should also be translatable to ^{19}F NMR tags.

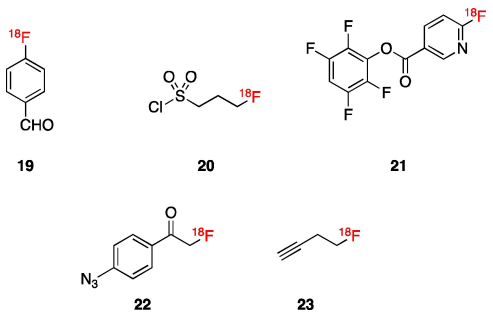

Figure 11. Examples of ^{18}F tags used for for ^{18}F-radiolabelling.

3. Summary and Outlook

This review aims to persuade molecular and chemical biologists of the value of ^{19}F NMR tagging as an additional instrument in the toolbox for studying proteins and biomacromolecules. Solid phase synthesis is limited to only the smallest proteins; and only a relatively limited set of fluorinated amino acids suitable for genetic incorporation are available compared with the potentially large number of conjugatable ^{19}F NMR reporters. In many cases the efficiency of genetic incorporation of the unnatural fluorinated amino acid is low or variable, and often requires the engineering of new aminoacyl-tRNA synthetases to accept the unnatural amino acids.[77] Chemical tags expand the range of tools available to researchers, providing a plug-and-play tool kit of interchangeable and optimizable tags with different chemical shifts, chemical shift responsiveness, different sensitivities, and in many cases, the ability to tag native proteins without mutagenesis. Moreover, tagging approaches are more broadly accessible to researchers without expertise or facilities for molecular biology, and should promote wider uptake within this field. Despite the advances made in chemical protein tagging, there remain several challenges to overcome to broaden the applications and uptake of this technology.

Compared to large organic fluorophores that are commonly used in chemical biology, fluorine tags tend to be smaller and less perturbing. However, this is not always the case, and we have seen earlier in this review that some fluorine tags are reasonably large and may impart steric effects upon the protein. Fluorine itself has been observed to affect the host protein behaviour when fluorinated amino acids are incorporated into a protein. C–F bonds are highly polarized due to the electronegative nature of fluorine, which can lead to electrostatic or dipolar interactions with nearby functional groups or solvent. The electron-withdrawing ability of fluorine atoms/groups can also lead to changes in the physicochemical properties of neighbouring amino acid side chains, such as hydrophobicity, polarity, ionizability and secondary structure propensity.[78] In some cases, fluorination of amino acids can greatly accelerate the folding or aggregation of proteins, often through increased hydrophobicity or 'fluorous' effects.[79–82] Counter to expectation, fluorination can often increase the polarity of hydrophobic amino acids. It is, therefore, reasonable to assume that these features may extend to fluorinated tags, which depending on the location of the tagged residue(s) in the protein of interest, may affect protein folding, interactions and ligand binding. As such, it is worth giving careful consideration to the placement of new tags and their compatibility with the systems under study, however, tagging at solvent exposed side chains may mitigate these effects somewhat. Those who are new to this area are directed to two excellent reviews[78,83] that discuss the impact of fluorine on protein structure in more detail.

There is not yet a consensus on the optimal design of ^{19}F NMR protein tags, however, some important observations have been reported and summarised in the following sections. The main features of fluorinated tags are a) a reporter composed of one or more normally equivalent fluorine atoms, b) an optional 'molecular amplifier' module that affects the chemical shift dispersion and chemical shift region of the spectrum, c) a linker that can affect the size, flexibility and physicochemical properties of the tag, and finally d) a conjugatable functional group or 'warhead' that is used to covalently bond the tag to a nucleophilic functional group in a protein of interest (Figure 12).

One of the current drawbacks of studying biomolecules using ^{19}F NMR is the limited sensitivity of NMR. In general, more fluorine affords a greater sensitivity reporter i.e. CF_3 groups can afford three times the signal-to-noise ratio of a C–F group in some cases. Similarly, significant enhancements can be achieved by incorporating multiple symmetrical fluorine atoms, such as the perfluoro-*tert*-butyl group with nine equivalent ^{19}F nuclei giving a single resonance.[84,85] However, structural perturbation may be especially notable for tags that contain higher numbers of fluorine atoms for sensitivity purposes, which will therefore, be larger, more lipophilic and more polarizing. Low signal-to-noise ratio can also be compensated by using advanced hardware such as cryoprobes or by developing new pulse sequences such as SHARPER, which collapses multiple fluorine signals into a singlet with greater signal-to-noise ratio,[86] albeit, this will be less useful for studying multiple species simultaneously.

However, the performance (signal-to-noise ratio and chemical shift dispersion) of the fluorine reporter is also dependent on what it is attached to. For example, aryl-CF_3 tags benefit from improved chemical shift sensitivity compared with alkyl-CF_3 groups.[63] Chemical shift anisotropy (CSA) is also approximately two times higher for some trifluoroalkyl groups compared with monofluoroalkyl groups.[61] The effect of CSA is also less pronounced in reporter groups comprising aryl-CF_3 groups compared to aryl-fluorines. There have been some recent developments in 'molecular amplifiers' of ^{19}F NMR chemical shifts, as discussed above.[62] The observation that aryl-CF_3 groups with increased polarizability and rapidly interconverting tautomers have increased dispersion between the signals of different conformational states will likely intensify research in this area.

An inherent challenge in this field comes from the increased chemical shift anisotropy of the fluorine tag bound to larger proteins, which results in signal broadening, especially at higher field strengths.[87] This can potentially be partly addressed by allowing increased flexibility in the linker group, (e.g. 5) giving narrower linewidths,[51,54] and indeed is a further advantage to the use of chemical tagging rather than incorporation of fluorinated amino acids that are normally more prone to broadening.[88] However, a fuller understanding of how the

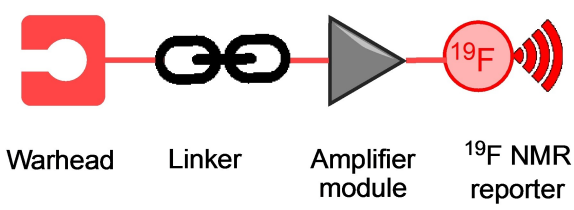

Figure 12. General schematic of the features in common ^{19}F NMR tags.

linker length, flexibility and physicochemical properties affects the sensitivity towards changes in chemical environment (and impacts protein behaviour) would be beneficial. An alternative solution to overcome CSA exploited the relaxation properties of the aromatic $^{19}F–^{13}C$ spin pair using a two-dimensional (2D) $^{19}F–^{13}C$ transverse relaxation-optimized spectroscopy (TROSY) experiment.[89]

As is evident from our earlier discussion, the most frequent targets for fluorine protein tagging are cysteine residues, whilst there are relatively few that target other residues such as tyrosine. New conjugation chemistries and 'warheads' that chemoselectively and site-selectively tag proteins would be beneficial. An intriguing possible future application of fluorine tagging could be in the labelling of patient derived materials, e.g., blood or tissue samples, to detect the presence of biomarkers of health or disease or to study proteins in their complex native environment. As briefly discussed earlier, Edwards *et al.* reported the simultaneous analysis of three orthogonally fluorine-tagged proteins: human serum albumin, transferrin, and immunoglobulin G in blood serum.[52] Importantly, protein samples were first individually tagged and then reconstituted as a mixture in serum (a crowded and complex environment) or buffer. The obvious extension of this to tagging patient samples, however, presents the challenge of achieving selective tagging at only a single target biomolecule. Albeit, given the wide chemical shift window of ^{19}F NMR, it may be still useful or even desirable to label multiple species for simultaneous analysis. Another major challenge to overcome here would be obtaining sufficiently high concentrations of the tagged species for NMR analysis, yet there are some proteins that are abundant in the blood e.g., human serum albumin (35–50 mg/mL),[90] whose levels can be diagnostic of e.g. liver and kidney function[91] and might represent a tractable initial target for fluorine-tagging *ex vivo*. Perhaps a future paradigm will see the development of target-specific conjugation chemistries that can be applied to a native biological sample to fluorine-tag only a single protein of interest to study its behaviour in the biological milieu or in-cell. This is a logical step and target selective electrophilic warheads have been widely explored especially in the field of covalent drug discovery, with particular success in the covalent protein kinase inhibitors field.[92]

It is a priority to develop the next generation of fluorine tags with improved signal-to-noise ratio and chemical shift sensitivity, affording a toolbox of tags with a range of characteristic chemical shifts in the same mould as fluorophores. We hope this review will be a useful resource for those who are considering exploring ^{19}F NMR tags for biology and who require guidance on the key considerations for choice of tag and compatibility with the protein of interest.

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Conflict of Interests

The authors declare no conflict of interest.

Keywords: ^{19}F NMR · fluorinated protein tags · protein tagging

[1] H. Chen, S. Viel, F. Ziarelli, L. Peng, *Chem. Soc. Rev.* **2013**, *42*, 7971–7982.
[2] A. M. Gronenborn, *Structure* **2022**, *30*, 6–14.
[3] N. G. Sharaf, A. M. Gronenborn, *Methods Enzymol.* **2015**, *565*, 67–95.
[4] J. L. Kitevski-LeBlanc, R. S. Prosser, *Prog. Nucl. Magn. Reson. Spectrosc.* **2012**, *62*, 1–33.
[5] G. F. Wang, C. G. Li, G. J. Pielak, *ChemBioChem* **2010**, *11*, 1993–1996.
[6] H. Li, C. Frieden, *Biochemistry* **2007**, *46*, 4337–4347.
[7] H. Li, C. Frieden, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 11993–11998.
[8] H. Li, C. Frieden, *Biochemistry* **2006**, *45*, 6272–6278.
[9] S. S. Stadmiller, J. S. Aguilar, C. A. Waudby, G. J. Pielak, *Biophys. J.* **2020**, *118*, 2537–2548.
[10] C. R. Buchholz, W. C. Pomerantz, *RSC Chem. Biol.* **2021**, *2*, 1312–1330.
[11] S. Shinya, R. Katahira, K. Furuita, T. Sugiki, Y. H. Lee, Y. Hattori, K. Takeshita, A. Nakagawa, Kokago, K. I. Akagi, M. Oouchi, F. Hayashi, T. Kigawa, M. Takimoto-Kamimura, T. Fujiwara, C. Kojima *RSC Med. Chem.* **2022**, *13*, 1100–1111.
[12] Z. Chai, C. Li, *Chem. Eur. J.* **2024**, *30*, e202303988.
[13] Z. Chai, Q. Wu, K. Cheng, X. Liu, Z. Zhang, L. Jiang, Z. Zhou, M. Liu, C. Li, *Angew. Chem. Int. Ed.* **2023**, *62*, e202300318.
[14] L. B. Pham, A. Costantino, L. Barbieri, V. Calderone, E. Luchinat, L. Banci, *J. Am. Chem. Soc.* **2023**, *145*, 1389–1399.
[15] W. Zhu, A. J. Guseman, F. Bhinderwala, M. Lu, X. C. Su, A. M. Gronenborn, *Angew. Chem. Int. Ed.* **2022**, *61*, e202201097.
[16] J. W. Lichtman, J. A. Conchello *Nat. Methods* **2005**, *2*, 910–919.
[17] E. A. Jares-Erijman, T. M. Jovin, *Nat. Biotechnol.* **2003**, *21*, 1387–1395.
[18] Y. Suzuki, J. R. Brender, M. T. Soper, J. Krishnamoorthy, Y. Zhou, B. T. Ruotolo, N. A. Kotov, A. Ramamoorthy, E. N. G. Marsh, *Biochemistry* **2013**, *52*, 1903–1912.
[19] G. F. Wang, C. G. Li, G. J. Pielak, *Protein Sci.* **2010**, *19*, 1686–1691.
[20] C. Li, G. F. Wang, Y. Wang, R. Creager-Allen, E. A. Lutz, H. Scronce, K. M. Slade, R. A. Ruf, R. A. Mehl, G. J. Pielak, *J. Am. Chem. Soc.* **2010**, *132*, 321–327.
[21] F. Tobola, M. Lelimousin, A. Varrot, E. Gillon, B. Darnhofer, O. Blixt, R. Birner-Gruenberger, A. Imberty, B. Wiltschi, *ACS Chem. Biol.* **2018**, *13*, 2211–2219.
[22] C. T. Gee, K. E. Arntson, E. J. Koleski, R. Staebell, W. C. K. Pomerantz, *ChemBioChem* **2018**, *19*, 963–969.
[23] K. E. Arntson, W. C. Pomerantz, *J. Med. Chem.* **2016**, *59*, 5158–5171.
[24] R. S. Norton, E. W. Leung, I. R. Chandrashekaran, C. A. MacRaild, *Molecules* **2016**, *21*, 860.
[25] C. T. Gee, E. J. Koleski, W. C. Pomerantz, *Angew. Chem. Int. Ed.* **2015**, *54*, 3735–3739.
[26] X. Ge, C. A. MacRaild, S. M. Devine, C. O. Debono, G. Wang, P. J. Scammells, M. J. Scanlon, R. F. Anders, M. Foley, R. S. Norton, *J. Med. Chem.* **2014**, *57*, 6419–6427.
[27] N. K. Mishra, A. K. Urick, S. W. J. Ember, E. Schoenbrunn, W. C. Pomerantz, *ACS Chem. Biol.* **2014**, *9*, 2755–2760.
[28] W. C. Pomerantz, N. Wang, A. K. Lipinski, R. Wang, T. Cierpicki, A. K. Mapp, *ACS Chem. Biol.* **2012**, *7*, 1345–1350.
[29] O. Binas, V. de Jesus, T. Landgraf, A. E. Völklein, J. Martins, D. Hymon, J. Kaur Bains, H. Berg, T. Biedenbänder, B. Fürtig, S. L. Gande, A. Niesteruk, A. Oxenfarth, N. S. Qureshi, T. Schamber, R. Schnieders, A. Tröster, A. Wacker, J. Wirmer-Bartoschek, M. A. W. Martin, E. Stirnal, K. Azzaoui, C. Richter, S. Sreeramulu, M. J. J. Blommers, H. Schwalbe *ChemBioChem* **2021**, *22*, 423–433.
[30] N. S. Troelsen, E. Shanina, D. Gonzalez-Romero, D. Danková, I. S. Jensen, K. J. Śniady, F. Nami, H. Zhang, C. Rademacher, A. Cuenda, C. H. Gotfredsen, M. H. Clausen, *Angew. Chem.* **2020**, *132*, 2224–2230.
[31] X. Wang, D Liu, L. Shen, F. Li, Y. Li, L. Yang, T. Xu, H. Tao, D. Yao, L. Wu, K. Hirata, L. M. Bohn, A. Makriyannis, X. Liu, T. Hua, Z.-J. Liu, J. Wang, *J. Am. Chem. Soc.* **2021**, *143*, 16320–16325.
[32] D. Gimenez, A. Phelan, C. D. Murphy, S. L. Cobb, *Beilstein J. Org. Chem.* **2021**, *17*, 293–318.
[33] L. Merkel, N. Budisa, *Org. Biomol. Chem.* **2012**, *10*, 7241–7261.
[34] E. W. Westhead, P. D. Boyer, *Biochim. Biophys. Acta* **1961**, *54*, 45–50.

[35] B. D. Sykes, H. I. Weingarten, M. J. Schlesinger, *Proc. Natl. Acad. Sci. USA* **1974**, *71*, 469–473.
[36] W. E. Hull, B. D. Sykes, *Biochemistry* **1974**, *13*, 3431–3437.
[37] C. Frieden, S. D. Hoeltzli, J. G. Bann, *Methods Enzymol.* **2004**, *380*, 400–415.
[38] M. H. Richmond, *Bacteriol. Rev.* **1962**, *26*, 398–420.
[39] P. B. Crowley, C. Kyne, W. B. Monteith, *Chem. Commun.* **2012**, *48*, 10681–10683.
[40] M. Wang, M. Lu, M. P. Fritz, C. M. Quinn, I. J. L. Byeon, C. H. Byeon, J. Struppe, W. Maas, A. M. Gronenborn, T. Polenova, *Angew. Chem. Int. Ed.* **2018**, *57*, 16375–16379.
[41] L. Wang, A. Brock, B. Herberich, P. G. Schultz, *Science* **2001**, *292*, 498–500.
[42] J. T. Hammill, S. Miyake-Stoner, J. L. Hazen, J. C. Jackson, R. A. Mehl, *Nat. Protoc.* **2007**, *2*, 2601–2607.
[43] S. Ye, A. Ann Berger, D. Petzold, O. Reimann, B. Matt, B Koksch, *Beilstein J. Org. Chem.* **2010**, *6*, 40.
[44] L. Merkel, M. Schauer, G. Antranikian, N. Budisa, *ChemBioChem* **2010**, *11*, 1505–1507.
[45] L. Fojtík, J. Fiala, P. Pompach, J. Chmelík, V. Matoušek, P. Beier, Z. Kukačka, P. Novák, *J. Am. Chem. Soc.* **2021**, *143*, 20670–20679.
[46] A. Miseta, P. Csutora, *Mol. Biol. Evol.* **2000**, *17*, 1232–1239.
[47] P. Ábrányi-Balogh, G. M. Keserű, *Warheads for designing covalent inhibitors and chemical probes, Advances in Chemical Proteomics*, Elsevier, **2022**, p. 47–73.
[48] A. Manglik, T. H. Kim, M. Masureel, C. Altenbach, Z. Yang, D. Hilger, M. T. Lerch, T. S. Kobilka, F. S. Thian, W. L. Hubbell, R. S. Prosser, B. K. Kobilka, *Cell* **2015**, *161*, 1101–1111.
[49] R. Horst, J. J. Liu, R. C. Stevens, K. Wüthrich, *Angew. Chem. Int. Ed.* **2013**, *52*, 10762–10765.
[50] U. A. Hellmich, N. Pfleger, C. Glaubitz, *Photochem. Photobiol.* **2009**, *85*, 535–539.
[51] J. M. Edwards, J. P. Derrick, C. F. van der Walle, A. P. Golovanov, *Mol. Pharm.* **2018**, *15*, 2785–2796.
[52] J. M. Edwards, P. Harris, J. T. Bukrinski, A. P. Golovanov, *ChemPlusChem* **2019**, *84*, 443–446.
[53] M. Vendruscolo, M. Fuxreiter, *Nat. Commun.* **2022**, *13*, 5550.
[54] J. M. Edwards, J. E. Bramham, A. Podmore, S. M. Bishop, C. F. van der Walle, A. P. Golovanov, *Anal. Chem.* **2019**, *91*, 4702–4708.
[55] H. R. Kalbitzer, G. Rohr, E. Nowak, R. S. Goody, W. Kuhn, E. Zimmermann, *NMR Biomed.* **1992**, *5*, 347–350.
[56] H. Hiscocks, A. Ung, G. Pascali, *AppliedChem* **2023**, *3*, 256–278.
[57] J. M. J. M. Ravasco, H. Faustino, A. Trindade, P. M. P. Gois, *Chem. Eur. J.* **2019**, *25*, 43–59.
[58] F.-J. Chen, J. Gao, *Chem. Eur. J.* **2022**, *28*, e202201843.
[59] R. Zhai, Z. Wang, Z. Chai, X. Niu, C. Li, C. Jin, Y. Hu, *Nat. Commun.* **2023**, *14*, 7865.
[60] Y. Huang, K. Reddy, C. Bracken, W. Zhang, D. Eliezer, O. Boudker, *J. Am. Chem. Soc.* **2023**, *145*(15), 8583–8592.
[61] S. L. Grage, U. H. Dürr, S. Afonin, P. K. Mikhailiuk, I. V. Komarov, A. S. Ulrich, *J. Magn. Reson.* **2008**, *191*, 16–23.
[62] G. A. Frere, A. Hasabnis, C. B. Francisco, M. Suleiman, O. Alimowska, R. Rahmatullah, J. Gould, C. Y. C. Su, O. Voznyy, P. T. Gunning, E. A. Basso, R. S. Prosser, *J. Am. Chem. Soc.* **2024**, *146*(5), 3052–3064.
[63] L. Ye, S. T. Larda, Y. F. Li, A. Manglik, R. S. Prosser, *J. Biomol. NMR* **2015**, *62*, 97–103.
[64] M. Somlyay, K. Ledolter, M. Kitzler, G. Sandford, S. L. Cobb, R. Konrat, *ChemBioChem* **2020**, *21*, 696–701.
[65] M. Judd, E. H. Abdelkader, M. Qi, J. R. Harmer, T. Huber, A. Godt, A. Savitsky, G. Otting, N. Cox, *Phys. Chem. Chem. Phys.* **2022**, *24*, 25214–25226.
[66] A. Bogdanov, V. Frydman, M. Seal, L. Rapatskiy, A. Schnegg, W. Zhu, M. Iron, A. M. Gronenborn, D. Goldfarb, *J. Am. Chem. Soc.* **2024**, *146*, 6157–6167.
[67] P. A. Luchette, R. S. Prosser, C. R. Sanders, *J. Am. Chem. Soc.* **2002**, *124*, 1778–1781.
[68] S. T. Larda, D. Pichugin, R. S. Prosser, *Bioconjugate Chem.* **2015**, *26*, 2376–2383.
[69] V. Vitali, F. Torricella, L. Massai, L. Messori, L. Banci, *Sci. Rep.* **2023**, *13*, 22017.
[70] Y. Hattori, D. Heidenreich, Y. Ono, T. Sugiki, K. Yokoyama, E. Suzuki, T. Fujiwara, C. Kojima, *J. Biomol. NMR* **2017**, *68*, 271–279.
[71] J. L. Sutcliffe-Goulden, M. J. O'Doherty, P. K. Marsden, I. R. Hart, J. F. Marshall, S. S. Bansal, *Eur. J. Nucl. Med. Mol. Imaging* **2002**, *29*, 754–759.
[72] M. Namavari, O. Padilla De Jesus, Z. Cheng, A. De, E. Kovacs, J. Levi, R. Zhang, J. K. Hoerner, H. Grade, F. A. Syud, S. S. Gambhir, *Mol. Imaging Biol.* **2008**, *10*, 177–181.
[73] G. Ma, J. W. McDaniel, J. M. Murphy, *Org. Lett.* **2020**. *23*, 530–534.
[74] Z. Li, L. Lang, Y. Ma, D. O. Kiesewetter, *J. Labelled Compd. Radiopharm.* **2008**, *51*, 23–27.
[75] D. E. Olberg, J. M. Arukwe, D. Grace, O. K. Hjelstuen, M. Solbakken, G. M. Kindberg, A. Cuthbertson, *J. Med. Chem.* **2010**, *53*(4), 1732–1740.
[76] O. Jacobson, D. O. Kiesewetter, X. Chen, *Bioconjugate Chem.* **2015**, *26*, 1–18.
[77] I. M. Robertson, B. A. Klein, B. D. Sykes, *J. Biomol. NMR* **2020**, *74*, 1–7.
[78] A. A. Berger, J. S. Völler, N. Budisa, B. Koksch, *Acc. Chem. Res.* **2017**, *50*, 2093–2103.
[79] E. S. Eberhardt, N. Panasik, R. T. Raines, *J. Am. Chem. Soc.* **1996**, *118*, 12261–12266.
[80] H. Iwai, A. Lingel, A. Pluckthun, *J. Biol. Chem.* **2001**, *276*, 16548–16554.
[81] B. C. Buer, J. L. Meagher, J. A. Stuckey, E. N. G. Marsh, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 4810–4815.
[82] M. A. Cejas, W. A. Kinney, C. Chen, J. G. Vinter, H. R. Almond, K. M. Balss, C. A. Maryanoff, U. Schmidt, M. Breslav, A. Mahan, E. Lacy, B. E. Maryanoff, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 8513–8518.
[83] S. A. Miles, J. A. Nillama, L. Hunter, *Molecules* **2023**, *28*, 6192.
[84] C. M. Tressler, N. J. Zondlo, *ACS Chem. Biol.* **2020**, *15*, 1096–1103.
[85] C. M. Tressler, N. J. Zondlo, *J. Org. Chem.* **2014**, *79*, 5880–5886.
[86] A. I. Silva Terra, M. Rossetto, C. L. Dickson, G. Peat, D. Uhrin, M. E. Halse, *ACS Meas. Sci. Au* **2023**, *3*(1), 73–81.
[87] J. T. Gerig, *Prog. Nucl. Magn. Reson. Spectrosc.* **1994**, *26*, 293–370.
[88] W. E. Hull, B. D. Sykes, *J. Mol. Biol.* **1975**, *98*, 121–153.
[89] A. Boeszoermenyi, S. Chhabra, A. Dubey, D. L. Radeva, N. T. Burdzhiev, C. D. Chanev, O. I. Petrov, V. M. Gelev, M. Zhang, C. Anklin, H. Kovacs, G. Wagner, I. Kuprov, K. Takeuchi, H. Arthanari, *Nat. Methods* **2019**, *16*, 333–340.
[90] V. Mishra, R. J. Heath, *Int. J. Mol. Sci.* **2021**, *22*, 8411.
[91] J. F. Xu, Y. S. Yang, A. Q. Jiang, H. L. Zhu, *Crit. Rev. Anal. Chem.* **2022**, *52*, 72–92.
[92] A. Abdeldayem, Y. S. Raouf, S. N. Constantinescu, R. Moriggl, P. T. Gunning, *Chem. Soc. Rev.* **2020**, *49*, 2617–2687.

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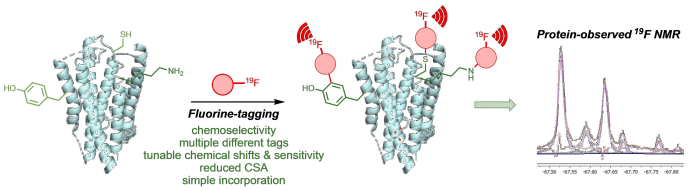

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'Tagging' proteins with fluorinated groups provides a tool to observe protein behavior at the atomic level using ^{19}F NMR. This background-free, environmentally-sensitive method allows researchers to study protein conformation, folding, aggregation and ligand/drug binding. Chemical tagging also allows the use of a much larger pool of fluorinated reporters with tunable chemical shifts and sensitivities compared to biosynthetic methods.