HHS Public Access
Author manuscript
*Chembiochem*. Author manuscript; available in PMC 2021 December 11.

Published in final edited form as:
*Chembiochem*. 2020 December 11; 21(24): 3451–3462. doi:10.1002/cbic.202000297.

# Perfluorocarbons in Chemical Biology

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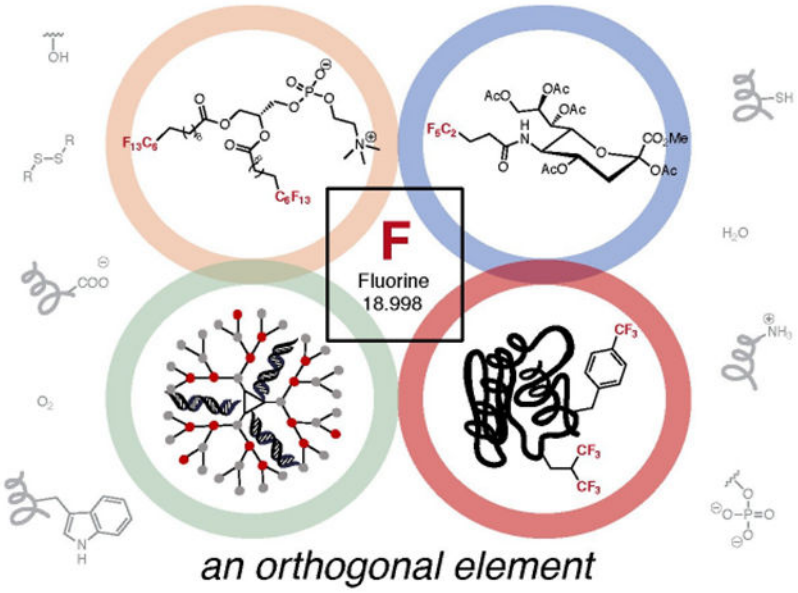
## Abstract

Perfluorocarbons, saturated carbon chains in which all the hydrogen atoms are replaced with fluorine, form a separate phase from both organic and aqueous solutions. Though perfluorinated compounds are not found in living systems, they can be used to modify biomolecules to confer orthogonal behavior within natural systems, such as improved stability, engineered assembly, and cell-permeability. Perfluorinated groups also provide handles for purification, mass spectrometry, and $^{19}$F NMR studies in complex environments. Herein, we describe how the unique properties of perfluorocarbons have been employed to understand and manipulate biological systems.

## Graphical Abstract




**Putting the 'F' into functional**: Perfluorocarbons are abiotic compounds that have unique properties, such as extreme hydrophobicity and the propensity to phase separate from aqueous and organic solutions. Herein, we review how perfluorinated biomolecules, materials, and probes function as orthogonal tools to study and manipulate biological systems.

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Conflict of Interest
The authors declare no conflict of interest.

**Keywords**

chemical biology; fluorine; orthogonal; perfluorocarbons; self-assembly

## 1. Introduction

Selectivity in complex biological environments is obtained through the coordination of many noncovalent interactions. Biomolecules have evolved such that they can efficiently function without interfering with one another. Complementary protein-substrate pairs have facilitated chemically induced protein dimerization,[1] protein-protein interactions,[2] and multi-color microscopy experiments.[3] Natural and synthetic membranes[4] form from amphiphilic lipids and define boundaries and compartments, mediating the entry and exit of ions and metabolites.[5] The exquisite specificity of DNA has resulted in an explosion of applications, including controlled cell-cell interactions,[6] biosensors,[7] and advanced nanomachines.[8]

Orthogonality has become an essential tool to allow chemical biologists to engineer selectivity in complex environments. Unique chemical handles enable natural systems to be probed and/or manipulated.[9–11] Abiotic functional groups with selective covalent reactivity are implicated in bioorthogonal chemistries to label, purify, and study biomolecules. Rare amino acid sequences confer binding to unnatural fluorophores and probes. In this review, we highlight orthogonality imparted by a single element, fluorine, and its implications in chemical biology.

## 2. Properties of Fluorine

Fluorine is rarely found in living systems,[12,13] yet it has unique characteristics due to its high electronegativity, steric profile, and low polarizability. Fluorine has a van der Waals radius of 1.47 Å, which is in between the radii of the hydrogen and oxygen nuclei (Figure 1A). Carbon-fluorine bonds are strong (110 to 130 kcal/mol) with a bond length of 1.3–1.4 Å, longer than a C–H bond but shorter than a C-O bond (Figure 1B). The small size of fluorine, high metabolic stability of the C–F bond, and fluorine's ability to dramatically change the electronics of a molecule have had the most widespread impact in the pharmaceutical industry, with 20–25% of current pharmaceuticals containing at least one fluorine atom.[14,15] The applications of fluorine to medicinal chemistry have been extensively reviewed.[16–21] The same fundamental properties of fluorine that render it prime for pharmaceutical optimization give rise to the distinct properties of perfluorocarbons, which we highlight herein. One further advantageous attribute of the fluorine atom is that the nucleus is NMR active with spin ½ and has 100% natural abundance, rendering fluorine an ideal contrast agent for magnetic resonance imaging (MRI).[22] Unlike $^{1}H$ NMR, $^{19}F$ chemical shifts can vary drastically (>350 ppm) making $^{19}F$ NMR a useful tool for sensing changes in conformation and environment.[23]

The effects of fluorine are intensified as the number of fluorine atoms on a carbon chain increases. Perfluorocarbons, molecules where all C–H bonds have been replaced by C–F bonds, create a separate phase from aqueous and organic solutions, demonstrating complete orthogonality to natural compounds. The separation of perfluorocarbons into the "fluorous"

phase is attributed to the high electronegativity of fluorine atoms precluding van der Waals interactions with neighboring molecules.[24,25] Perfluorocarbons would rather form a separate non-interacting phase than participate in induced dipole-induced dipole interactions with hydrocarbons or induced dipole-dipole interactions with water. Their low propensity for intermolecular interactions also explains the decreased boiling points of perfluorocarbons relative to their hydrocarbon counterparts (Figure 1C). It is important to note that the fluorous phase does not arise from enthalpically favorable fluorine-fluorine interactions, but rather the disinclination for perfluorinated molecules to interact with all other species.[26]

When perfluorinated amphiphiles are placed in water, another intriguing effect is observed. The perfluorinated surfactants self-assemble into micelles and/or vesicles with a $\Delta G$ that is lower than analogous hydrocarbon surfactants.[28] This phenomenon, referred to as the fluorous effect, originates from the large size of fluorine as compared to hydrogen (Figure 1C).[29] Thus, perfluorinated amphiphiles have more high-energy water molecules associated with them than hydrocarbon amphiphiles of the same length. Upon self-assembly, more water molecules are released, resulting in an extreme version of the hydrophobic effect.

As demonstrated with the fluorous phase and the fluorous effect, the unique properties of perfluorocarbons can generally be attributed to the high electronegativity and increased size of fluorine compared to hydrogen. These simple, yet powerful attributes provide orthogonality in complex environments that allow for protein-protein stabilization, self-assembly in membranes, enhanced permeability, altered cell surfaces, and biomolecule purification and immobilization.[30,31] In this review, we primarily describe applications of perfluorocarbons in chemical biology as molecules containing $CF_3$ groups or longer perfluorinated chains. We note that while trifluoromethyl groups do not strictly fall under the definition of perfluorocarbons, multiple $CF_3$ groups on one structure can often convey similar properties to a single, longer perfluoroalkyl chain.[32–35] Other fluorinated compounds, such as those containing single fluorine atoms or $sp^2$ C–F bonds, also have intriguing properties, which have been described elsewhere.[36–38]

## 3. Perfluorination of Peptides and Proteins

### 3.1. Incorporation of perfluorinated amino acids

Hydrophobicity mediates protein folding, formation of protein secondary structure, and protein-protein interactions. Perfluorocarbons are known to be even more hydrophobic than their hydrocarbon analogues (as discussed above) without significant changes in shape. As such, they provide a unique tool for designing and improving structural and functional features of many proteins. Fluorination of proteins can be achieved by unnatural amino acid (AA) incorporation or protein semisyn-thesis. Tirrell, Degrado, Kumar and others have demonstrated that fluorinated amino acids can be tolerated by the natural translation machinery. Trifluorovaline **1**,[39,40] trifluoroleucine **2**,[39,41–43] trifluoroisoleucine **3**,[44] trifluoromethionine **4**,[45–47] and hexafluoroleucine **5**[48] can be incorporated into proteins *in vitro* with auxotrophic *Escherichia coli* strains; though **1** and **5** require engineered hosts that overexpress the appropriate tRNA synthetase for efficient incorporation (Figure 2). Trifluoroethylglycine **6** can be incorporated with a mutated isoleucyl-tRNA synthetase.[49]

Unnatural trifluoromethyl phenylalanine **7**[50–52] and $OCF_3$Phe **8**[53] have been incorporated site-specifically using orthogonal tRNA/aminoacyl-tRNA synthetase pairs. Alternatively, fluorinated proteins can be achieved by the incorporation of fluorinated amino acids (such as **9**–**15**) by solid-phase peptide synthesis (SPPS) and/or native chemical ligation (NCL).[54–62] Although AAs with longer fluorinated chains ($\geq C_3F_7$) have been synthesized, their incorporation into peptides through biosynthetic methods is less common.[63–66]

### 3.2. Chemical methods for perfluorination of peptides and proteins

Perfluorination of amino acids to alter protein properties and facilitate $^{19}$F NMR analysis (more details in Section 3.3) was originally pioneered through unnatural amino acid incorporation; however, due to its success, more recent work has focused on installing fluorine atoms by chemical modification of proteins (Figure 3). Using classic bioconjugation reagents such as α-haloacetamides (**16** and **17**),[67–69] maleimides (**18**),[70] and N-hydroxysuccinimide (NHS) ester (**19**),[70] nucleophilic cysteine or lysine resides can be modified with trifluoromethyl groups. Fluorinated tags with different linkers (**19** versus **16**) provide distinct fluorine chemical shifts that could be differentiated by $^{19}$F NMR and be used to track proteins in a complex mixture.[70]

Perfluoroalkyl radicals provide methods to modify amino acids without the need for spacers between the fluorinated group and the amino acid. Inspired by a demonstration of trifluoromethylation with Langlois reagent **20** in cell lysate,[71] Davis, Gouverneur, and co-workers generated trifluoromethyl radicals under aqueous conditions and labeled aromatic amino acids and free cysteine.[72] Improved specificity could be achieved in the absence of free cysteine and at pH 6 to provide >50% conversion to $CF_3$-tryptophan (Figure 3B and C). Using fluoroalkylated iodine reagents (i.e., derivatives of Togni's reagent, **21**), tryptophan residues have also been modified with functionalized perfluoroethyl groups (Figure 3D).[73] With this method, minor modifications of other amino acids or other positions on the indole heterocycle are also observed. For cysteine modification, Umemoto's trifluoromethylation reagent[74] and **21** have been explored.[75,76] Though perfect selectivity for one amino acid has not yet been achieved, others have pursued similar strategies for trifluoromethylation of cysteine, tyrosine,[77] tryptophan, and/or histidine, which have recently been reviewed.[78]

These recent advances in fluorination of intact proteins with both classic bioconjugation chemistries and reactivity specific to perfluorinated reagents have enabled 19F NMR studies. Further advancements are still necessary to improve selectivity; however, once the chemistries are optimized, applications toward engineered proteins and assemblies can be pursued.

### 3.3. Perfluorinated proteins studied by $^{19}$F NMR

Given the absence of fluorine in biological systems and its high NMR sensitivity, incorporation of fluorinated AAs can be used to study proteins by $^{19}$F NMR. The addition of multiple, chemically equivalent fluorine atoms (i.e., trifluoromethyl, hexafluoroisopropyl, or perfluoro-*tert*-butyl) enhances the sensitivity of $^{19}$F protein NMR experiments. Many have used the above protein fluorination methods to monitor protein dynamics and protein-membrane interactions by NMR.[67–69,79,80] Mehl, Pielak, and co-workers have used site-

specific incorporation of trifluoromethylphenylalanine (**7**) to study protein conformation and binding,[50] as well as detect proteins in *E. coli*.[51] Whereas monofluorinated AAs resulted in broadened fluorine signal that was unable to be detected in large proteins, the fluorine signal from **7** could be detected even for proteins up to 100 kDa, demonstrating the need to incorporate perfluorinated groups.

Protein fluorination can also be used to study protein-small molecule interactions with $^{19}$F NMR.[81,82] Changes to protein conformation or hydration upon the introduction of a small molecule can be detected, which provides a unique assay for drug discovery. This technique, deemed protein-observed $^{19}$F NMR (PrOF), is an alternative to the more commonly performed ligand-observed $^{19}$F NMR methods, which rely on monitoring changes in fluorinated small-molecule fragments in the presence of a non-fluorinated protein target.[83] The molecular information obtained in PrOF NMR makes this approach particularly advantageous for screening small-molecule fragments and combining them together to invent higher affinity inhibitors (Figure 4). PrOF NMR was initially demonstrated using a monofluorinated tyrosine, but has since been extended to trifluoromethyl groups installed via protein modification as shown in Figure 3B.[72] By modifying lysozyme with $CF_3$ groups, the binding affinity of a known lysozyme inhibitor was determined by $^{19}$F NMR. With a trifluoromethylated myoglobin, chemical shift changes in the presence of various ligands were also observed. This approach holds promise as ligands discovered with PrOF can be further modified to create strong and selective inhibitors for interesting protein targets, as has been done for bromodomains.[84,85]

### 3.4. Perfluorination stabilizes protein-protein interactions

Beyond structural 19F NMR studies, fluorination can confer stability in proteins as well as drive protein folding and arrangement. Marsh, Kumar, and Tirrell have all investigated the effects of incorporation of fluorinated amino acids on protein structure and function.[26,39,48] They have found that introduction of fluorinated amino acids minimally perturbs secondary and tertiary structure. For example, Marsh and co-workers designed a 27-residue peptide which formed a tetrameric antiparallel four-helix bundle (Figure 5). In this design, leucine or hexafluoroleucine (**5**) residues would point toward the center of the bundle forming the hydrophobic core. The authors found that perfluorination did not disrupt the overall structure, and that fluorination provided a stabilizing effect - increasing $\Delta G_{\text{unfold}}$ by more than 10 kcal/mol when all leucine residues were replaced by **5**.[86,87] The replacement of hydrophobic amino acids with their fluorinated analogues can increase protein stability to both chemical and thermal denaturation. The stabilizing effect of these replacements can be attributed to the unique ability of fluorination to increase size and hydrophobicity of a side chain, while maintaining its shape. This ability is in contrast to traditional efforts to increase side chain hydrophobicity, which normally also alters side chain shape (i.e., valine to isoleucine) and thus sterically disrupts protein-protein interactions.

Ultimately, enhanced stability is important in the context of the utility of proteins and enzymes. Recently, the Montclare laboratory has shown that different fluorinated coiled-coil proteins (containing **2**) can self-assemble into robust nanofibers that bind metals or a small molecule[88] or if designed appropriately, form micelles (Figure 5B).[89] It is thought that

fluorination plays a role in the stability of these protein micelles, as the critical micelle concentration (CMC) was determined to be smaller than that of a non-fluorinated control. These nanostructures represent initial steps toward leveraging fluorination as a tool for responsive biomaterial design.[88] Protein fluorination also provides an alternative method to stabilize enzymes against denaturation by organic solvent or high temperature for industrial applications and biocatalysis.[90]

In a therapeutic context, fluorinated proteins have been shown to retain or to have improved activity in comparison to non-fluorinated variants. Tirrell and co-workers reported that a fluorinated coiled-coil peptide maintains its ability to bind target DNA similar to its natural counterpart.[91] Marsh and co-workers have shown that fluorinated antimicrobial peptides can retain their activity as therapeutic agents, while gaining improved stability to hydrolysis.[92] Although not yet as broadly used as fluorination in small-molecule drug discovery, protein fluorination is a strategy for stable nanostructures, catalysts, and therapeutics.

## 4. Enhanced Membrane and Cell Permeability with Fluorous Tags

### 4.1. Perfluorination facilitates self-assembly in membranes

Given that the replacement of hydrophobic amino acids with their fluorinated counterparts can enhance the stability of proteins in solution, others have looked to how fluorination of amino acids can be used to drive the formation of protein assemblies within membranes. Perfluorocarbons are the key to designing orthogonal protein assemblies as their hydrophobicity allows for partitioning into the membrane, and their lipophobicity allows for separation from natural hydrocarbon lipids. Kumar and co-workers have shown that rationally designed fluorinated peptides can insert into vesicle bilayers[93] or micelles[94] and self-assemble to form larger defined complexes driven by the unfavorable interactions of the fluorinated side chains with natural lipids.

Adding perfluorocarbons to membranes can also be achieved through the addition of fluorinated lipids. Kumar and co-workers demonstrated that the orthogonality of fluorinated lipids to hydrocarbon lipids allows for the formation of distinct perfluorocarbon domains in supported lipid bilayers (Figure 6A).[95] The affinity of perfluorocarbons for membranes can also be used to display molecules of interest on membrane surfaces to study how ligand clustering affects cell-surface interactions and alter cell adhesion.[96]

### 4.2. Fluorinated lipids and tags for cellular internalization

An extension of self-assembly and clustering of fluorinated lipids within membranes is the formation of artificial lipid rafts. Lipid rafts are rigid microdomains, generally formed by cholesterol enrichment. The formation of lipid rafts has been connected to enhancing endocytosis.[97,98] Given the tendency of fluorinated lipids to form rigid phase-separated domains in the presence of hydrocarbon lipids (Figure 6A), Kumar and co-workers postulated that artificial fluorinated lipids could be used to form domains within biological membranes and enhance internalization into cells.[95,99]

Cells incubated with partially fluorinated phospholipids bearing a biotin or fluorophore head group were more readily taken up than their hydrocarbon counterparts in an energy

dependent manner (Figure 6B). To demonstrate how this strategy could be used for delivering cargo into cells, partially fluorinated phospholipids with a biotin headgroup **22** were prepared and shown to more readily carry FITC-labeled avidin into the cytosol than the hydrocarbon analogue.[99]

Fluorinated tags have also been conjugated to oligonucleotides to facilitate their internalization into cells.[100] In some cases, it is thought that fluorination leads to self-assembly prior to internalization and that larger fluorinated oligonucleotide aggregates are preferentially endocytosed rather than internalized by lipid-raft-mediated mechanisms or passive diffusion.[101,102]

### 4.3. Fluorinated activators of cell-penetrating peptides

Perfluorinated fatty acids (ionic amphiphiles) have been employed in concert with cell-penetrating peptides (CPPs) to promote cellular internalization (Figure 6C). Perfluorocarbon fatty acids like **23**, which readily embed in cell membranes, are more basic than their hydrocarbon counterparts. Clustered perfluorinated acid **23** interacts with positively charged arginine residues to minimize charge repulsion and deliver fluorescently labeled, cationic CPPs (i.e., **24**) into cells more effectively than their non-fluorinated counterparts.[103]

## 5. Transfection agents and protein delivery

The enhanced uptake of fluorinated chains has made perfluorocarbons valuable components in the design of delivery vehicles. Cationic polymers are commonly employed to encapsulate negatively charged DNA and siRNA. Two of the common issues with the use of cationic polymers and amphiphiles for gene delivery are inefficient transfection and cytotoxicity of the material due to excessive positive charge.[104] The implementation of perfluorocarbon containing polymers and lipids to encapsulate nucleic acids mitigates both of those problems due to perfluorocarbons' affinity for membranes[105] and presumably the electronegativity of fluorine altering the p$K_a$ of cationic polymers.[106,107]

In the formulation of the delivery vehicle, cationic fluorinated polymers pack tightly around nucleic acids since fluorinated chains preferentially associate with each other and not the nucleic acids. Good packing improves the stability of these materials *in vivo* and requires lower polymer to DNA charge ratios than cationic hydrocarbon polymers.[108] Highly stable materials, which are not excessively cationic, result in decreased cytotoxicity. For example, when a G5 poly(amidoamine) (PAMAM) dendrimer is more than 50% fluorinated, efficient DNA and siRNA delivery can be achieved with minimal cytotoxicity when compared to a commercial transfection agent Lipofectamine 2000.[109]

More recently, charged fluorinated tags have also been used to encapsulate proteins for delivery to the cytosol. In 2018, Cheng and co-workers prepared a library of fluorinated and alkylated polymers from branched poly(ethyleneimine). In the presence of protein, these amphiphiles complex with proteins and assemble into uniform nanoparticles, which deliver protein into cells more effectively than a non-fluorinated polymer control.[110] Protein cargo has also been encapsulated inside perfluorocarbon nanoemulsions by using noncovalent fluorous tags that electrostatically interact with the protein surface to disperse protein inside

the perfluorocarbon core of nanoemulsions. In the presence of ultrasound, perfluorocarbons can undergo a liquid-to-gas phase transition causing the emulsions to rupture and release their protein cargo.[111]

## 6. Perfluorinated Artificial Cell Surfaces

Although many have found the addition of fluorine atoms to proteins and lipids to be advantageous, perfluorination of one of the other major classes of biomolecules - carbohydrates - has been less explored. Carbohydrates on cell surfaces are well known to play important roles in cell recognition, binding events, adhesion, and viral infections. Abnormal glycosylation can be implicated in tumor progression and metathesis. Given the hydrophobic and lipophobic nature of perfluorocarbons, cell-surface fluorination could provide a means to disrupt normal interactions of carbohydrates and lectins. The Kumar laboratory has explored the effects of cell-surface fluorination and introduced unnatural fluorinated carbohydrates by hijacking the sialic acid biosynthetic and salvage pathways, common approaches to introduce unnatural functionality into cell-surface glycans (Figure 7). Mannosamine derivatives can be converted to their corresponding sialic acid analogues through the sialic acid biosynthetic pathway, while unnatural sialic acids can be incorporated into cellular glycoproteins through the salvage pathway. In the latter case, there are fewer enzymatic steps that must be traversed, leading to higher levels of incorporation.

Kumar and co-workers prepared a library of fluorinated unnatural mannosamine (F-ManNAc, **25**) and sialic acid derivatives (F-Neu5Ac, **26**) which were then incubated with mammalian cell lines.[112] Fluorinated acetylated mannosamine derivatives with trifluoromethyl groups were metabolically incorporated and comprised 18% (**25a**) and 63% (**25b**) of the total membrane sialic acids respectively; however, longer fluoroalkyl groups on the mannosamine (**25c**–**e**) were not well tolerated. To incorporate longer chains, sialic acid derivative **26** was tested and resulted in 7% incorporation of $C_2F_5$ groups (**26d**). The metabolic incorporation of compounds **25** and **26** into the glycocalyx allowed for the display of $10^7$–$10^8$ $CF_3$ groups on the cell surface. Cells modified with trifluoromethyl groups showed less adherence to fibronectin and selectins, demonstrating that fluorination can be used to modulate cell adhesion.[113,114] The orthogonality of perfluorocarbons provides a tool for engineering new adhesion interactions between cells, biomolecules of interest, and unnatural surfaces.

## 7. Perfluorination for Purification and Immobilization

The ability of perfluorocarbons to self-associate has been used in synthetic chemistry as a way to quickly purify products and recycle catalyst and reagents since the 1990s when fluorous biphasic catalysis was introduced by Horvath.[115] In this approach, a molecule of interest (i.e., an expensive metal catalyst) can be modified with a fluorinated tag or ligand, such that it can be easily recovered from a reaction mixture by liquid fluorous extraction or by separation on fluorous silica gel, known as fluorous solid-phase extraction (FSPE).[116,117] Compounds with fluorous tags interact with fluorous silica gel and can be separated from both non-fluorinated and differentially fluorinated components of a mixture by washing with different solvent systems. This idea was quickly extended to the purification

and/or enrichment of both small molecules and biomolecules, including peptides, oligonucleotides, and carbohydrates.[118] Strategies for “capping” unreacted intermediates during peptide synthesis or tagging the desired product (or an undesired reagent) with fluorous chains allows for the simple removal of the tagged component by fluorous extraction (liquid or solid-phase) or even by centrifugation.[119–121]

### 7.1. Small-molecule library synthesis

Fluorous tags can aide in drug discovery through the preparation of large compound libraries. In 2001, Curran and co-workers introduced the concept of “fluorous mixture synthesis” (Figure 8A).[116,122] This strategy relies on the separation of compounds by their fluorous content on fluorous silica gel. In fluorous mixture synthesis, each substrate is modified with a different fluorous tag (i.e., $C_4F_9$, $C_6F_{13}$, $C_8F_{17}$, etc. or a mixture of these tags). The fluorinated substrates are then mixed together for a series of synthetic steps. The final compounds can be “unmixed” using FSPE and the tags can be removed in a deprotection step. The fluorous tag serves not only as a tool to separate the final products, but also as a barcode as the fluorous chain length relates to the retention time on the fluorous column and, thus, the identity of the initial substrate. Fluorous mixture synthesis has been employed to access stereoisomer libraries of macrolactones, oligoisoprenoids, and macrosphelides as well as a large number of natural product analogues.[123–126]

Fluorous tags have also been used to simplify purifications in diversity-oriented synthesis (DOS).[127] Initial substrates were modified with a fluorous tag and used in a variety of transformations. After each step, the fluorous-tagged product was separated from the reagents simply by FSPE, thereby avoiding column chromatography over multiple steps. In comparative studies between “homogenous” DOS with fluorous tags and “heterogenous” DOS with traditional solid-supports, it was found that the fluorous-tag strategies were preferred due to the ease and speed of purification with FSPE.[128]

### 7.2. Proteomics with fluorous tags

In proteomics and molecular biology, researchers often use separation and enrichment techniques to accurately detect, identify, and quantify proteins and post-translational modifications. Traditional techniques include biotin tags for detection with avidin resins and oligohistidine peptides (His tags) for Ni-NTA affinity chromatography. In 2005, Peters and co-workers applied a fluorous tagging strategy to proteomics (Figure 8B). Upon protein (or cell lysate) digest, peptides of interest were tagged with fluorous chains. Cysteines were detected with fluorous iodoacetamides (**27**), amines were tagged with fluorous NHS esters (**28**), and *O*-phosphorylation could be detected by β-elimination and Michael addition with a fluorous thiol (**29**).[129] Mixtures of tagged and untagged peptides were loaded onto a FSPE cartridge, isolated, and analyzed by mass spectrometry (MS). It was found that enrichment could be obtained based on differing fluorine content (i.e., two fluorous tags in one peptide), allowing for separation of peptides with multiple tagged residues or post-translation modifications.

Compared to biotin-based reagents, fluorous-tagged peptide fragments were easily removed from fluorous-tagged silica, facilitating product recovery. These fluorous tags are also inert

and stable under MS analysis as no fragmentation is observed leading to less complicated tandem mass spec MS/MS analysis. This approach has been extended to the enrichment of peptides with other side chain functionalities and small-molecule metabolites by using perfluoroalkyl chains with different functional handles,[130] many of which are now commercially available or have additional functionality, such as photocrosslinkers.[131,132] Fluorous tags installed through direct radical trifluoromethylation of proteins can also be used for MS protein profiling applications.[133,134] Recently, noncovalent fluorous-tagging has been explored for isolating proteins, however this has yet to be demonstrated in a complex biological environment.[135]

### 7.3. Fluorous microarrays

Another area where fluorous tags associating with fluorinated solid supports has been leveraged is the creation of microarrays (Figure 8C). Generally, microarrays require covalent modification of the surface with molecules of interest.[136,137] However, Pohl and co-workers found that fluorous tagged carbohydrates could be patterned onto a fluorinated glass slide, eliminating the need for the installation of functional handles onto slides. When exposed to a fluorescently labeled lectin, the authors observed selective binding even with repeated washes with detergent containing buffer, demonstrating the robustness of the fluorous-derived microarray.

Since this initial report, fluorous microarrays have been used to present DNA and proteins. These works have explicitly demonstrated minimal nonspecific binding on fluorous microarrays.[138,139] Fluorous-tagged DNA can be used for micropatterning in a reversible manner through five cycles of immobilization and removal.[138] Peptides can be presented for protease screening; careful design of the linker between the peptide and the fluorous tag leads to negligible enzyme inhibition.[140] Similarly, small molecules have been displayed to identify histone deacetylase inhibitors.[141]

Crude cell-lysates containing enzymes of interest can be assessed by treating fluorous-tagged enzyme substrates immobilized on a fluorous surface. Cellular material can be washed away such that only fluorous-tagged substrates and products are retained on the surface. Using nanostructure-initiator mass spectrometry (NIMS), which is a soft desorption and ionization method, a ratio between substrates and products can be measured. The noncovalent nature of fluorous microarray formation is thought to provide flexibility to the immobilized substrates, enhancing enzyme activity when compared to covalent surface attachment, and also allow for ionization of the substrates.[142,143] Kiessling and co-workers have generalized the NIMS platform to less promiscuous enzymes with a clickable variant where the initial substrates are modified with an azide. Post reaction, substrates and products are treated with perfluorinated terminal alkynes and then immobilized. With this enzyme-activity assay, P450 mutants can be screened for the oxidation of small terpenes.[144] In the future, one could envision combining fluorous tag library synthesis (Section 7.1) with fluorous microarrays, where instead of removing the fluorous tags from the final compounds, the compounds could be directly displayed on a microarray for target identification or enzyme activity determination by NIMS.

## 8. Perfluorinated MRI Tags and Probes

Given the utility of perfluorocarbons for $^{19}$F NMR, they are also promising molecules for magnetic resonance imaging (MRI) applications. The high sensitivity of fluorine to magnetic resonance provides opportunities for multiplexing and responsive probes. Additionally, the absence of fluorine from biological tissue makes for little background signal. Ideally, highly symmetric perfluorocarbons (perfluoro-15-crown-5 ether **30**, PERFECTA **31**, etc.) with many chemically equivalent nuclei can be leveraged for greater signal (Figure 9A).[145] However, perfluoro-15-crown ether and PERFECTA are insoluble in water, necessitating their formulation within nanomaterials, such as emulsions, for imaging applications in cellulo and in vivo. The use of fluorinated nanomaterials for medical imaging has been reviewed extensively elsewhere.[146]

Complementary to this work, we describe recent examples of small-molecule fluorinated tags, which can be used to probe cellular environments by MRI. The Pomerantz group has devised a solution to have high signal from fluorinated molecules that are still aqueous soluble. Disordered peptide **32** with trifluoroacetylated lysines among multiple unmodified lysines was synthesized (Figure 9B).[147] In designing a highly disordered peptide without significant secondary structure, resonance degeneracy of the fluorine nuclei was achieved, providing thirty approximately “equivalent” fluorine atoms without significant line broadening. The placement of many lysine residues within this peptide conferred water solubility to the probe. The fluorinated oligo(lysine) peptide could then be conjugated to BSA without observing significant signal broadening that is typical for large biomolecules. Minimal signal broadening was attributed to the conformational flexibility of the fluorinated side chains appended to the peptide. This work suggests that high signal $^{19}$F MRI probes can be obtained with fluorinated molecules of high structural flexibility and disorder, as an alternative to high symmetry. Ultimately for these probes to be applicable to animal models, further gain of signal could be obtained by using different fluorinated AAs with perfluoro-*tert*-butyl groups or multiple disordered peptides could be conjugated to a protein or nanomaterial.

The high sensitivity of fluorine nuclei to its environment has also been capitalized on by the Que group. Que and co-workers have shown that fluorinated redox-sensitive metal complexes can be employed to study changes in biological environments, like enzyme activity, redox events, and the presence of reactive species or ions (Figure 9C).[148] A known copper (PET) probe was modified to alter the ligand scaffold by fluorination to access **33**.[149] In this system, paramagnetic $d^9$ $Cu^{2+}$ shortens the spin-spin relaxation time ($T_2$) of nearby $^{19}$F nuclei, weakening the signal of the fluorine nuclei providing an “off” state for the probe. In a hypoxic environment, $Cu^{2+}$ is reduced to diamagnetic $d^{10}$ $Cu^{+}$, resulting in a lengthening of $T_2$ and turning the $^{19}$F signal “on”. Ligand dissociation upon copper reduction also results in signal “turn on”. Increasing the number of equivalent fluorine atoms in the probe improves the signal intensity (**33** to **34**). In addition, altering the distance between the copper center and the fluorine atoms with ethylene glycol linkers (**34**, $n$=1–4), improves aqueous solubility and modulates the $Cu^{2+}/Cu^{+}$ reduction potential - improving sensitivity and selectivity for hypoxia.[150]

By choosing different paramagnetic/diamagnetic metal cycles (i.e., $Co^{2+}$ to $Co^{3+}$, etc.) and changing the fluorinated ligand scaffolds (**35**), the Que group has also been able to monitor reactive oxygen species, cysteine presence, and peroxidase activity by $^{19}F$ MRI.[151] Careful understanding and improved ability to design stimuli-responsive probes will enable new opportunities to bring chemical biology tools to an array of biological systems.

## 9. Summary and Outlook

In the past decade, many have explored how abiotic, hydro-and lipophobic fluorocarbons can be integrated with naturally occurring systems. Both seemingly small ($CF_3$) and larger changes ($C_nF_{2n+1}$) can alter self-assembly, improve biomolecule stability, and facilitate cellular entry. Perfluorinated groups can interact with or be incorporated into all four classes of biomolecules. Tools to design and access fluorinated proteins have been developed, and their structures and high stability have been extensively explored. These fundamental studies provide the basis for new applications of highly stable fluorinated proteins in the therapeutic realm. With careful engineering one could also use fluorination to design better stabilized coiled-coil protein interactions that function as improved peptide tags for protein labeling.[152,153]

Direct perfluorination of biomolecules has been explored, though excellent selectivity remains difficult. Given their increased hydrophobicity, perhaps extended perfluoroalkyl radicals[154,155] could improve chemoselectivity or be harnessed for selective labeling of hydrophobic proteins and membrane-bound protein domains, which has been difficult with current radical protein modifying techniques.[134] Fluorous tagging has been explored for proteomics and microarray formation, spanning a range of biomolecules and small molecules. New fluorous-tagging strategies are poised to be used as an orthogonal handle, alongside more traditional chemistries, for multiplexed proteomics and microarray formation.[156]

$^{19}F$ NMR and MRI techniques have enabled researchers to study the conformational dynamics of many isolated proteins as well as identify small-molecule binders. In the future, simple and direct methods for RNA perfluorination could extend $^{19}F$ NMR discovery techniques to small-molecule binders of RNA.[157–159] Responsive fluorinated probes containing paramagnetic metals facilitate the study of redox changes in cellular environments by $^{19}F$ NMR. Continued development of tags and probes with enhanced fluorine signal, like the perfluoro-*tert*-butyl group, could facilitate further 19F NMR studies in mammalian cells and animals.[160]

Looking forward, the significant work towards incorporating perfluorinated groups into proteins, peptides, carbohydrates, and lipids should facilitate new tools for manipulating and studying biological systems in research laboratories and in clinical settings. Perfluorination could be a tool for subcellular localization of proteins and increase membrane affinity as palmitoylation does[161,162] or to help therapeutic biomolecules and diagnostics permeate the hydrophobic blood-brain barrier.[18,163–166] Beyond directing events in cellulo and in vivo, perfluorination could provide exciting opportunities for interfacing biological systems with unnatural hydrophobic materials.

## Acknowledgments

We thank D.A. Estabrook, M. Pengshug, and H.L. van de Wuow for critical reading of this manuscript. M.A.M. is supported by the Chemistry-Biology Interface Training Program (NIH 5T32GM008496). E.M.S. acknowledges the following for support for fluorous chemical biology and nanotherapeutics work within the group: The University of California Cancer Research Coordinating Committee (UC CRCC, grant no. CNR-18-524809), the American Chemical Society Petroleum Research Fund (ACS PRF, grant no. 57379-DNI4), the Alfred P. Sloan Award (FG-2018-10855), and the Hellman Fellows Award.

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Prof. Ellen Sletten received her BSc in chemistry from Stonehill College in 2006 and PhD in chemistry in 2011 from UC Berkeley. Her graduate work in Carolyn Bertozzi's laboratory involved the development of new bioorthogonal chemistries. Ellen performed postdoctoral studies in Timothy Swager's group at MIT working on dynamic fluorous materials. In late 2015, she began her independent career as an assistant professor in the Department of Chemistry and Biochemistry at UCLA. Her group focuses on methods to control and detect chemistries in living systems and has been recognized with an Alfred P. Sloan Award and NIH New Innovator Award.

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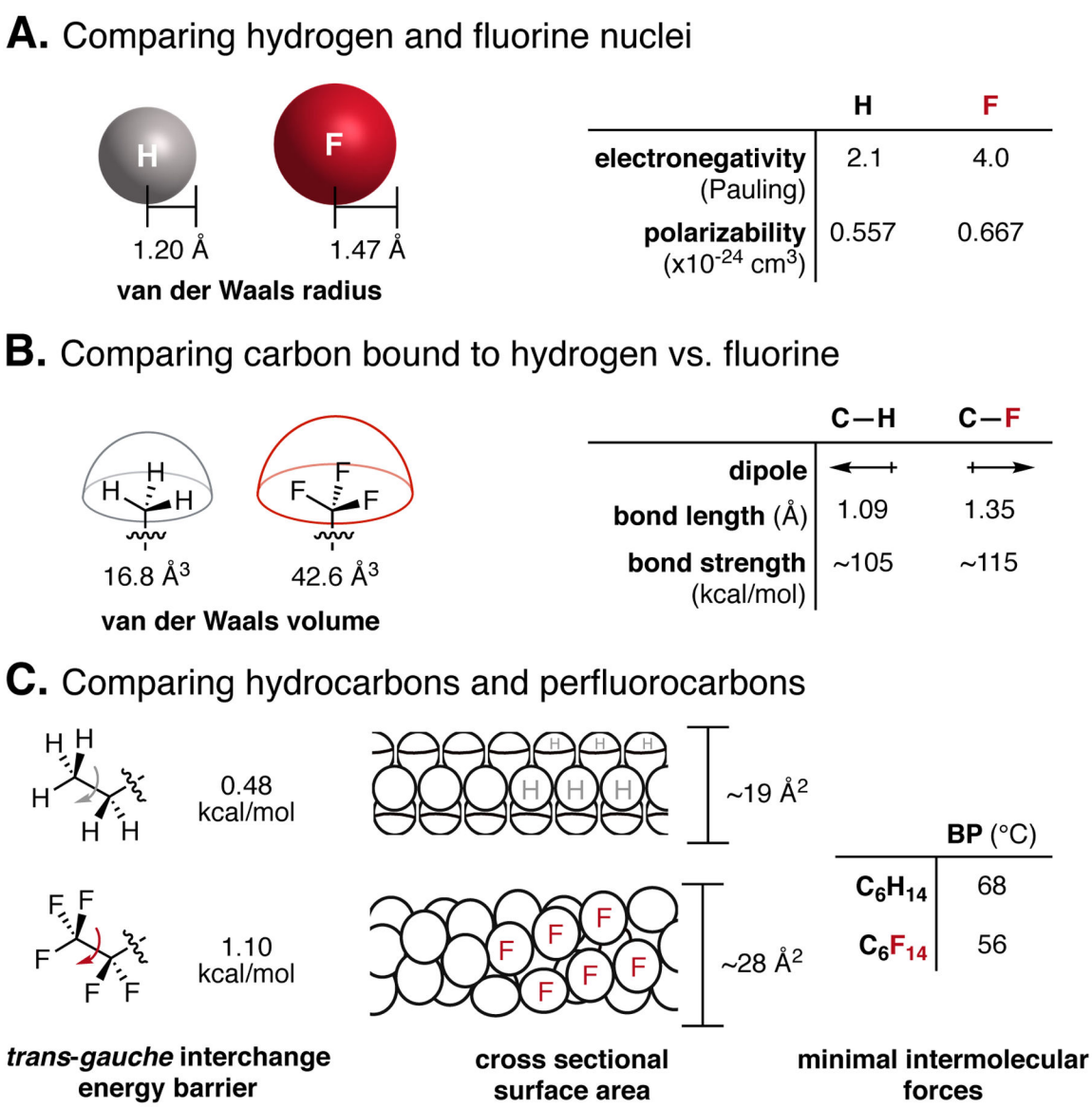



**Figure 1.**

Characteristics of perfluorocarbons as they relate to the C–F bond. A) Comparing hydrogen and fluorine nuclei. B) Comparing the C–H with the C–F bond and the methyl with the trifluoromethyl group. C) Comparing hydrocarbons and perfluorocarbons. The larger size of fluorine increases the rotational barrier in perfluorocarbons making them more rigid. The larger size of fluorine also increases the overall surface area of perfluorocarbons making them more hydrophobic. The low polarizability of fluorine and C–F bonds leads to few intermolecular forces and low boiling points.[24,25,27]

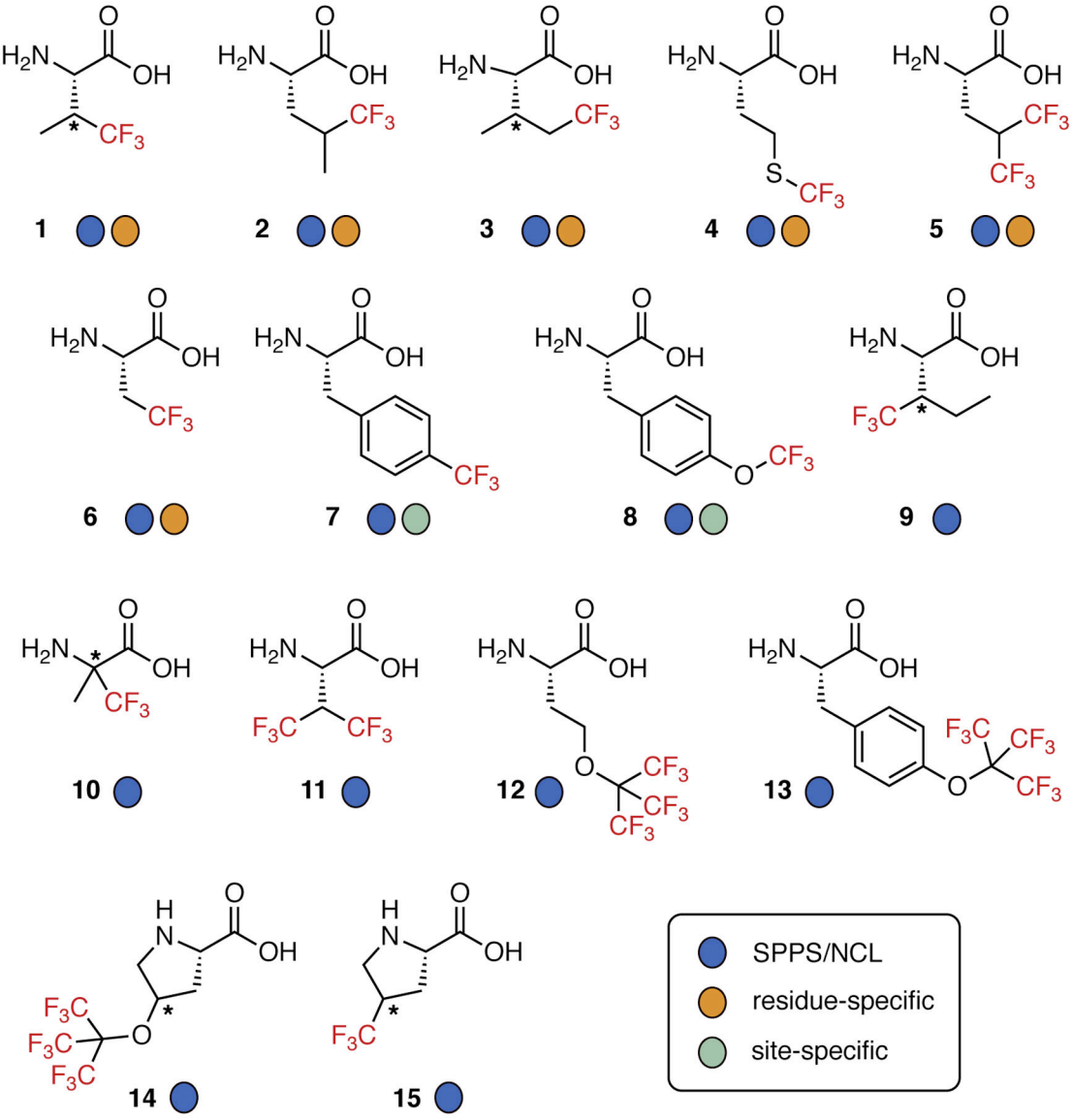



**Figure 2.**

Selected examples of fluorinated amino acids and their modes of incorporation into peptides and proteins. Fluorinated residues can be incorporated by using auxotrophic cell lines (orange circle) or site-specifically with amber stop codon suppression and an orthogonal aminoacyl-tRNA synthetase/tRNA pair (green circle). All the fluorinated amino acids can be incorporated through solid-phase peptide synthesis (SPPS) or native chemical ligation (NCL; blue circle).

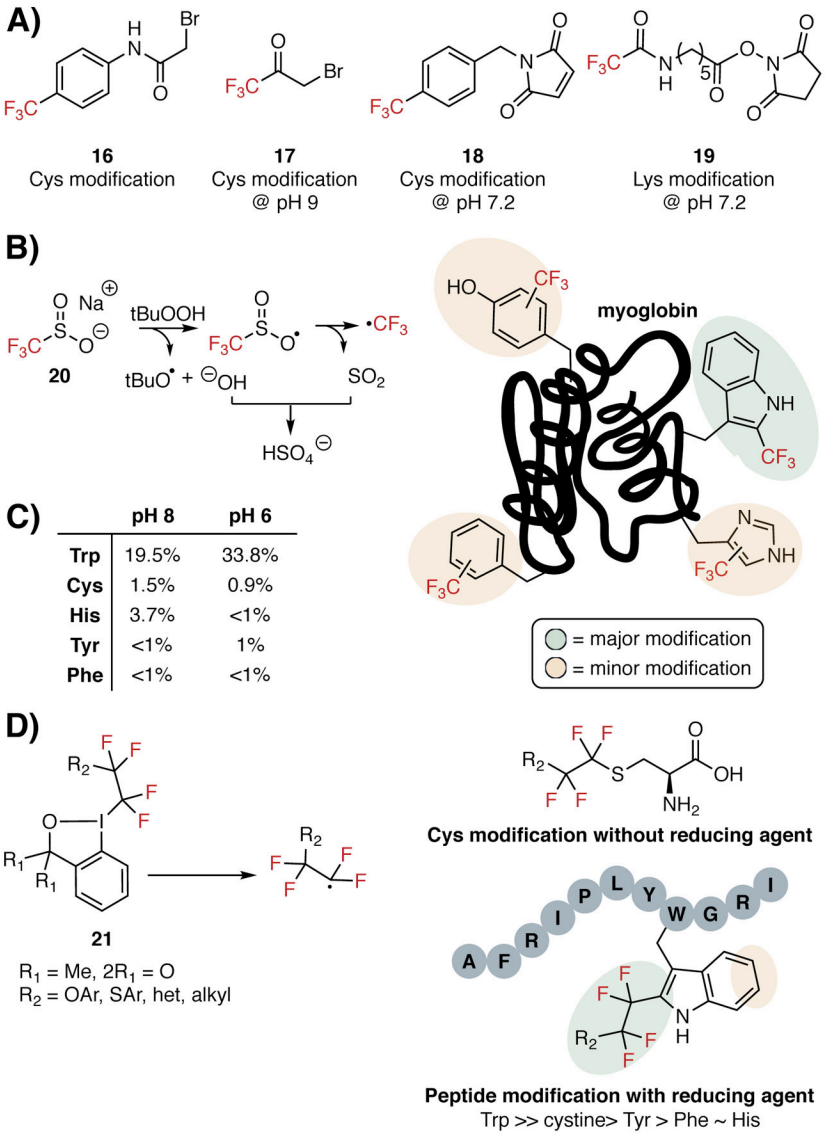



**Figure 3.**

Chemical methods for perfluorination of peptides and proteins. A) Classic bioconjugation reagents used to add trifluoromethyl groups to cysteine and lysine residues in proteins. B) Generation of a trifluoromethyl radical from Langlois reagent **20** under aqueous conditions leads to direct protein modification. Major trifluoromethylation occurs on tryptophan residues, though minor modification is observed on other aromatic residues and free cysteines, if present. C) Results from the reaction of an equimolar mixture of five amino acids and **20** run to 40% conversion to determine residue specificity at different pH values. [72] D) Hypervalent iodine reagents (Togni's reagent, **21**) can be used to add functionalized perfluoroethyl groups to amino acids such as cysteine. Tryptophan residues can also be perfluorinated in the presence of **21** and a reducing agent. Modification of tryptophan under these conditions occurs primarily at the indole-C2 position with minor modifications on the other indole carbons or other aromatic residues.

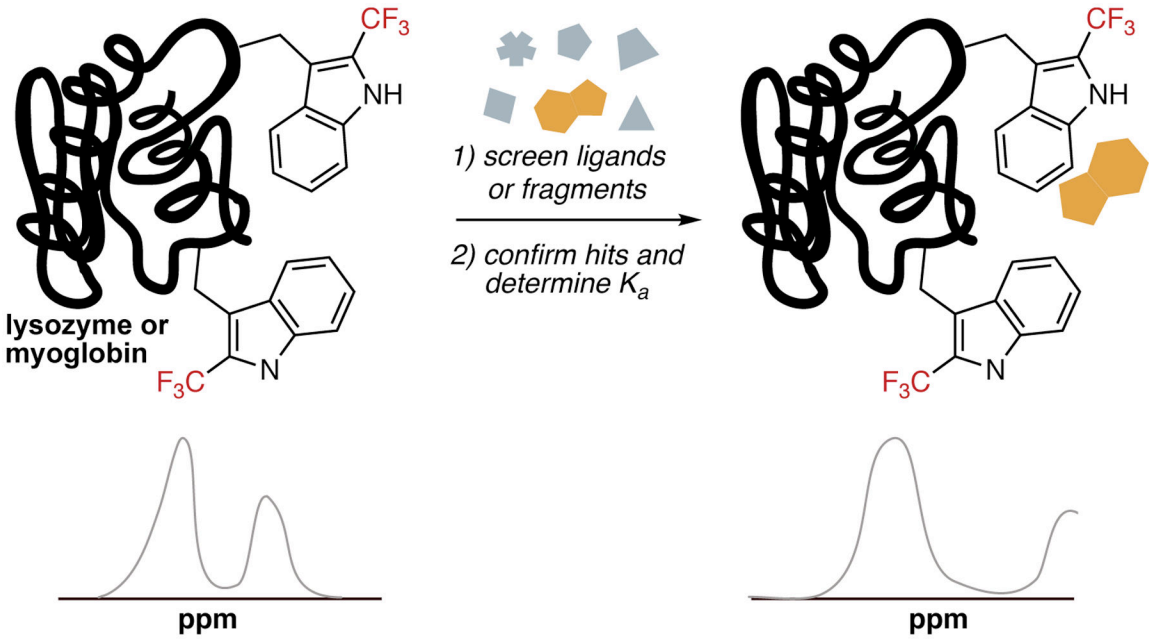
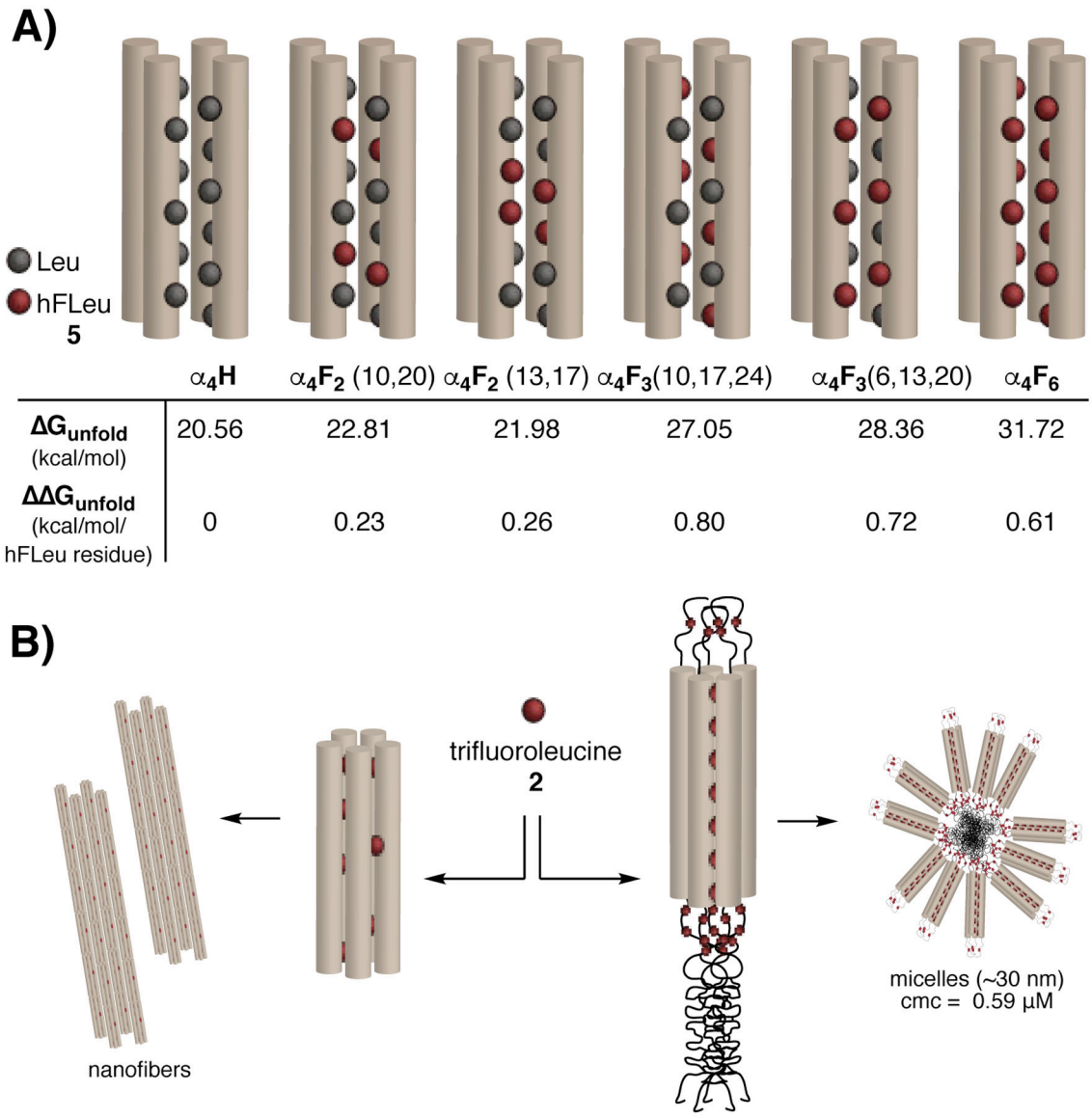



**Figure 4.**

Protein-observed $^{19}$F NMR (PrOF NMR). This method uses the incorporation of fluorinated amino acids to monitor protein conformation changes in the presence of small-molecule fragments for drug discovery. Proteins with trifluoromethylated tryptophan residues have been used in these types of studies.[72]




| | $\alpha_4H$ | $\alpha_4F_2$ (10,20) | $\alpha_4F_2$ (13,17) | $\alpha_4F_3$(10,17,24) | $\alpha_4F_3$(6,13,20) | $\alpha_4F_6$ |
|---|---|---|---|---|---|---|
| $\Delta G_{unfold}$ (kcal/mol) | 20.56 | 22.81 | 21.98 | 27.05 | 28.36 | 31.72 |
| $\Delta\Delta G_{unfold}$ (kcal/mol/ hFLeu residue) | 0 | 0.23 | 0.26 | 0.80 | 0.72 | 0.61 |

**Figure 5.**

Understanding the thermodynamics of fluorinated proteins and applications. A) Substituting fluorinated amino acids for their hydrocarbon counterparts can improve the stability of peptide bundles toward chemical denaturants. Both the number and placement of fluorinated hexafluoroleucine (hFLeu, **5**) residues can affect stability. Figure adapted and data from Marsh and co-workers.[86,87] B) Incorporation of trifluoroleucine **2** into different coiled-coil proteins allows the formation of nanostructures such as fibers and micelles. Fluorination confers stability on the fibers[88] and lowers the CMC.[89]

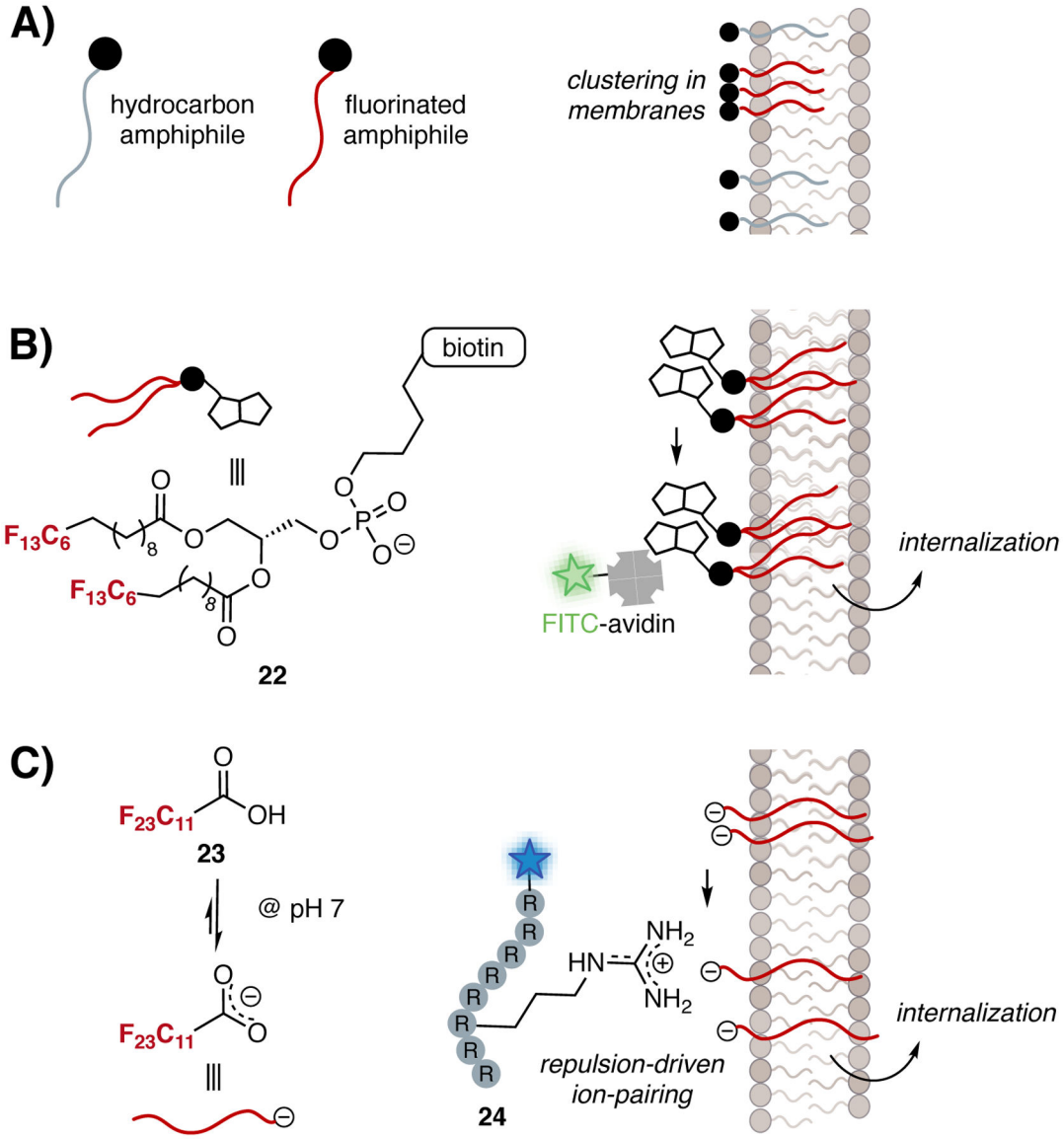



**Figure 6.**

Use of fluorinated amphiphiles for cellular internalization. A) Fluorinated amphiphiles tend to aggregate in membranes. B) Fluorinated phospholipids modified with biotin first cluster together in membranes. Addition of FITC-avidin allows the internalization of avidin cargo into the cytosol (FITC=fluorescein isothiocyanate).[99] C) Perfluorinated carboxylic acids (like **23**) assemble in membranes. Due to charge repulsion, the perfluorinated acids preferentially associate with arginine-containing, cationic peptides rather than remaining aggregated, thus facilitating the entry of these payloads into the cytosol.[103]

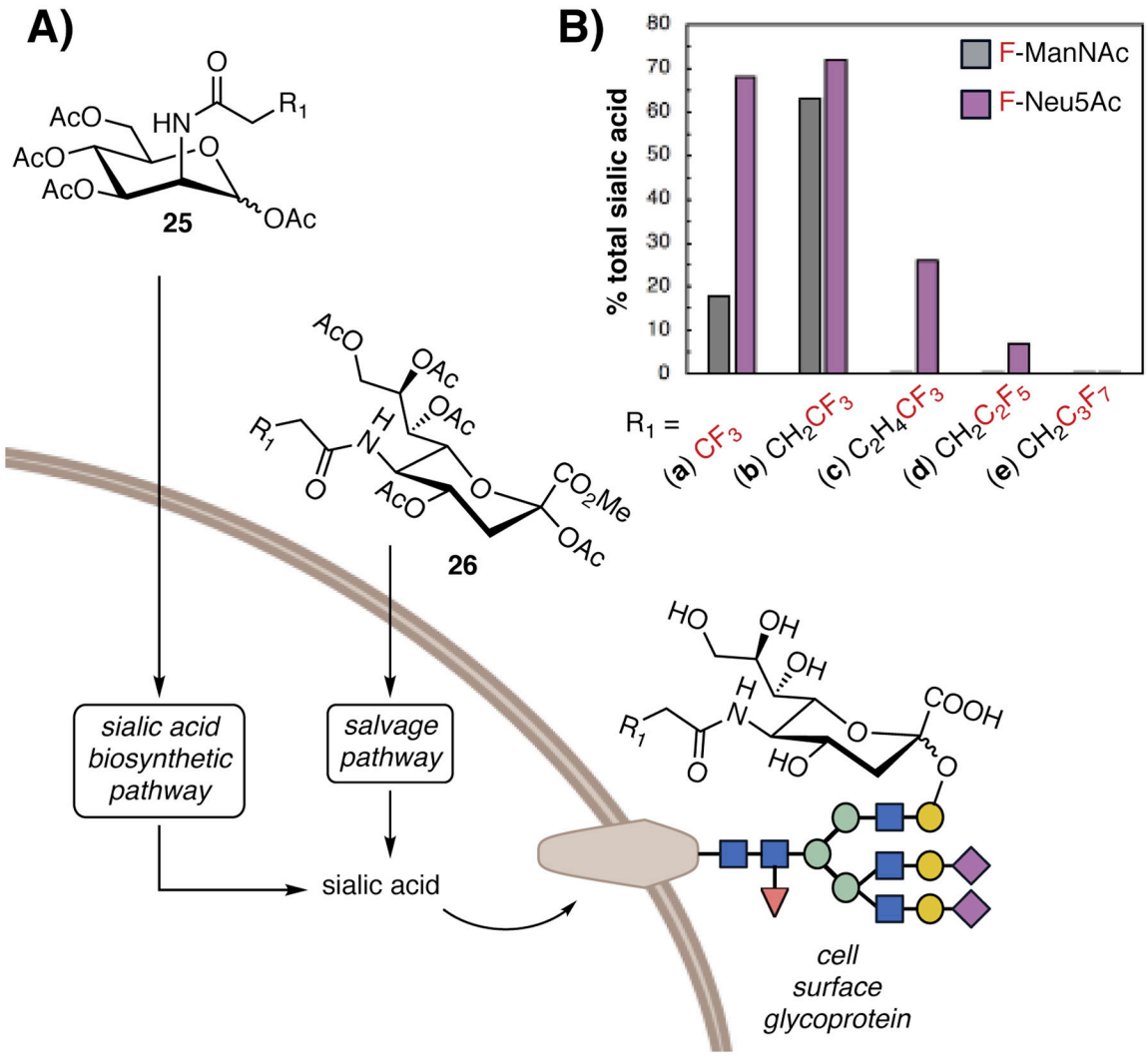



**Figure 7.**

Incorporation of unnatural fluorinated carbohydrates. A) Metabolic incorporation of fluorinated monosaccharides through hijacking the sialic acid biosynthetic and salvage pathways. B) After incubation with mannosamine derivative **25** (F-ManNAc) or sialic acid derivative **26** (F-Neu5Ac), the percent of total sialic acid was obtained by acid hydrolysis of cell-surface sialic acids, derivatizing with fluorogenic 1,2-diamino-4,5-methylene-dioxybenzene, and HPLC analysis. Data shown are for incorporation in HL60 cells.[112]

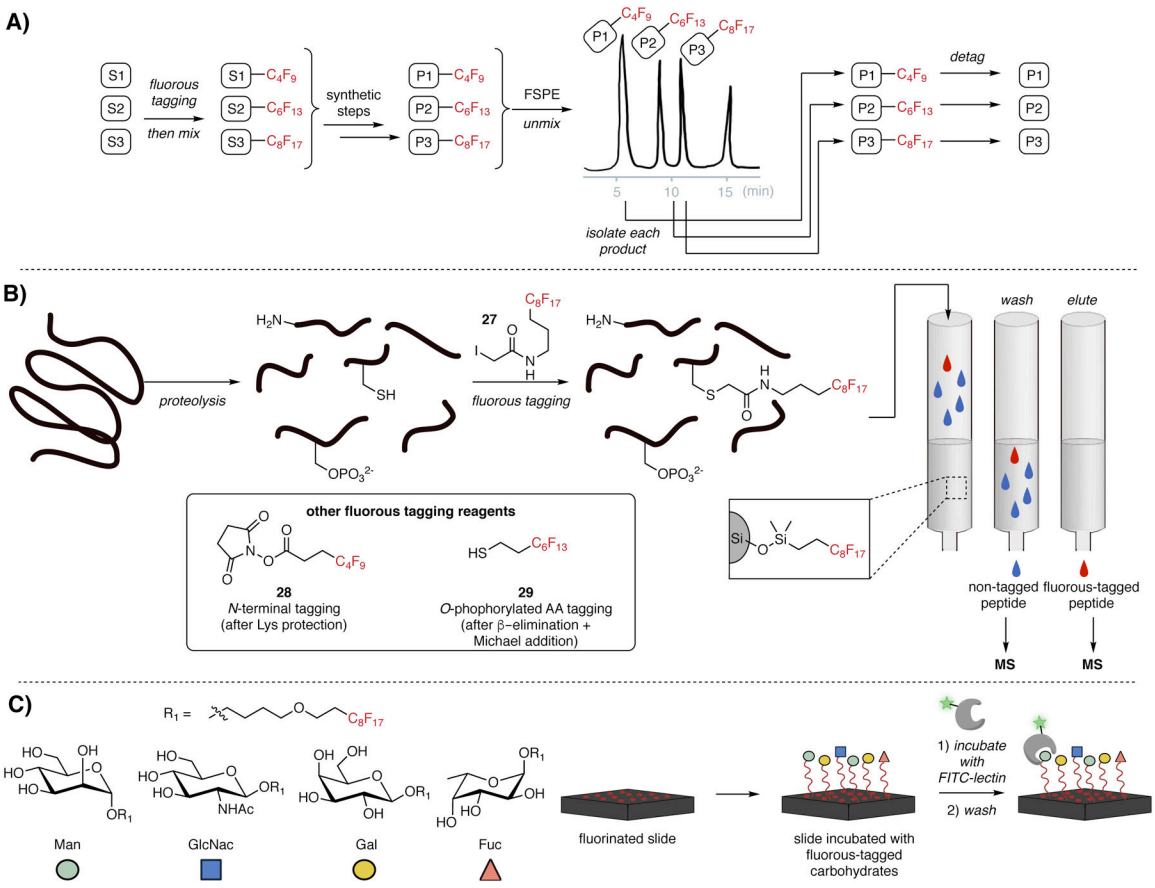



**Figure 8.**

Fluorous-mediated purification, immobilization, and characterization. A) Fluorous mixture synthesis. Substrates (S) modified with fluorous tags of different chain lengths can be mixed for reaction sequences to form fluorous-tagged products (P), which can be unmixed by solid-phase fluorous (FSPE). B) Fluorous tagging of proteins and separation of fluorous-tagged peptide fragments by FSPE. C) Fluorous microarrays for immobilization of fluorous-tagged carbohydrates and studying carbohydrate-binding.

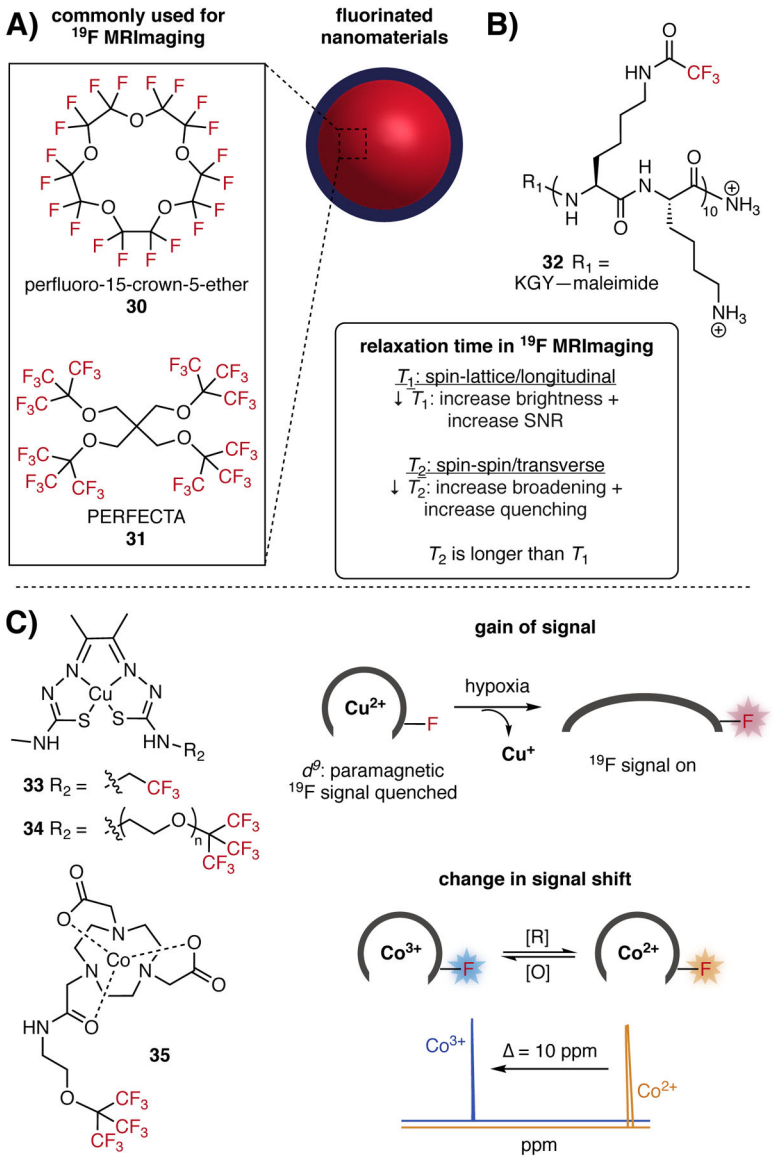



**Figure 9.**

Fluorinated molecules for $^{19}$F MRI. A) Perfluorocarbons with many chemically equivalent fluorine atoms for increased $^{19}$F MRI signal when formulated as nanoemulsions. B) Disordered peptide used by Pomerantz and co-workers to achieve degenerate fluorine signal by NMR and MRI. C) Examples of metal coordinated fluorinated probes reported by Que and co-workers.