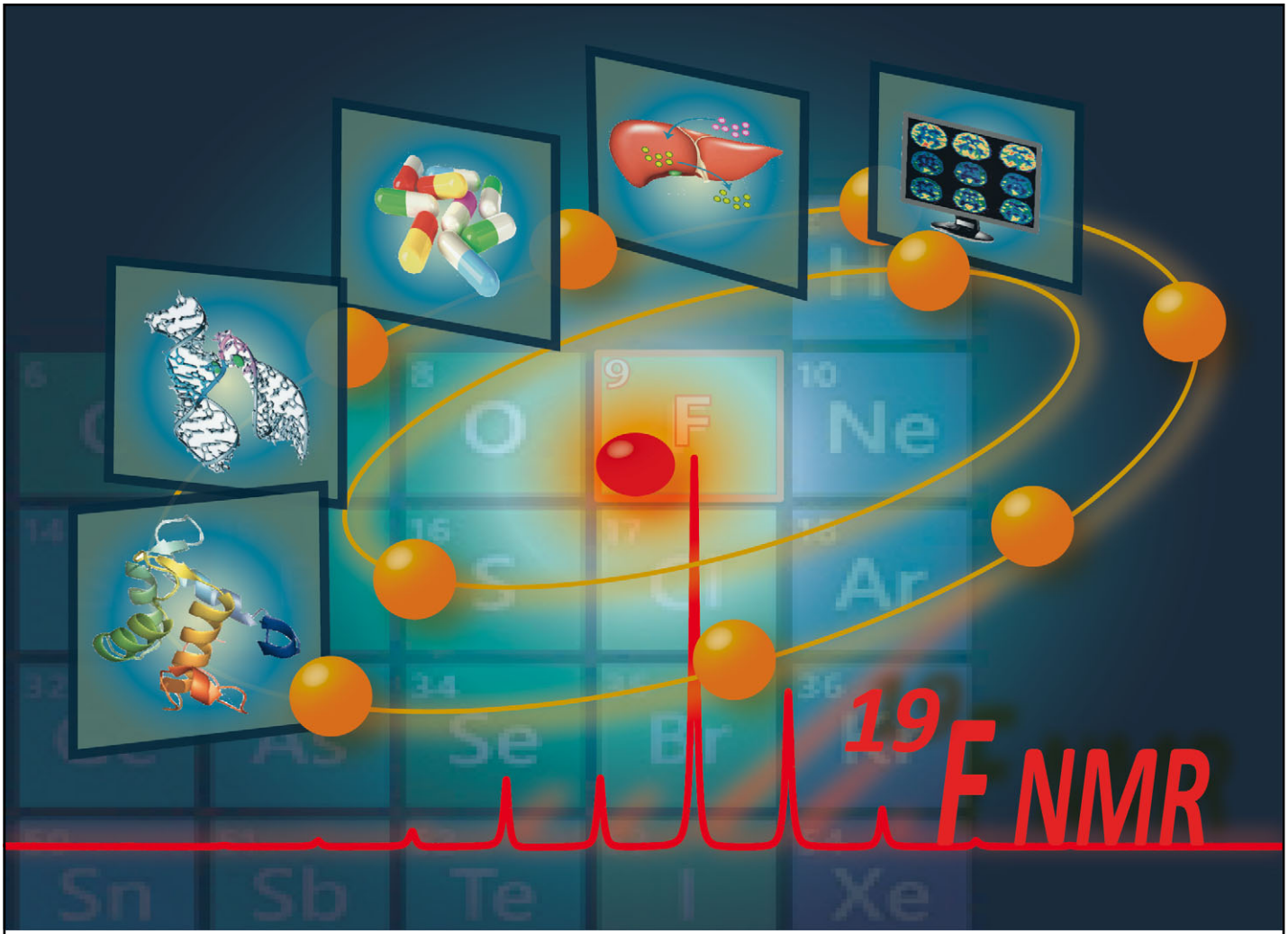

Featuring work from the research groups of Drs Stéphane Viel, Fabio Ziarelli and Ling Peng at Aix-Marseille University, Marseille, France.

^{19}F NMR: a valuable tool for studying biological events

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^{19}F NMR: a valuable tool for studying biological events

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With the spectacular advancement of NMR techniques and the flourishing of fluorine chemistry allowing the synthesis of various fluorinated molecules, ^{19}F NMR represents a compelling option for studying myriad biological events ranging from the structure and function of biomolecules, enzymatic mechanisms, and metabolic pathways, through to drug screening and discovery as well as medical imaging. In this *tutorial review*, we aim to provide readers with a brief overview of the recent applications of ^{19}F NMR in various areas relating to biological and biomedical research, together with a brief introduction of specific hardware improvements permitting the practical implementation of ^{19}F NMR.

Key learning points

(1) Favorable NMR properties make fluorine a special nucleus for studying biological systems.
(2) Recent hardware advances make the ^{19}F NMR study of biological systems convenient and a reality.
(3) Recent applications of ^{19}F NMR in studying the structure and function of biological macromolecules such as proteins and nucleic acids *etc.*
(4) Implementation of ^{19}F NMR in drug screening, hit discovery and target identification.
(5) ^{19}F NMR for studying the metabolism of drugs, agrochemicals and biologically relevant molecules.
(6) Using ^{19}F NMR for assessing the environmental impact of fluorinated agrochemicals or other relevant compounds.

Introduction

Since as early as 1970, the use of ^{19}F NMR spectroscopy in combination with fluorinated molecular probes, has been recognized as a non-invasive and attractive approach to investigating the structure and function of proteins, a very important class of biomolecules.[1] Ongoing improvements in resonance magnetic technology alongside innovations in data acquisition and advancements in fluorine chemistry continue to broaden the range of applications of ^{19}F NMR to study biological phenomena with fluorine labeled molecules.[2–4]

In this review, we present our perspective on ^{19}F NMR developments for the investigation of biological systems focusing mainly on work carried out over the past five years. Readers are advised to refer to previously published reviews for extensive summaries of earlier results.[1–4] In the first section of this review, we recapitulate the specific advantages of the fluorine nucleus for NMR investigations, giving some insights into recent hardware developments that have contributed to further reinforcing its intrinsic interest as a molecular probe for the investigation of biological systems. The following sections mainly focus on the use of ^{19}F NMR in combination with fluorinated molecular probes in structural and functional studies of proteins and nucleic acids,[5] drug screening and discovery,[6,7] metabolism analysis and *in vivo* tracking[8] of bioactive molecules, as well as in other applications such as gene transfection reporting and molecular imaging. Overall, in the interest of tutorial utility, we have strived to give a balanced and concise yet informative overview of this broad topic. As such, the description of some important works has voluntarily been made short, irrespective of their individual significance.

Why and how is fluorine NMR used?

^{19}F nuclear magnetic properties

The ^{19}F nucleus presents many favorable magnetic properties. Firstly, it is a spin-1/2 nucleus and has no quadrupolar moment, thereby greatly simplifying the spectral analysis. Its 100% natural abundance as well as a high gyromagnetic ratio make ^{19}F only about 17% less sensitive than ^{1}H but much more

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informative from a chemical viewpoint. Indeed, its much broader chemical shift range (over 400 ppm for organofluorine compounds) allows the detection of even subtle modifications in the chemical environment. Compared to other typically investigated NMR receptive nuclei, the sensitivity of ^{19}F is also much greater (roughly by a factor of 8, 27 and 250 with respect to ^{31}P, ^{13}C, and ^{15}N, respectively). When analyzing non-isotopically enriched samples, the advantage of using ^{19}F over ^{13}C or ^{15}N becomes even more obvious because the natural abundance of ^{13}C and ^{15}N is only 1.1% and 0.4%, respectively. The high gyromagnetic ratio of ^{19}F also results in rather strong dipolar couplings, leading to ^{19}F–^{19}F and ^{19}F–^{1}H nuclear Overhauser enhancement (NOE) effects that can be used as distance constraints in structural elucidation. In addition to exhibiting reasonably narrow resonances, fluorine atoms in organofluorine compounds have relatively short longitudinal magnetic relaxation times, allowing relatively fast NMR experiments. An additional advantage of using ^{19}F NMR for the investigation of biological processes relates to the relatively simple NMR spectra that can be obtained with no interfering background signals due to the absence of endogenous fluorinated compounds in most biological systems. Dynamic range complications are also avoided due to the absence of the strong ^{1}H NMR water signal. Collectively, these remarkable properties enable the straightforward execution of most one-dimensional and multi-dimensional ^{19}F NMR experiments,[9] thus adding to their appeal for the study of biological systems.

^{19}F NMR hardware developments

The close vicinity of the ^{19}F and ^{1}H Larmor frequencies (only 6% difference between them) requires the use of elaborate radio-frequency (RF) circuitry designed to prevent RF resonant

Han Chen

Han Chen received her BS in 2000 from China University of Geosciences and her PhD in 2005 in organic chemistry from Wuhan University. During her PhD, she developed a series of fluorinated, non-metabolisable molecular probes for investigating in vivo the signaling role of 2-oxoglutarate acid, a key Krebs cycle intermediate. Currently, she is a post-doctoral researcher at Harvard Medical School, where she is working on drug-resistant herpesvirus mutants to search for new antivirals for the treatment of herpesvirus infections.

Stéphane Viel

Stéphane Viel graduated in 1999 from an engineering school (Hautes Etudes Industrielles) in Lille (France) and received his PhD in nuclear magnetic resonance (NMR) spectroscopy from Université Paul Cézanne (Marseille) in 2004, after performing most of his doctoral studies in Prof. Annalaura Segre's NMR laboratory in Rome (Italy). He is now Associate Professor at Aix-Marseille Université and works in the Institut de Chimie Radicalaire, where he is in charge of the NMR group. His research activities lie at the interface of analytical chemistry and molecular spectroscopy, and aim at designing methodologies in order to investigate chemically and biologically relevant systems.

Fabio Ziarelli

Dr Fabio Ziarelli completed his undergraduate studies in chemistry at Perugia University (Italy) and received his "Laurea" degree in 1997 with honors cum laude. He then did an intensive training internship at the CNRS-IRC in Lyon (1997–1999), and at the CNR-IMC in Rome (1999–2001), specializing in the field of solid-state nuclear magnetic resonance (NMR). In 2004, he obtained his PhD in solid-state NMR spectroscopy from Université de Provence (Marseille) and he joined the CNRS as a Research Engineer. In this context, he manages the solid-state NMR instrumentation of the Fédération Sciences Chimiques, the analytical facility of Aix-Marseille Université.

Ling Peng

Dr Ling Peng received her BS in chemistry from Nanjing University, China in 1986 and her PhD under the direction of Professor Albert Eschenmoser from the Swiss Federal Institute of Polytechnic in Zurich in 1993. She then spent three years as a post-doc in the group of Prof. Maurice Goeldner at Louis Pasteur University in Strasbourg in France. In 1997, she was recruited as a research scientist at the French National Scientific Research Center (CNRS) and is now a research director. Her current research is focused on the development of chemical tools for exploring biological events, drug discovery and drug delivery.

signals at one frequency from interfering with those at the other frequency. NMR instrumentation (probeheads and spectrometers) has for many years been mostly optimized for ^{1}H observation, which has globally hampered the development of ^{19}F NMR. Today, several manufacturers offer dedicated ^{19}F NMR probeheads, and modern spectrometers typically incorporate electronic modules used to enable high-band RF amplifiers to be shared between ^{1}H and ^{19}F channels. Specifically, this allows the NMR carrier frequency to be switched back and forth between the ^{19}F and ^{1}H resonance frequencies during the course of the NMR experiment, thereby allowing altogether highly sensitive double-resonance ^{19}F/^{1}H NMR experiments to be recorded, including when ^{1}H decoupling is applied. In parallel to the recent commercialization of cryogenically-cooled probeheads optimized for liquid-state ^{19}F NMR, a ^{19}F/^{1}H microcoil probehead was proposed for performing solid-state NMR experiments with small amounts of F-labeled biological molecules.[10] In addition, ^{19}F high resolution magic angle spinning (HRMAS) NMR has been shown to be of interest for the investigation of mass-limited samples in the liquid state.[11] Another recent development is the introduction of technical solutions to minimize sample heating within the probehead when performing ^{19}F NMR experiments involving multi-pulse acquisition schemes on solids.[12]

While rare in biological systems, fluorine is present in a large range of plastic materials in the form of fluoropolymers. These polymers are quite commonly used in RF circuits for their optimal electronic characteristics. However, the presence of such fluoropolymers in the proximity of the detection coil leads to a very large signal in the ^{19}F NMR spectrum (with a typical line-width of several hundreds of Hz). Eliminating this so-called background signal requires either careful selection of the materials used for the electronic circuitry of the NMR probehead, a solution that is not routinely implemented by manufacturers, or an appropriate signal filtration using specific pulse sequences. Several propositions have been made in the literature, none of which is completely satisfactory, especially when quantitative results are sought. Recently, one specific method has been proposed which could potentially be useful in the context of ^{19}F NMR, involving the electronic removal of the background signals in solid state NMR.[13]

Structural and functional study of F-labeled proteins

Studying the behavior of proteins is fundamental to understanding the biological processes in which they are involved. ^{19}F NMR has been demonstrated as a powerful tool to describe the conformations and functions of proteins, their interactions with binding partners, and their response to external stimuli or environmental changes. Compared with other widely used techniques, such as fluorescence and circular dichroism, ^{19}F NMR can provide more detailed information on the local structure and properties of proteins, thus adding a new dimension to our understanding.

Conformational and structural analysis

Most ^{19}F NMR studies on protein structure and function require F-labeled proteins as a primary prerequisite. While many methods can be used to fluorine label a protein of interest, the most widely used strategy is the biosynthetic incorporation of F-labeled amino acids by microbial protein expression. It should be noted that site-specific incorporation is extremely important since non-specific multiple-site labeling might affect the global protein stability and complicate the assignment of each signal in the NMR spectrum. In this regard, Schultz and co-workers pioneered the use of orthogonal tRNA/aminoacyl-tRNA synthetase pairs to incorporate ^{19}F-labeled amino acids site-specifically.[14] This technique was further improved by Mehl and co-workers.[15] Another approach to introducing fluorine probes at specific positions within a protein is to chemically modify existing amino acids, such as cysteine.[16] A number of chemical reagents can selectively react with protein cysteines even when the proteins are in a detergent-solubilized or column-bound state, without perturbing their function or leading to their misfolding.

^{19}F NMR has been exploited to obtain information about local conformational changes within specific regions of a protein during its folding process. Protein folding is a spontaneous process, the dysfunction of which can be related to various human diseases. Therefore, elucidation of the structural and conformational changes during protein folding is a hot area of research. In this context, ^{19}F NMR spectroscopic analysis dominates over the commonly utilized ^{1}H, ^{13}C, and ^{15}N NMR. One recent example is the ^{19}F NMR study of F-labeled α-synuclein during its fibrillation induced by sodium dodecyl sulfate (SDS), which is used to mimic the membrane environment.[17] α-Synuclein is a 140-amino-acid-residue protein, the fibrillation of which relates to Parkinson's disease and other neurodegenerative disorders. By incorporating 4-trifluoromethylphenylalanine (tfmF) into various positions of α-synuclein, detailed conformational changes at these residues following SDS addition were illuminated very clearly based on ^{19}F NMR analysis, thus revealing the stages of α-synuclein fibril formation. As shown in Fig. 1, in the absence of SDS, α-synuclein was disordered. Changes in the ^{19}F NMR chemical shift with increasing concentrations of SDS from 0 to 0.8 to 2.4 mM, indicated the induction of a partial helical conformation in the first several residues including F4tfmF. Above 2.4 mM SDS, a more folded α-synuclein with a higher helix-content form was revealed by the changes in the ^{19}F NMR chemical shift of V70tfmF. The C-terminal region remained disordered, as revealed by the spectral recording at position 133. The obtained data suggested that α-synuclein adopted, in an SDS-mimicked membrane environment, a metastable, aggregation-prone state, which was apt to intermolecular interactions that progress towards disease-associated fibrillation. This work illustrates the value of ^{19}F NMR in enhancing our understanding of the molecular mechanism underlying α-synuclein fibrillation.

Recently, Sakakibara *et al.* have shown that high-resolution heteronuclear three-dimensional NMR experiments can be used to examine isotopically enriched (^{2}H/^{13}C/^{15}N) labeled proteins "at work" at the atomic level in living cells (so-called in-cell NMR).[18] However, routine ^{15}N enrichment is insufficient for detecting most globular proteins in cells due to the high

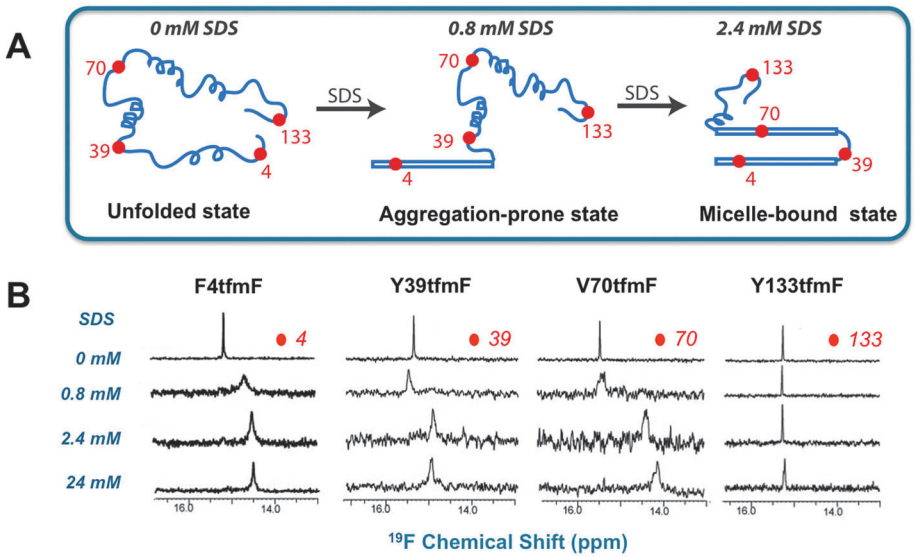

Fig. 1 ^{19}F NMR study of the fibrillation of α-synuclein induced by sodium dodecyl sulfate (SDS). (A) Conformational forms, at different concentrations of SDS, of α-synuclein protein with 4-trifluoromethylphenylalanine (tfmF)-labeled at positions 4, 39, 70 and 133. The red dots represent tfmF-labeled sites. (B) ^{19}F NMR spectra of α-synucleins with a tfmF residue at positions 4, 39, 70 and 133 respectively, with increasing concentrations of SDS (0, 0.8, 2.4 and 24 mM). Reproduced and modified with permission from *ChemBioChem*, 2010, **11**, 1993. Copyright (2010) Wiley.

viscosity and weak interactions in cytoplasm. ^{19}F NMR based on F-labeled proteins is a compelling solution to this problem owing to its high sensitivity, little background contamination and broad chemical shift range. The incorporation of 3-fluorotyrosine (3FY) or 4-trifluoromethylphenylalanine (tfmF) into proteins allows the study of not only globular proteins but also disordered proteins within cells using ^{19}F NMR.[19] Moreover, F-labeling enables ^{19}F NMR to provide site-specific structural and dynamic information on proteins based on chemical shifts and line-widths. For example, in the case of α-synuclein, ^{19}F NMR revealed that the line-width of the ^{19}F resonance in tfmF at position 39 was larger than that at position 133 in cells (Fig. 2), indicating that the side chain at position 133 is much more flexible.[19] Such dynamic information on disordered proteins in cells could not be obtained by NMR with other nuclei (for instance with two-dimensional ^{1}H–^{15}N correlation NMR experiments), hence demonstrating the unique advantage of ^{19}F NMR.

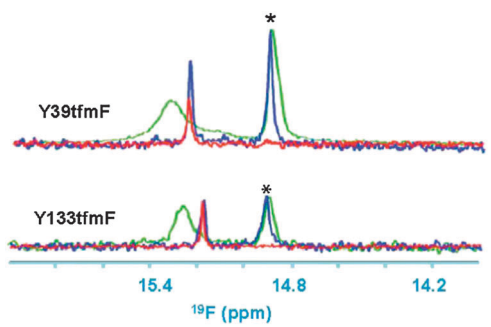

Fig. 2 ^{19}F NMR spectra of α-synuclein protein with 4-trifluoromethylphenylalanine (tfmF)-labels at positions 39 and 133. Spectra from cell slurries, clear lysates, purified tfmF-labeled protein and supernatant are shown in green, blue and red, respectively. The asterisks indicate the free tfmF resonances. Reprinted with permission from *J. Am. Chem. Soc.*, 2010, **132**, 321. Copyright (2010) American Chemical Society.

Furthermore, ^{19}F NMR can unveil intermediates in the protein-folding pathway that are otherwise difficult to capture and investigate due to their very short transient nature and extremely low concentration. In this context, the imposing power of ^{19}F NMR mainly relates to its time resolution (in the order of seconds), which allows the real-time monitoring of a wide range of sites in the same folding time frame. This enables the direct detection of intermediates and is crucial to understanding the folding mechanism of a whole molecule. An example of such an application was reported by Freiden and Li in which they studied the sequential intermediates during the folding of a fatty acid binding protein mutant G121V in real time with 4-fluorophenylalanine incorporated protein.[20]

^{19}F NMR investigations of protein folding and conformational changes often rely on the use of fluorinated aromatic amino acids since they are either commercially available or easily accessible by synthesis. However, there are also examples of fluorinated aliphatic amino acids being utilized in this area. One recent example was reported by Marx *et al.* who labeled a DNA polymerase (*KlenTaq* DNA polymerase) with trifluoromethionine (TFM).[21] In their study, the F-labeled polymerase, which displayed similar enzymatic activity and fidelity to the parent protein, was then used to investigate the different states of DNA polymerase on the way to nucleotide incorporation. In this case, ^{19}F NMR provided useful information to elucidate conformational changes and dynamics during enzyme catalysis.[21]

^{19}F NMR has been widely applied to elucidate structural and site-specific conformational changes in proteins since the effect of the fluorine substitution is usually minimal with regards to its native state. However, in some rare cases, F labeling may

disrupt the protein structure due to the high electronegativity of fluorine. Therefore, carefully checking the influence of F-substitution is crucial to ensure that all ^{19}F NMR observations are truly representative of conformational change processes occurring in the native proteins.

Dynamic and functional investigation

Many protein functions are governed by their dynamics. Thus "watching" proteins in action in real time is a dream shared by many scientists. ^{19}F NMR represents an attractive option for studying protein functions and interactions with their partners, including ions, ligands, peptides or nucleic acids. Developing a detailed understanding of how biological molecules bind and interact with one another is essential to reveal their function and mechanisms of action.

One example of the application of ^{19}F NMR in this area was to study the impact of Zn^{2+} on the stability and enzymatic activity of a 40 KDa protein murine adenosine deaminase (mADA), a key enzyme in purine metabolism, which plays a crucial role in normal immune competence.[22] Zn^{2+} is tightly bound to mADA and is required for the protein activity. Usually, investigating the role of a metal cofactor is very difficult since its absence may lead to protein instability and aggregation, thus precluding NMR or X-ray studies. However, the combined use of the high-resolution structure of the mADA protein and real-time ^{19}F NMR with 6-fluorotryptophan incorporated at positions 117, 161, 264 and 272, allowed the observation of Zn^{2+}-induced structural changes propagating throughout the whole protein which helped explain the low stability of the Zn^{2+}-free apo-enzyme. Moreover, real-time ^{19}F NMR further measured the relationship between the structural change and the decrease in enzymatic activity that occurred with Zn^{2+} gradual loss (Fig. 3).

Another successful application of ^{19}F NMR in the study of protein dynamics was recently reported by Pielak's group to examine whether volume exclusion dominates the protein crowding effect in cells.[23] The crowding effect refers to the fact that proteins typically perform their functions at high concentrations within cells. However, whether this crowding effect mainly arises from volume exclusion, which favors native proteins with compact structures, has been debated for many years. In their work, Pielak *et al.* introduced 3-fluorotyrosine (3-FY) into a variant of the L protein (a 7 kDa globular protein), which can fold reversibly upon addition of salt. If volume exclusion were to dominate the crowding effect, the variant protein would be expected to preferentially remain in its native state in cells as opposed to in dilute solution. Accordingly, these authors used ^{19}F NMR to study the variant protein both in phosphate buffer and in cells. Their results portrayed that the unfolded F-labeled variant gradually folded in buffer with increasing NaCl concentration (Fig. 4A). However, this protein remained in its unfolded state within cells throughout, even at high salt concentrations (Fig. 4B). These results indicate that the volume exclusion provided by the highly crowded intracellular environment alone is insufficient to overcome the unfavorable free energy of folding, and that the protein might be less stable in living cells than *in vitro*. Due to the poor resolution of ^{19}F NMR for 3-FY labeled protein in cells, Pielak *et al.* could not quantitatively assess such a stability change. Recently, Hamachi and co-workers utilized a chemical biology method to incorporate a ^{19}F-probe into an endogenous protein (please see the principle of this method[24] in the "drug screening and discovery" section and Fig. 9), and quantitatively compared protein dynamics in living cells and *in vitro* using ^{19}F NMR.[25] They also found that the intracellular proteins showed greater conformational fluctuations with respect to those *in vitro*, which was consistent with the finding observed by Pielak's group.

Ligand–protein binding dissociation constants can also be assessed using ^{19}F NMR. Prosser's group determined the value of the equilibrium dissociation constant K_d for the interaction of the (2-hydroxynicotinic-NH)-Arg-Ala-Leu-Pro-Pro-Leu-Pro-diaminopropionic acid-NH_2 peptide and the labeled SH3 domain of Fyn tyrosine kinase by monitoring the chemical shift of different F-labeled residues in relation to the binding peptide.[26] They found that the introduction of 3-F-tyrosine as a probe into the protein kinase increased the value of K_d due to the decreased binding interaction with the peptide.

Another hot topic that has been intensively studied by ^{19}F NMR is the interaction between membrane proteins and lipid bilayers. In living cells, membrane-active proteins act as gates, channels, pumps, transporters and enzymes in various biological events including signal transduction, nutrient assimilation and energy conversion, *etc.* ^{19}F NMR has evolved as an important complementary tool for use in combination with traditional computational approaches and experimental cross-linking methods to study the structures of membrane proteins. A successful application of ^{19}F NMR in the field of membrane proteins is the *in situ* study of an *E. coli* membrane protein diacylglycerol kinase (DAGK) labeled with 4-trifluoromethylphenylalanine (tfmF) using magic angle spinning (MAS) solid state ^{19}F NMR. Results from this study showed that the chemical shift of each residue at different locations in the protein was closely related to the interactions between the membrane protein and its surrounding lipid or lipid-mimicking environment.[27] Although ^{19}F NMR is still an emerging tool in this field,

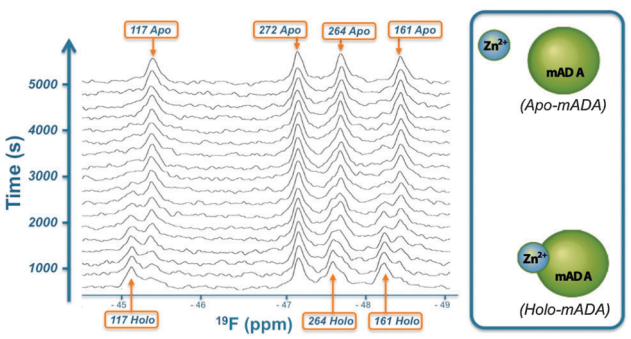

Fig. 3 ^{19}F NMR spectral recording of the dipicolinic acid (DPA)-induced real-time structural changes of the F-labeled murine adenosine deaminase (mADA) from holo- to Zn^{2+}-free apo-enzyme. The tryptophan residues of mADA at positions 117, 161, 264 and 272 are labeled with 6-fluorotryptophan. Reprinted and modified with permission from *J. Phys. Chem. B*, 2010, **114**, 16156. Copyright (2010) American Chemical Society.

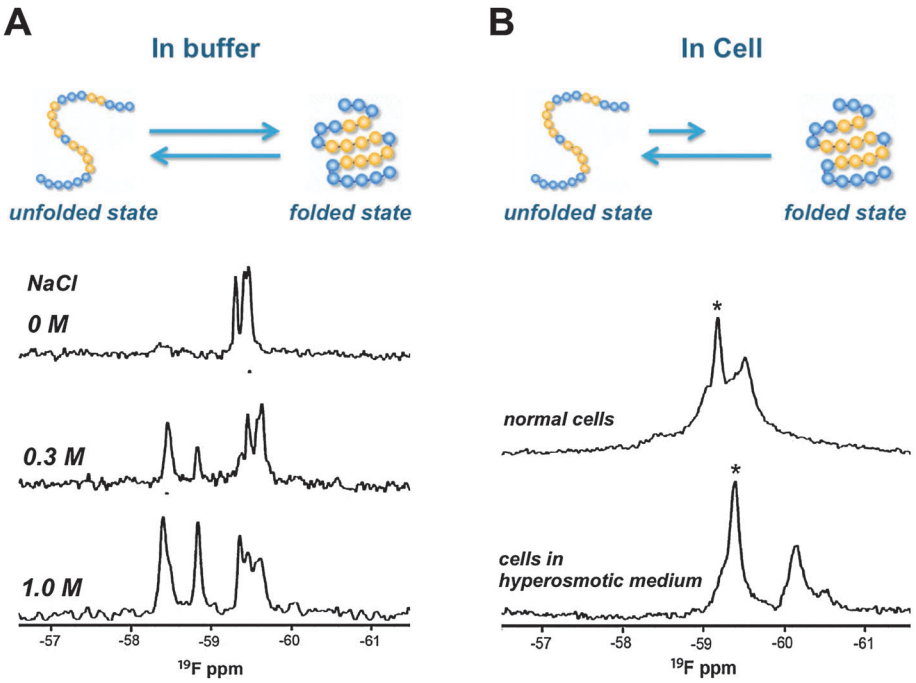

Fig. 4 Dynamics and folding of 3-fluorotyrosine (3-FY) labeled L protein in buffer (A) and in cells (B) studied using ^{19}F NMR. (A) F-labeled L protein gradually folded with increasing NaCl concentration in 20 mM phosphate buffer at pH 6.0. (B) F-labeled L protein could not fold even when the *Escherichia coli* cells expressing the protein were induced in hyperosmotic medium. The asterisks denote the resonance due to free 3-FY. Reprinted and modified with permission from *J. Am. Chem. Soc.*, 2011, **133**, 8082. Copyright (2011) American Chemical Society.

several articles have reported its successful use in studying the dynamics of membrane-active peptides and proteins.[28,29] We believe more and more applications will be explored with further development of the technique.

Structural and functional study of F-labeled nucleic acids

The use of fluorinated probes in combination with NMR spectroscopy also represents a useful tool for studying structures and functions of nucleic acids. ^{19}F NMR has been exploited to monitor conformational changes and folding of nucleic acids,[30] and to investigate their binding with either small molecular ligands or macromolecular partners.[31]

The incorporation of fluorine labels into nucleic acids is mainly achieved by two methods: one is chemical synthesis *via* easily accessible F-labeled nucleoside phosphoramidites on solid phase, and the other is enzymatic synthesis *via* T7 RNA polymerase using F-labeled nucleoside building units.[32] One particular series of F-labeled nucleosides modified either on the ribose sugar component (*e.g.* 2′-F-ribose[30,31]) or on the nucleobase unit (*e.g.* 5-F-pyrimidine[32]) is currently widely used in investigating nucleic acids.

^{19}F NMR has been successfully implemented in the study of DNA–carcinogen adduct formation. As a hallmark signature of mutation, DNA–carcinogen adduct formation is one of the leading inducers of mutagenesis and carcinogenesis. DNA adducts with aromatic amines, probably the most studied mutagens, have been detected in various human tissues and have hence been implicated in the etiology of human cancer. Although ^{1}H NMR and crystallography have proven valuable for studying these kinds of DNA adducts, neither technique is competent or sensitive enough to study the complex dynamic equilibrium in which DNA adducts adopt multiple conformations. ^{19}F NMR can overcome this problem and has been successfully applied by Cho's group to study the conformational heterogeneities of a 2-aminofluorene–DNA adduct.[33] The data provided by ^{19}F NMR demonstrate that the conformational distribution and thermodynamic properties of various F-labeled DNA are responsible for determining the final mutagenic outcome of this type of DNA adduct.[33]

Defining higher order DNA structures is essential for understanding their functions and interactions in biological systems and here again ^{19}F NMR demonstrates its advantages. Not only can ^{19}F NMR monitor the duplex formation for DNA, but it can also trace DNA triplex formation *via* Hoogsteen hydrogen interactions. For example, Nishimoto and colleagues prepared a series of F-labeled oligodeoxynucleotides with 5-fluoro-2′-deoxyuridine (5-FU) as the ^{19}F NMR reporter, and successfully monitored the structural changes of DNA from single strand to triplex at different temperatures by ^{19}F NMR (Fig. 5).[34]

In addition to its use in the study of DNA, ^{19}F NMR has also been widely used to investigate RNA secondary structure and RNA-mediated biological events. RNA offers a functional repertoire that often relies on its intrinsic conformational flexibility to adopt alternative secondary structures. This conformational interplay is a very complex process and the quantification of

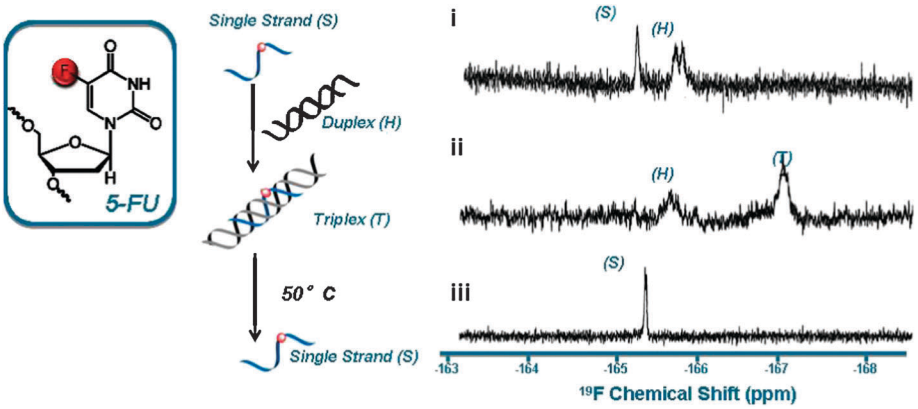

Fig. 5 ^{19}F NMR spectra of an oligodeoxynucleotide (ODN) possessing a 5-fluoro-2′-deoxyuridine unit (5-FU), denoted by a red dot. (i) Single strand (S) ODN at 25 °C; (ii) single strand ODN formed triplex (T) in the presence of DNA duplex at 25 °C; (iii) triplex (T) changed back to single strand (S) ODN at 50 °C. Higher order structure (H) of ODN was also detected at 25 °C. Reprinted and modified with permission from *Bioorg. Med. Chem.*, 2010, **18**, 6690. Copyright (2010) Elsevier.

individual conformations is difficult even for short RNA oligonucleotides. ^{19}F NMR analysis has already shed some light on the process and was developed by Micura *et al.* to discriminate RNA secondary structure populations.[30,31,35] A recent study carried out by this group presented an excellent example of how temperature-dependent shifts of ^{19}F resonances can be used to analyze the dynamic equilibrium of RNA secondary structures.[35]

Recently, Virta and Kiviniemi reported the first example of using ^{19}F NMR to survey viral RNA invasion,[36] a more complex process compared with the examples mentioned above. The authors introduced 5-[4,4,4-trifluoro-3,3-bis(trifluoromethyl)-but-1-ynyl]-2′-deoxyuridine into the HIV-1 TAR RNA model, and studied the ^{19}F resonance shift as the RNA structure changed from its initial hairpin structure A to the structural forms C, D or B during invasion or thermal denaturation (Fig. 6). They further reported that conjugation of oligoribonucleotides with aminoglycoside can promote RNA invasion, and the increasing dissociation constant K_d can be easily determined by ^{19}F NMR.[37] These studies demonstrate that ^{19}F NMR provides a straightforward and non-perturbing method to access detailed structural information about RNA folding.

Drug screening and discovery

The identification of compounds displaying potent biological activity is a key step in drug development. Many drug discovery processes rely heavily on high-throughput screening (HTS) based on homogeneous fluorescence spectroscopy or on the radioactive ligand displacement assay. However, the structural modification due to the bulky fluorescent tags and their tedious incorporation into parent molecules, as well as the susceptibility to background

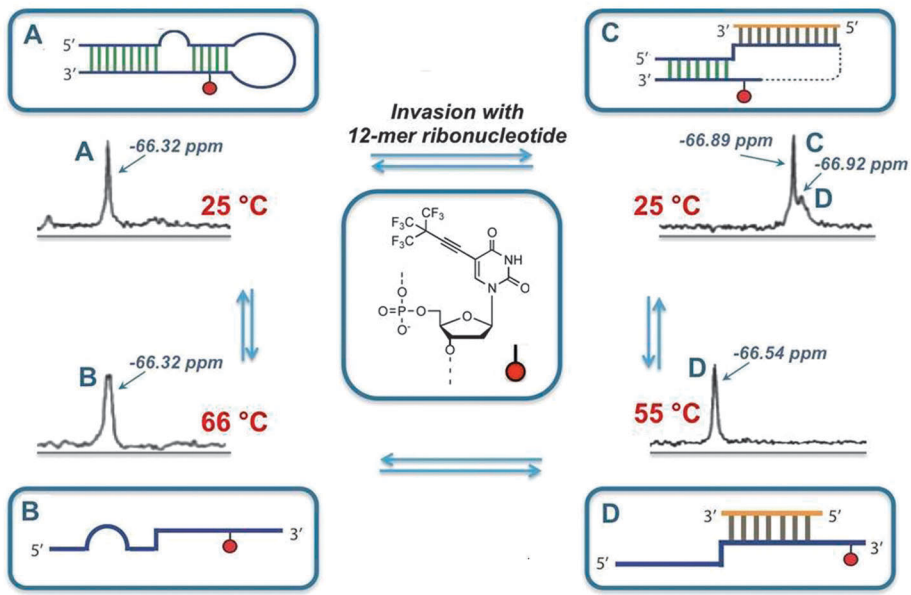

Fig. 6 ^{19}F NMR spectral recording of the F-labeled TAR model A and its invasion complexes C, D and B at different temperatures. Red balls represent F-labeled nucleotide. Reprinted and modified with permission from *J. Am. Chem. Soc.*, 2010, **132**, 8560. Copyright (2010) American Chemical Society.

effects together with radioactivity safety concerns, all constitute important limitations of these methods. Raising the quality and reliability of drug screening in the identification of new lead molecules is the primary challenge facing pharmaceutical development. Consequently, there is a growing need for efficient and robust methods that can identify compounds with the desired binding affinity in a relatively short timespan. Over the years, a number of advances in ^{19}F NMR-based screening have made it a useful tool for the identification of lead compounds, in combination with HTS. The widely used ^{19}F NMR-based screening, which was developed by Dalvit *et al.*, includes fluorine chemical shift anisotropy and exchange for screening (FAXS)[38] and fluorine atoms for biochemical screening (FABS).[39] Both methods have been successfully used for ligand-based screening, functional screening, fragment-based screening and dynamic library screening.[6]

In the FAXS method, an F-labeled ''spy'' molecule (with medium to weak binding affinity to the target) and a control molecule (with no binding affinity to the target) are implemented to monitor the screening process. After binding to the protein, the line-width of the ^{19}F NMR signal originating from the ''spy'' molecule drastically increases because of slow tumbling and/or fast on–off exchange, hence resulting in a concomitant large loss in signal intensity. After adding the test compounds, a hit is indicated by the reappearance of the sharper (and more intense) signal of the ''spy'' molecule at the same distinctive resonance frequency as that observed in the absence of protein. The relative change in fluorine signal intensity demonstrates that the new ligand in the library has a higher binding affinity compared to that of the spy molecule (Fig. 7). This method was successfully applied in screening novel fragment compounds targeting the phosphotyrosine binding site of the Src homology 2 (SH_2) domain of the protein tyrosine kinase v-Src, resulting in the identification of a novel phosphotyrosine mimetic fragment.[40] Further thermodynamic studies indicated that the hit had a highly favorable binding enthalpy, which may

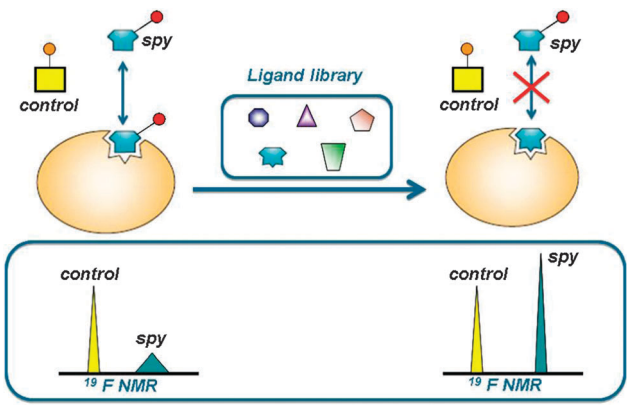

Fig. 7 Schematic presentation of the principle of fluorine chemical shift anisotropy and exchange for screening (FAXS). The broad signal of the ''spy'' molecule becomes sharp due to its displacement from the receptor by a competitive ligand. The sharp signal of the control molecule represents an internal reference. The red and orange dots represent the F-labeled sites in the ''spy'' and control molecules, respectively.

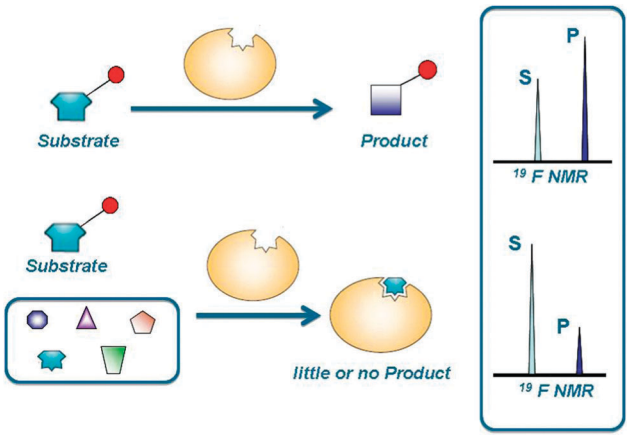

Fig. 8 Schematic presentation of the principle of fluorine atoms for biochemical screening (FABS). The red dots represent the F-labeled sites in the substrate and in the product. Letters ''S'' and ''P'' refer to substrate and product, respectively.

contribute to its enhanced selectivity. The main advantages of FAXS are: (i) only the spy molecule needs to be fluorinated (the screened ligands need not); (ii) a very low protein concentration is required (which is especially useful for poorly soluble proteins or proteins that are difficult to purify); and (iii) even ligands with weak binding activity (which are typically ignored in routine HTS) can be detected. Although FAXS is a very powerful method, it can only be used to study ligands that bind to previously known binding sites targeted by the spy molecule.

Alternatively, the FABS method requires the substrate to be labeled with a fluorinated moiety (usually a $-CF_3$ moiety), and uses ^{19}F NMR spectroscopy to detect the initial and enzymatically-modified substrates (Fig. 8). This method can be used not only to screen inhibitors of a wide range of enzymes, but also to simultaneously screen ligands of multiple proteins. This advantage has been harnessed to screen selective inhibitors from plant extracts of traditional Chinese medicine, of prolyl oligopeptidase and dipeptidyl peptidase.[41] In addition, FABS has been used to determine the function of novel proteins and explore their potential as drug targets,[39] an important step in the pharmaceutical development of new drugs to treat various diseases.

Searching for and identifying drugs that specifically bind to proteins of interest in living systems is one of the important stages in drug development. Recent progress in biomolecular imaging constitutes a considerable stride towards this goal. A new chemical labeling scheme, termed ligand-directed sulfonyl (LDT) chemistry, was developed by Hamachi's group for *in vivo* labeling of ''endogenous'' proteins.[42,43] In this approach, an affinity ligand and a probe are connected by an electrophilic arylsulfonyl ester group, which is attacked by a nucleophilic side chain of amino acids near the active site of the protein. The protein is thus chemically labeled by the probe with the concomitant release of the ligand molecule (Fig. 9). The LDT reagents can be designed in different ways according to the protein target and detection methods. Among them, the biosensor containing a 3,5-bis(trifluoromethyl)benzene derivative and arylsulfonamide for human carbonic anhydrase (CA) is quite attractive. The 3,5-bis(trifluoromethyl)benzene probe bears six

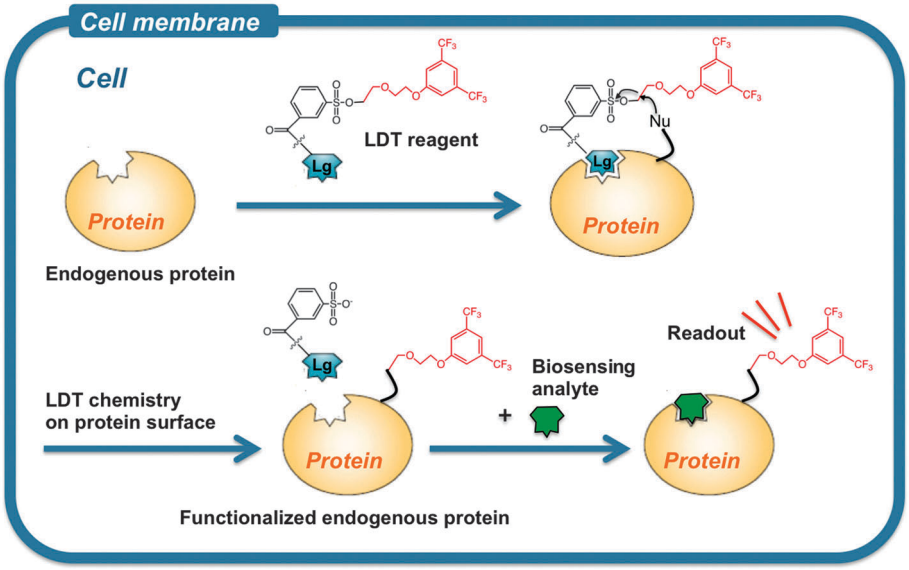

Fig. 9 Schematic illustration of ligand-directed sulfonyl (LDT) chemistry for labeling endogenous proteins in living cells. ''Lg'' denotes ligand. Reproduced and modified with permission from *Nat. Chem. Biol.*, 2009, **5**, 341. Copyright (2009) Macmillan Publisher Ltd.

magnetically equivalent fluorine nuclei which allows the binding process of this probe towards CA to be conveniently monitored by ^{19}F NMR. The high selectivity of this LDT reagent allowed the detection of the targeted protein CA in human red blood cells despite the existence of a large amount of hemoglobin. In addition, using the ^{19}F NMR signal as a readout, this intracellular F-labeled CA can act as a biosensor to screen CA ligands *in vivo*. In the same way, it is possible to detect the ligands for different proteins in the same cells by various combinations of probes and ligands in LDT reagents with the possibility of using other readouts than ^{19}F NMR. Although this cell-based screening system cannot be used as yet in HTS due to the affinity and cell permeability of ligand molecules, the versatility of this method should prove useful in many applications, including drug discovery and medical diagnosis.

Study of organofluorine metabolism

The use of organofluorine compounds in the agrochemical, pharmaceutical and plastic industries has increased steadily over the past 30 years.[44] Today, more than 40% of agrochemicals currently on the market are fluorinated. Such widespread use of organofluorine compounds has resulted in their accumulation in the environment. The biodegradation of fluorinated xenobiotics therefore has considerable importance in the bioremediation of environmental contaminants. In this context, ^{19}F NMR has proven to be an extremely useful and non-invasive tool for monitoring the biotransformation of these fluorinated compounds in *ex vivo* and *in vivo* studies. Certain species of bacteria or fungi can utilize fluoroaromatic compounds as a carbon and energy source and ^{19}F NMR can monitor the biodegradation of these compounds effectively. For the degradation of non-fluorinated compounds, fluorinated analogs can be used as model compounds due to the small radius and high electronegativity of fluorine atoms. Nevertheless, fluorinated compounds are often very stable and cannot be easily metabolized by enzymes, hence the need for ^{19}F NMR to detect the accumulation of non-metabolizable intermediates in the culture medium or in the cells. The use of ^{19}F NMR with a fluorinated non-metabolizable analog, 2,2-difluoropentanedioc acid (Fig. 10A) successfully demonstrated the signaling role of a Krebs cycle intermediate, 2-oxoglutaric acid in cyanobacterium *Anabaena*. This work provided the first *in vivo* experimental evidence that Krebs cycle intermediates also play signaling roles in living systems, hence adding to their traditional roles of simply providing precursors for biosynthesis.[45]

^{19}F NMR has also been used to study the metabolism of fluorinated xenobiotics in animals, for example, in the biotransformation of 2,3,3,3-tetrafluoropropene (HFO-1234yf, Fig. 10B).[46] HFO-1234yf is a non-ozone-depleting fluorocarbon replacement and has been developed as a refrigerant. Evaluation of HFO-1234yf in rats and mice revealed only a very low toxicity potential. Nevertheless, lethality was observed in rabbits exposed to high concentrations. ^{19}F NMR revealed several metabolic intermediates with qualitative and quantitative differences in the metabolic transformation pathways of HFO-1234yf in rabbits when compared with rats, which may account for the differences in toxicity shown between the two species. It should be noted that ^{19}F NMR alone cannot give an overview of the whole metabolic profile due to its low sensitivity in the micromolar range and possible defluorination during metabolism. Its combination with additional techniques such as HPLC-^{1}H NMR, UPLC-MS and radiometry is necessary to firmly identify the products of metabolism.

Aside from the increasing number of fluorine compounds in the agrochemical field, there has also been a surge of fluorine-containing drugs in the pharmaceutical industry (from 2% in 1970 to over 20% today). ^{19}F NMR represents a very useful tool

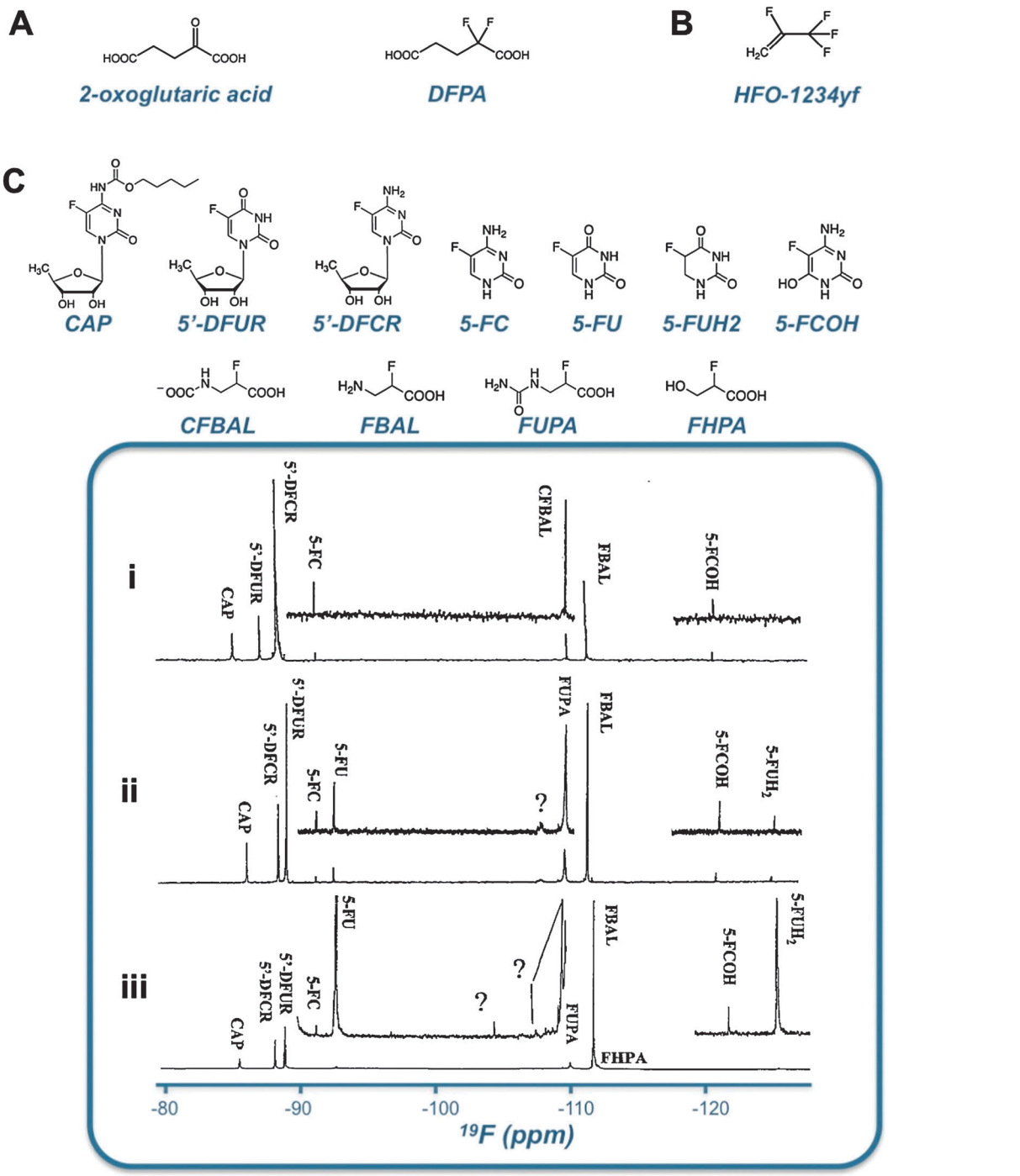

Fig. 10 (A) 2-Oxoglutaric acid, a key intermediate in the Krebs cycle, and its fluorinated non-metabolizable analog, 2,2-difluoropentanedioc acid; (B) 2,3,3,3-tetrafluoropropene (HFO-1234yf), a non-ozone-depleting fluorocarbon refrigerant; (C) typical ^{1}H-decoupled ^{19}F NMR spectra of urine samples from rats (i), mice (ii) and patients (iii) treated with capecitabine (CAP). Capecitabine and its metabolites are listed with the spectra. Reprinted with permission from *Drug Metab. Dispos.*, 2002, **30**, 1221. Copyright (2002) American Society for Pharmacology and Experimental Therapeutics.

for determining the metabolic fate of these fluorine-containing drugs in humans. Capecitabine (CAP, N_4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine, Fig. 10C) is an orally available prodrug of 5′-deoxy-5-fluorouridine (5′-DFUR) with limited side effects, which has recently been introduced for the treatment of breast and colorectal cancers. Although most of the pharmacokinetic and metabolic studies on CAP were carried out using liquid chromatography as the main analytical tool, ^{19}F NMR allowed the simultaneous detection and quantification of fluorine-containing compounds, even unknown substances.[47] Indeed, in urine samples collected from rats, mice and humans treated with CAP, different amounts of metabolites were identified by ^{19}F NMR. In addition to several metabolites such as 5′-deoxy-5-fluorouridine (5-DFUR), 5-fluorouracil (5-FU), α-fluoro-β-ureidopropionic acid (FUPA),

α-fluoro-β-alanine (FBAL) and 5,6-dihydro-5-fluorouracil (5-FUH2), which have been identified previously by liquid chromatography, some new catabolites such as 5-fluorocytosine (5-FC), 5-fluoro-6-hydroxycytosine (5-FCOH), fluoride ion, 2-fluoro-3-hydroxypropionic acid (FHPA) and fluoroacetate (FAC) were observed and identified accordingly (Fig. 10C).

The ever growing number of applications for organofluorine compounds in the agrochemical, pharmaceutical and material sciences, has stimulated the search for versatile and sustainable methods to generate new fluorinated chemical entities. The biosynthesis of organofluorine compounds starting with naturally abundant inorganic fluoride is of great expectation. In 2002, O'Hagan *et al.* first reported the identification of a fluorinase enzyme[48] in the bacterium *Streptomyces cattleya*, which can utilize fluoride to synthesize a series of organofluorine compounds. This discovery offered a new avenue towards the eventual preparation of novel organofluorine compounds *via* biotechnology.[49] In this context, ^{19}F NMR has proven to be an extremely useful tool for monitoring the formation and biotransformation of fluorinated compounds in the bioengineered microorganisms. Impressively, measuring the activity of the fluorinase enzyme and its engineered mutants in the strain was only made possible using continuous ^{19}F NMR analysis, indicating the essential role of ^{19}F NMR in investigating fluorometabolite biosynthetic pathways. This discovered fluorinase, together with its engineered mutant forms, clearly holds huge potential for the synthesis of various fluorinated building units and/or innovative fluorine compounds and offers truly exciting perspectives.

Other applications

In addition to its ability to explore the structure, function and metabolic pathways of biologically relevant molecules, ^{19}F NMR also has wide applications in monitoring gene reporters and in molecular/cellular imaging, all with considerable clinical relevance.

Gene therapy has emerged as a promising strategy for the treatment of diseases, provided safe and efficient delivery vectors are available. With the view to establishing a non-invasive method to detect transgene activity *in situ* during gene delivery, Mason and co-workers developed a series of fluorinated glycosylated derivatives as *lac*Z gene reporters. For many years, the *lac*Z gene encoding β-galactosidase (β-gal) has been the primary choice of reporter gene to ensure effective transfection. With their fluorinated glycosylated derivatives, Mason and colleagues were able to detect β-gal activity on the basis of ^{19}F NMR chemical shift associated with the release of fluorophenyl aglycons from galactopyranoside conjugates.[3] This assay system has been applied to the detection of *lac*Z gene expression in transfected prostate cancer PC-3 cells.[50] Although the concept of ^{19}F NMR reporter molecules for detecting gene activity is still in its infancy, we believe it holds great potential and promise for the development of gene therapy for the treatment of various diseases.

By allowing the visualization of labeled cells *in vivo* in real time, magnetic resonance imaging (MRI) has provided new insights into the dynamics of cell tracking and migration. Usually, this technique is used to track immune cells and other cell types using metal-ion based ^{1}H contrast agents. However, ^{1}H contrast agents present an inherent challenge relating to common difficulties in interpreting subtle changes, and they also suffer from intense background signals originating from biological systems. The lack of endogenous fluorine in living organisms gives rise to the superior contrast-to-noise features displayed by ^{19}F MRI and permits definitive localization of the uniquely labeled cells. This has opened up new research avenues in molecular and cellular imaging, and increased the number of applications in biomedicine, as summarized in a recent review.[51]

Conclusions

We have reviewed how ^{19}F NMR has played an important role in molecular biology and biochemistry investigations including the structure and function of proteins and nucleic acids, enzymatic mechanisms, metabolic pathways and biomolecular interactions. In addition, ^{19}F NMR-based drug screening promises to be of tremendous benefit for the triage of hits resulting from HTS, and as such should help medicinal chemists in the identification of qualified leads and thereby facilitate the development of new pharmaceutical and agrochemical products. Although ^{19}F NMR still suffers from a lack of sensitivity, thus imposing the need for relatively highly-concentrated biological molecules when studying biological systems, the emergence of new techniques and advances in hardware are expected to give rise to additional exciting applications.[52]

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