

# Two-field nuclear magnetic resonance: spectroscopy and relaxation

Samuel Cousin

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### Thèse présentée par

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### **Two-Field Nuclear Magnetic Resonance:**

### Spectroscopy and Relaxation

Directeur de thèse : Fabien Ferrage

préparée à l'École Normale Supérieure et l'Université Pierre et Marie Curie

UMR 7203, Laboratoire des Biomolécules

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### Résumé court

Cette thèse traite de la RMN, en phase liquide à champs multiples, pour la détermination de la structure et la dynamique de petites molécules et de protéines.

Dans une première partie, la dynamique des chaînes latérales des protéines aux échelles de temps ps-ns a été étudiée grâce à l'observation du <sup>13</sup>C des groupes méthyles marqués sélectivement  $\{^{13}C^{1}H^{2}H_{2}\}$  sur les isoleucines- $\delta_{1}$ . Un jeu de vitesses de relaxation exceptionnel a été obtenu en combinant les données acquises à haut champ magnétique (R<sub>1</sub>, R<sub>2</sub>, NOE) et les vitesses de relaxation longitudinale R<sub>1</sub> obtenues par relaxométrie haute résolution à 18 champs magnétiques, de 0,2 T à 18,8 T. Ces dernières données ont été corrigées à l'aide du programme ICARUS, adapté pour les groupes méthyle à cette occasion. La nécessité de simuler les expériences de relaxation nous a conduit à calculer la matrice de relaxation par un programme que nous avons nommé REDKITE. En outre, un modèle de fonction de densité spectrale a été proposé pour prendre en compte les différentes sources de rotation incluant la diffusion globale, la rotation des groupes méthyle, et les mouvements de l'axe CC, décrits avec un ou deux temps de corrélation effectifs. Il nous a alors été possible d'accéder à une description de la dynamique des groupes méthyle sur trois ordres de grandeur d'échelles de temps (de quelques ps à quelques ns).

Dans une deuxième partie, la spectroscopie RMN à deux champs magnétiques a été développée en collaboration avec les équipes d'ingénieur de Bruker. Ce système permet le contrôle des spins des noyaux de <sup>1</sup>H, <sup>13</sup>C et <sup>15</sup>N dans deux centres magnétiques (à 0,33 T et 14,1 T) avec une homogénéité suffisante et le transfert entre ces deux centres. Combiné à l'utilisation des cohérences à zéro-quantum, le spectromètre RMN à deux champs nous a permis d'effectuer des spectres de corrélation homoet hétéronucléaires à haute résolution avec les expériences 2F-INAZEQUATE et 2F-HZQC. Cette séquence hétéronucléaire a été utilisée pour réduire considérablement la contribution de l'échange chimique à la relaxation transverse, ce qui a permis d'observer des signaux de noyaux en échange qui étaient à la coalescence et invisibles à haut champ.

Le système à deux champs a également été utilisé pour effectuer un transfert de polarisation grâce à une expérience TOCSY où la période de transfert de polarisation est effectuée à 0.33 T. La corrélation entre des noyaux de carbone-13 dont les signaux sont séparés par plus de 150 ppm (appartenant à des groupes carbonyle et méthyle) a été observée tout en conservant la résolution offerte par les hauts champs magnétiques.

## Acknowledgment

I would like to thank all the members of my thesis committee who accepted to review the work performed during the last three years. I am grateful that experts in various areas of NMR agreed to evaluate the work presented in this manuscript.

### Recette pour une thèse épicée, cuite à point et parfumée :

### 1) Préparation de la pâte à tarte :

La pâte doit être réalisée avec minutie pour éviter la déformation, voire l'explosion de la tarte.

- Pétrissez la pâte longuement en ajoutant successivement les différentes farines de céréales : Jerem, Boris, Alex, Rémi, Nina, Julien, Pauline, Solène, Baptiste, Julie, Erwan, Sévan et Paul.
- Ajoutez à cela deux ou trois doses de cours abscons et quelques centilitres de débats pour ne perdre ni le goût, ni la souplesse de la pâte.
- Très rapidement, passez dans un plat de type *Teddy's bar* pour que votre pâte ne perde pas une once de cohésion.
- Enfournez-la dans un stage de  $M_2$  pendant 6 mois sous la surveillance de **Dimitrios**, qui vous aidera à choisir la garniture.

### 2) Appareil :

La garniture est un choix fondamental : sélectionnez les ingrédients avec minutie, puis lancez-vous dans la tâche sans vous décourager.

- Dans un plat rond, versez Shahid, Ludo et Jean-Jacques, pour un label bio naturel.
- Pour plus de complexité dans le goût, ajoutez une grande dose de Fabien, et une once de Daniel et Marco.
- Assaisonnez ensuite avec un peu de code : je vous conseille la marque **Cyril** qui est de très bonne qualité.
- Pour obtenir un goût prononcé de RMN, ajoutez 100mL de **Philippe** et au minimum 2L de **Pavel**. Oui, effectivement c'est beaucoup mais vous ne serez jamais déçu·e !
- Cette préparation peut être longue à réaliser. Faites de nombreuses pauses avec **Baptiste** et **Alexandra**. S'ils sont occupés, utilisez 12 g d'une **famille** proche et soudée, bien sûr toujours équipée de portables en cas de coup dur.
- N'hésitez pas également à être assisté : j'ai pour ma part été aidé dans une grosse première partie de cette tambouille par **Veronica**.

### 3) Garniture :

- Une fois l'appareil réalisé, choisissez les fruits. Je recommande fortement d'ajouter une barquette de **Thorsten** et de **Jean-Max**, fruits robustes et goûteux qui apporteront une subtilité inégalée à votre tarte. Étant de toutes saisons, ils pourront être ajoutés à n'importe quel moment, ce qui leur octroie une valeur inestimable pour la confection d'une tarte de qualité.
- Attention : si la préparation prend une tournure que vous n'attendiez pas et que vous n'arrivez pas à faire les tâches supplémentaires correctement, utilisez un robot de cuisine. Le robot Guillaume effectue les tâches à merveille et dispose d'une autonomie de batterie incroyable. Je l'utilise personnellement depuis 10 ans et il n'a toujours pas montré de faiblesse !

### 4) Décoration :

- Toute bonne tarte se doit d'être impeccable dans sa présentation. Ajoutez-y des colorants chimiques pour plus de folie : **E.Ernestophone**, **E.CRF** et **E.DumbAndBrass** donnent des couleurs absurdes et opportunes.
- Vous pouvez également ajouter des petits éléments décoratifs se trouvant proches de votre cuisine : **collègues** divers·e·s et varié·e·s pour donner un peu de tonus à votre préparation.

### 5) Cuisson :

Les cuissons intermédiaires sont nécessaires tout au long de la préparation, mais pour la cuisson finale, faites cuire la tarte à l'un des thermostats suivants : *Estas Tonne, Olafur Arnalds, Nils Frahm, GoGo Penguin, Mono, Silent Whale Becomes a Dream, Matthew Halsall*, ou encore *Esbjörn Svensson Trio.* 

### 6) Démoulage et dégustation

Probablement l'étape la plus complexe de cette recette. Réunir pour cela un grand nombre d'ami·e·s et collègues

- Tandis que la tarte était encore chaude, j'ai, pour ma part, demandé à **Hugues**, **Julie**, **Julia**, **Nicolas**, **Pavel** et plus encore à **Thibaut**, de m'aider pour démouler cette tarte, afin d'éviter qu'elle ne perde sa belle allure.
- Juste avant de servir, faites flamber avec un verre de Fabien, chauffé au préalable.
- La tarte est enfin terminée. Servez-la avec un petit verre de Whisky (de la Scotch Malt Whisky Society). Partagez-la alors avec les plus grands cuisiniers européens : **Pr. Redfield**, **Dr. Kupce**, **Dr. Bonhomme**, **Dr Dumez**, **Dr Schanda**, **Dr. van Heijenoort** et **Dr. Ferrage**.

### Le mot du chef

Petites mains, marmitons, sauciers, un immense merci à vous. Nous savons que la thèse, c'est n'est pas que de la tarte... Merci à toi **Julie**, pour ta fraîcheur et ta compréhension, pour ton enthousiasme et ta franchise.

Rien ne peut être "éclairé" dans la luminance totale. Il faut beaucoup de silence pour entendre une note. Il faut beaucoup de nuit pour qu'un éclair puisse jaillir, pour qu'une couleur neuve soit perçue, soit reçue.

Alain Damasio, Aucun souvenir assez solide

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# Avant-propos

Utilisant la liberté qui m'est accordée ici, il me semble intéressant de commencer ce document en évoquant mon sujet de recherche de manière peu habituelle et probablement un peu vindicative. Au cours de mes enseignements et de mes rencontres externes au laboratoire, on m'a demandé à de nombreuses reprises d'expliciter le sujet de ma thèse. Un choix se présentait alors à moi : soit décrire mon sujet comme une étude qui mènera à comprendre puis à guérir la maladie xeroderma pigmentosum (ou maladie des enfants de la lune); soit dire que je code des programmes pour comprendre la dynamique de groupements d'atomes, tout en participant au développement d'une machine qui permettra de confirmer mes calculs.

Naturellement, un bon nombre d'audit-eurs-trices m'accorderait plus d'intérêt si j'expliquais mes recherches de la première façon. Si l'un-e d'eux doit choisir de financer un projet plutôt qu'un autre, il-elle s'engagera sans hésitation vers ce qui a trait aux enfants de la lune. Après trois années de réflexion sur la façon de présenter mon sujet, je pense qu'il ne faut pas essayer de justifier nos choix de recherche par leurs applications. Leur en donner une, c'est affirmer que nous cherchons autre chose que la connaissance. Essayer de légitimer nos recherches par les applications, c'est progressivement verrouiller la recherche fondamentale.

Au même titre que l'art ou la culture, la recherche fondamentale est une nécessité pour notre société. Cependant, la complexité des molécules qui composent le vivant est, de mon point de vue, l'une des choses les plus fascinantes en science à l'heure actuelle. C'est donc avec grand plaisir que j'ai effectué mes recherches en utilisant comme objet d'étude les biomolécules. Me présentant comme biophysicien moléculaire, j'ai fait le choix dans ce manuscrit de ne décrire que brièvement les objets biologiques sur lesquels j'ai travaillé. Je laisserai donc à d'autres chercheur·e·s passionné·e·s la possibilité d'en tirer des applications techniques.

# Résumé

### Description générale des champs d'expertise explorés

**Biophysique moléculaire.** Après la découverte du maintien du code génétique dans la structure en double hélice de l'ADN, les scientifiques ont recherché la façon dont l'organisme traduit cette information. Une partie essentielle de l'information génétique code pour l'expression de protéines qui remplissent un grand nombre des fonctions essentielles du vivant : entre autres, messagers, marqueurs, régulateurs, et catalyseurs. L'étude de ces fers de lance de la machinerie moléculaire du vivant repose sur les compétences de chercheur·e·s de toutes disciplines. La compréhension des biomolécules à l'échelle atomique s'est, dans la majorité des cas, cantonnée à un espace à trois dimensions. De nombreuses techniques ont été développées, utilisées et combinées pour étudier la structure des protéines, comme la diffraction aux rayons X,<sup>1</sup> la résonance magnétique nucléaire (RMN)<sup>2</sup> et la cryo-microscopie électronique à transmission.<sup>3</sup> La description de la structure des protéines a été la clé pour comprendre les fondements moléculaires de la fonction des protéines. Cette relation forte entre structure et fonction est au fondement de la biologie structurale.

Cependant, le paradigme de la relation structure-fonction est en pleine évolution. Elle tend ainsi à inclure une quatrième dimension à la description des protéines : le temps. On évoque dorénavant la relation structure-dynamique-fonction. L'étude de l'évolution dans le temps de la structure des protéines est particulièrement utile pour l'étude des chaines latérales des protéines, en particulier dans le contexte de la reconnaissance moléculaire.<sup>4-6</sup> De par sa résolution atomique et sa grande sensibilité aux processus dynamiques, la RMN est devenue une technique essentielle pour l'étude des fondements moléculaires de la fonction des protéines, de leur structure, de leur dynamique, de leurs interactions, de leur modifications post-traductionnelles, etc.

**Détails sur la RMN.** La spectroscopie de résonance magnétique nucléaire est une technique aux applications multiples.<sup>7</sup> Elle est utilisée pour explorer les trois états usuels de la matière (liquide, solide et gazeux) avec une résolution atomique. La RMN permet l'étude d'un spectre extrêmement large de domaines d'application, allant de l'étude des piles à combustibles<sup>8</sup> à la progression d'une cellule tumorale chez l'humain.<sup>9</sup> Elle peut être employée pour ses qualités analytiques,<sup>10</sup> ou pour sa capacité d'imagerie (IRM<sup>11</sup>). En biophysique, elle est utilisée pour la caractérisation de la structure des macromolécules biologiques,<sup>12</sup> et la détermination de leur dynamique.<sup>13</sup>

Dans toutes ses formes, la RMN consiste à introduire les spins nucléaires au sein d'un champ magnétique intense. Plus celui-ci est intense, plus le signal obtenu sera intense et résolu. Depuis son introduction en 1946 par Felix Bloch et Edward Mills Purcell,<sup>14, 15</sup> les champs magnétiques de nos spectromètres n'ont cessé de croître (Figure 1).<sup>16</sup> Un nouveau spectromètre à 1,2 GHz (28,2 T) est en construction, bravant un nombre incroyable de barrières technologiques.



FIGURE 1 – Évolution du champ magnétique des spectromètres de RMN au cours des années

**Relaxation.** Le retour à l'état d'équilibre des spins nucléaires, appelé relaxation, est induit par les fluctuations du champ magnétique local exercées sur chaque noyau. Ces fluctuations sont locales et sont liées aux mouvements moléculaires à l'échelle atomique. Elles sont souvent causées par les principales interactions qui impliquent les spins nucléaires : l'interaction dipôle-dipôle, le déplacement chimique et le cas échéant, le couplage quadrupolaire. Dans le cas des petites molécules en solution, la rotation globale extrêmement rapide (quelques picosecondes pour une molécule d'eau, quelques nanosecondes pour une petite protéine) tend à orienter statistiquement ces interactions de façon isotrope. La résultante de ces interactions est donc nulle et le signal RMN est souvent très fin.

Dans le cas des macromolécules, le temps caractéristique de la diffusion rotationnelle est plutôt de l'ordre de la nanoseconde. L'effet des fluctuations des interactions évoquées précédemment sur la relaxation est différent. Afin de quantifier l'effet des fluctuations de ces interactions, deux fonctions sont nécessaires.

La fonction de corrélation (C(t)) décrit la perte de la corrélation d'une interaction (souvent avec elle-même) au gré des mouvements de réorientation des axes pertinents pour cette interaction, par exemple, le vecteur qui relie deux noyaux couplés pour l'interaction dipolaire. Ainsi si le mouvement du vecteur est décrit par des mouvements rapides ou lents de plus ou moins grande amplitude, la fonction de corrélation sera différente (Figure 2.(a,b)).

La deuxième fonction quantifiant les interactions et celle qui peut être déterminée par des mesures de RMN, est appelée fonction de densité spectrale ( $\mathcal{J}(\omega)$ ).  $\mathcal{J}(\omega)$  et  $\mathcal{C}(t)$  sont réciproques par transformée de Fourier, et deux exemples différents sont indiqués Figure 2.(c,d).

Étude de la relaxation des spins nucléaire. Afin d'évaluer  $\mathcal{J}(\omega)$ , il est possible de mesurer indirectement sa valeur en plusieurs points à l'aide des expériences de mesure de vitesses de relaxation



FIGURE 2 – Influence de l'amplitude et des échelles de temps des mouvements internes sur la fonction de corrélation C(t) (a et b) et sur celle de densité spectrale  $J(\omega)$  (c et d) dans les liquides. (a et b) Influence de l'amplitude des mouvements internes sur la fonction de corrélation dans un liquide isotrope pour (a) les mouvements rapides et (b) les mouvements nanosecondes avec un temps de corrélation identique à celui du temps caractéristique global (ici  $\tau_c = 5 \text{ ns}$ ). (c et d) Influence de l'amplitude des mouvements internes sur la fonction de densité spectrale dans le cas d'un liquide isotrope, dans le cas (c) de mouvements rapides et (d) pour des mouvements nanosecondes avec un temps de corrélation similaire à celui du temps de rotation globale.

(relaxation transverse, relaxation longitudinale ou relaxation croisée). Ces vitesses dépendent de la fonction de densité spectrale à certaines fréquences, qui sont des fréquences propres du système de spin au champ magnétique de l'étude. La combinaison de ces trois vitesses permet de reconstituer la fonction de densité spectrale en trois points. Il est souhaitable de vouloir caractériser cette fonction à de nombreuses fréquences, en particulier si la nature des mouvements est assez complexe. Afin d'acquérir suffisamment de points expérimentaux pour avoir un échantillonnage statistique, il faut évaluer la fonction de densité spectrale à un maximum de fréquences et donc travailler à un grand nombre de champs magnétiques.

La relaxométrie à haute résolution permet de profiter de la sensibilité et de la résolution d'un spectromètre RMN conventionnel, mais d'effectuer la relaxation à un champ magnétique différent de celui employé pour la polarisation et la détection.<sup>17</sup> Pour ce faire, l'échantillon est déplacé dans le champ de fuite de l'aimant supraconducteur durant la période de relaxation (Figure 3). Ainsi, de nombreux temps de relaxation sont obtenus, sur une large gamme de champs magnétiques, ce qui permet de reconstituer  $\mathcal{J}(\omega)$  sur un grand intervalle de fréquences.



FIGURE 3 – Principe de la relaxométrie haute résolution : le champ magnétique est changé par déplacement dans le champ de fuite de l'aimant pour une position donnée.

Cependant les vitesses de relaxation mesurées « naturellement » par cette approche souffrent d'erreurs systématiques. En effet, la relaxation des spins nucléaires se fait par de nombreuses voies (on parle de relaxation croisée) et celles-ci doivent être contrôlées par des impulsions radiofréquence (rf) afin de mesurer correctement une vitesse de relaxation. Dans le cas de la relaxométrie haute résolution, l'échantillon RMN n'est plus situé au sein de la bobine rf, et le système de spins ne peut être contrôlé durant la période de relaxation. Ainsi, il est nécessaire de prendre en compte les différents phénomènes de relaxation croisée durant la période de relaxation, dans notre cas la relaxation de  $I_z$  vers d'autres termes de spins. Cette étape, assurée par le programme ICARUS (Iterative Correction for the Analysis of Relaxation Under Shuttling<sup>18</sup>), nécessite la connaissance de la matrice de relaxation - matrice qui indique toutes les vitesses de relaxation de toutes les voies de relaxation dans un système de spin. Il est donc nécessaire de calculer ces matrices, mais aussi de trouver un modèle de fonction de densité spectrale afin de calculer convenablement les termes qui la composent.

Marquage sélectif et protéine de grande taille. Ici, nous allons décrire nos études de la dynamique des chaînes latérales de protéines. Les chaînes latérales sont généralement étudiées en diluant au maximum le nombre de spins sur ces chaînes dans les protéines. Par exemple, on utilisera un marquage uniforme  $\{^{12}C^2H\}$  couplé à un marquage spécifique des groupes méthyles (leucine, alanine ou isoleucine). Afin de diminuer les vitesses de relaxation croisée, nous avons décidé de marquer sélectivement les groupes méthyles des isoleucines  $\delta_1$  avec un groupement  ${}^{13}C^2H_2{}^1H$ . Nous avons commencé le développement méthodologique de cette approche sur l'ubiquitine, protéine modèle largement étudiée par RMN<sup>19-21</sup> et qui possède uniquement sept isoleucines.

**Spectroscopie RMN à deux champs.** Une façon élégante de s'affranchir de la correction des données est de pouvoir contrôler le système de spins à la position où s'effectue la relaxation. Cette approche permet, en outre, de valider les résultats obtenus avec les vitesses de relaxation obtenues grâce à ICARUS. Il peut donc être très intéressant de créer un système RMN à deux champs magnétiques, dans lequel une seconde zone de travail est homogénéisée en champ magnétique et de plus équipée

d'une sonde rf pour le contrôle du système de spins. Un tel système, le spectromètre à deux champs développé par Bruker, a rendu possibles, dans le cadre de ce travail, des études inédites de relaxations.

Outre la possibilité d'étudier précisément la relaxation des spins dans un champ magnétique faible, un tel appareil permet l'analyse de systèmes de spins qui souffrent des champs magnétiques intenses. C'est le cas des noyaux de carbone-13 carbonyles dont la relaxation transverse peut être très rapide, en raison de l'action du déplacement chimique anisotrope.

**Plan du résumé.** Dans ce contexte, mes travaux de thèse ont exploré une large gamme de champs d'expertise. Ainsi, afin de résumer au mieux ma contribution dans ces domaines, ce chapitre sera axé sur la présentation de trois points spécifiques :

- La compréhension des voies de relaxation dans un système de spins complexe, avec pour objectif la création d'un programme appelé REDKITE pour déterminer la matrice de relaxation de systèmes variables, notamment <sup>13</sup>C<sup>2</sup>H<sub>2</sub><sup>1</sup>H.
- L'étude de l'ubiquitine sélectivement marquée sur les groupes méthyles des isoleucines  $\delta_1$  avec le groupement  ${}^{13}C^2H_2{}^1H$ , au moyen de la relaxométrie haute résolution.
- Le développement et les premiers résultats obtenus sur le spectromètre RMN à deux champs magnétiques, notamment l'utilisation des cohérences à zéro quantum pour obtenir des spectres résolus sous l'évolution du déplacement chimique à bas champ.

### Étude de la matrice de relaxation en utilisant REDKITE

La relaxométrie à haute résolution consiste à transporter l'échantillon RMN hors de la zone homogène du champ magnétique afin de mesurer la vitesse de relaxation longitudinale des spins nucléaires à des champs magnétiques différents. Hors de cette zone d'homogénéité, il est impossible de contrôler le système de spins par des impulsions radio fréquences, et cela induit des imprécisions dans la mesure des vitesses de relaxation. Le programme ICARUS<sup>18</sup> permet de prendre en compte les voies de relaxation croisées afin d'extraire la constante de temps de l'autorelaxation uniquement (élément diagonal de la matrice de relaxation). Cela nous amènera à décrire de manière précise la dynamique pico- à nanoseconde de la macromolécule. Ce programme nécessite cependant la connaissance de la forme algébrique de la matrice de relaxation.

La théorie de la relaxation est relatée dans de nombreux livres<sup>22, 23</sup> et de nombreuses revues.<sup>24, 25</sup> Cependant, la complexité des calculs des vitesses de relaxation dans des systèmes de plusieurs spins rend son évaluation ardue. SPINDYNAMICA<sup>26</sup> propose déjà d'évaluer les termes de la matrice de relaxation et ce, pour des systèmes de spins complexes. Néanmoins, ce programme ne donne pas accès aux étapes de calcul analytique. Un second groupe propose quant à lui de calculer les termes de la matrice de relaxation de manière analytique,<sup>27</sup> mais leurs calculs ne prennent pas en compte les interactions dipolaires et quadrupolaires de manière simultanée. Malgré une recherche approfondie, il ne m'a pas été possible de trouver un programme permettant de calculer systématiquement chaque terme de la matrice de relaxation analytiquement et quel que soit le système de spins, et les interactions qui le gouvernent. REDKITE a donc été écrit en suivant les simplifications analytiques proposées dans le programme d'Ilia Kuprov (notamment l'utilisation des propriétés de symétrie, afin de diminuer considérablement le temps de calcul).

Pour le système de spins  ${}^{13}C^2H_2{}^{1}H$ , la matrice de relaxation est de grande taille (1296 × 1296). Par souci d'efficacité, seules les vitesses de relaxation longitudinale et croisée correspondant à la polarisation longitudinale  $\hat{C}_z$ , directement impliquée dans la relaxométrie haute résolution, ont été calculées. Ces termes ont été évalués pour un champ magnétique entre 0,33 et 14,1 T (gamme d'opération du système de relaxométrie) et seuls les termes qui possèdent une vitesse de relaxation supérieure à cinq pour cent du terme d'autorelaxation à au moins un champ magnétique ont été conservés. Le résultat de cette étude est résumé par la figure 4. Une matrice de relaxation réduite (Figure 5) a été utilisée dans ICARUS en considérant les termes :  $\hat{C}_z$ ,  $\hat{C}_z\hat{H}_z$ ,  $\hat{C}_z\hat{H}_z\hat{D}_z^{1,2}$ .



FIGURE 4 – Simulation de vitesse de relaxation croisée dépendant du champ magnétique, normalisée par la vitesse d'autorelaxation du  $\hat{C}_z$ . Sont représentées les vitesses de relaxation relatives entre les termes  $\hat{C}_z$  et  $\hat{D}_z$  (rose),  $\hat{C}_z$  et  $\hat{C}_z\hat{H}_z$  (violet), ainsi que  $\hat{C}_z$  et  $\hat{C}_z\hat{H}_z\hat{D}_z$  (vert). La relaxation croisée entre les termes  $\hat{C}_z$  et  $\hat{D}_z$  (rose) a été négligée pour le calcul de la matrice de relaxation.

Ce programme a été généralisé pour prendre en compte jusqu'à six atomes dans le système de spins, avec des orientations relatives des tenseurs d'interaction différentes et modulables pour des spins de nombre quantique magnétique  $|m_s| \ge 1$ ; il a notamment été utilisé dans le cadre des calculs de longue durée de vie dans un système  ${}^{13}C^2D_2$  en collaboration avec l'équipe travaillant sur la polarisation nucléaire dynamique. Dans la volonté de rendre le REDKITE disponible pour la communauté scientifique, il reste à inclure la possibilité de choisir la base dans laquelle la relaxation des termes de spin est calculée. Cela permettra par exemple de trier les termes de relaxation en fonction du nombre de cohérences du terme de spin associé.



FIGURE 5 – Représentation graphique de la matrice de relaxation pour le groupement méthyle { ${}^{13}C^{2}H_{2}{}^{1}H$ }. Les vitesses de relaxation apparaissent sous forme de pixels dont la couleur code la valeur. Les paramètres utilisés pour la fonction de densité spectrale sont les paramètres typiques obtenus pour le groupement  $\delta_1$  de l'isoleucine 36 de l'ubiquitine

### Étude de la dynamique des chaînes latérales de protéines par RMN

La relaxométrie à haute résolution permet une étude très approfondie des paramètres dynamiques d'une protéine. Une première étude a été publiée par le laboratoire : les vitesses de relaxation du squelette polypeptidique de l'ubiquitine y ont été évaluées sur dix champs magnétiques différents (de 0,5 à 22,3 T).<sup>18</sup> Les groupements {<sup>15</sup>N<sup>1</sup>H} relaxent cependant très rapidement à bas champ, et permettent uniquement d'échantillonner les mouvements du squelette peptidique.

Les noyaux de carbone des groupements méthyles, quant à eux, relaxent plus lentement grâce à la rotation du groupe méthyle autour de l'axe de la liaison CC à une échelle de temps de quelques picosecondes.<sup>28</sup> De plus, les groupes méthyles apparaissent sur un grand nombre de chaînes latérales de protéines car ils sont présents dans de nombreux acides aminés (Ala, Val, Leu, Ile, Thr, Met). Avec comme objectif l'étude de systèmes de haut poids moléculaire, un marquage sélectif a été effectué sur les groupements méthyles { $^{13}C^2H_2^1H$ } des isoleucines- $\delta_1$  (Iles- $\delta_1$ ) de l'ubiquitine, tandis que le reste de la protéine est marqué { $^{2}H$ ; $^{15}N$ }. Pour ce faire, un  $\alpha$ -keto acide marqué sélectivement (2-keto-3-d<sub>2</sub>-4- $^{13}C$ ,D<sub>2</sub>-butyrate) est ajouté peu avant l'induction afin de remplacer systématiquement le pyruvate dans les voies métaboliques.<sup>29</sup>

#### Ubiquitine

**Expérience de relaxation.** Des mesures de vitesses de relaxation ont été effectuées à haut champ, sur les noyaux du squelette peptidique ainsi que les chaînes latérales. Les expériences de relaxation longitudinale (R<sub>1</sub>) ont été effectuées à 9,4, 14,1, 18,8 T. Les expériences de R<sub>1</sub>,  $\rho$  et de mesure des vitesses de relaxation croisée dipolaire par effet Overhauser nucléaire (NOE) ont été effectuées à 14,1 et 18,8 T. Les expériences sur l'azote-15 ont permis de déterminer le temps de corrélation de la diffusion rotationnelle de la protéine ( $\tau_c$ ), à l'aide du programme ROTDIFF.<sup>30</sup> L'échantillon a par la suite été transféré dans D<sub>2</sub>O. Le temps de corrélation  $\tau_c$  a été évalué en utilisant la relation de Stokes-Einstein et les valeurs tabulées de la viscosité de H<sub>2</sub>O et D<sub>2</sub>O à 298 K.<sup>31</sup>

Les vitesses de relaxation à bas champ ont été mesurées en employant la relaxométrie à haute résolution.<sup>17</sup> Cette approche permet d'évaluer la relaxation des spins nucléaires à des champs magnétiques inférieurs à 10 T, en déplaçant l'échantillon dans le champ de fuite de l'aimant supraconducteur. Les vitesses de relaxation longitudinale ont été mesurées à 18 champs magnétiques, entre 0,2 et 7 T. L'analyse des données de relaxation a été réalisée à l'aide d'une nouvelle version du programme ICARUS qui prend en compte les voies de relaxation croisée spécifiques aux groupes méthyles. Le facteur correctif maximum appliqué sur les vitesses de relaxation est de l'ordre de 5% pour les champs magnétiques les plus faibles (Figure 6).



FIGURE 6 – Vitesses de relaxation longitudinale du carbone-13 mesurées à haut champ (9,4 T, 14,1 T, 18,8 T) et bas champ magnétique pour les sept Iles- $\delta_1$ -{<sup>13</sup>C<sup>2</sup>H<sub>2</sub><sup>1</sup>H} de l'ubiquitine marquée {<sup>15</sup>N,<sup>2</sup>H} dans D<sub>2</sub>O, pH 4,6 avec 50 mM d'acide acétique.

**Fonction de densité spectrale et sélection des modèles de mouvement.** Les vitesses de relaxation mesurées sont utilisées pour ajuster les paramètres de plusieurs modèles de la fonction de densité spectrale, de complexité croissante. Une version de la fonction de densité spectrale « model-free » a été adaptée pour les groupements méthyles, à la manière de Tugarinov<sup>32</sup> en partant de la fonction de corrélation suivante :

$$\mathcal{C}(t) = e^{-t/\tau_c} \left( S_m^2(\theta) + \left( \mathcal{P}_2(\cos \theta_{i,j}) - S_m^2(\theta) e^{-t/\tau_m} \right) \left( S^2 + (1 - S_f^2) e^{-t/\tau_f} + (S_f^2 - S) e^{-t/\tau_s} \right) \right)$$
(1)

dont la transformée de Fourier donne la fonction de densité spectrale :

$$\mathcal{J}_{5}(\omega) = \frac{2}{5} \left( S_{f}^{2} S_{s}^{2} S_{m}^{2}(\theta) \frac{\tau_{c}}{1 + (\omega\tau_{c})^{2}} + S_{m}^{2}(\theta)(1 - S_{f}^{2}) \frac{\tau_{1}}{1 + (\omega\tau_{1})^{2}} + S_{m}^{2}(\theta) S_{f}^{2}(1 - S_{s}^{2}) \frac{\tau_{2}}{1 + (\omega\tau_{2})^{2}} + S_{s}^{2} S_{f}^{2}(\mathcal{P}_{2}(\cos\theta_{i,j}) - S_{m}^{2}(\theta)) \frac{\tau_{3}}{1 + (\omega\tau_{3})^{2}} + (1 - S_{f}^{2})(\mathcal{P}_{2}(\cos\theta_{i,j}) - S_{m}^{2}(\theta)) \frac{\tau_{4}}{1 + (\omega\tau_{4})^{2}} + (\mathcal{P}_{2}(\cos\theta_{i,j}) - S_{m}^{2}(\theta)) S_{f}^{2}(1 - S_{s}^{2}) \frac{\tau_{5}}{1 + (\omega\tau_{5})^{2}} \right)$$

$$(1 - S_{f}^{2})(\mathcal{P}_{2}(\cos\theta_{i,j}) - S_{m}^{2}(\theta)) \frac{\tau_{4}}{1 + (\omega\tau_{4})^{2}} + (\mathcal{P}_{2}(\cos\theta_{i,j}) - S_{m}^{2}(\theta)) S_{f}^{2}(1 - S_{s}^{2}) \frac{\tau_{5}}{1 + (\omega\tau_{5})^{2}} \right)$$

Dans cette équation,  $\mathcal{P}_2(\cos \theta_{i,j})$  est le polynôme de Legendre, pour l'angle entre les interactions I et J,  $S_i^2$  est le paramètre d'ordre du mouvement *i*, avec l'échelle de temps associée  $\tau_i$ . De plus,  $\tau_1^{-1} = \tau_c^{-1} + \tau_f^{-1}$ ,  $\tau_2^{-1} = \tau_c^{-1} + \tau_s^{-1}$ ,  $\tau_3^{-1} = \tau_c^{-1} + \tau_m^{-1}$ ,  $\tau_4^{-1} = \tau_c^{-1} + \tau_m^{-1} + \tau_f^{-1}$ ,  $\tau_5^{-1} = \tau_c^{-1} + \tau_m^{-1} + \tau_s^{-1}$ ,

et le paramètre d'ordre du mouvement de rotation est :

$$S_m^2(\theta) = \mathcal{P}_2(\cos\theta_{\vec{i}\ \vec{CC}})\mathcal{P}_2(\cos\theta_{\vec{CC}\ \vec{i}}) \tag{3}$$

Cette fonction de densité spectrale prend en compte le mouvement de rotation globale (termes d'indice c), le mouvement de rotation du groupe méthyle (termes d'indice m), deux échelles de temps caractérisées par deux paramètres d'ordres pour le mouvement de l'axe CC, un rapide (termes d'indice f) et un plus lent (termes d'indice s). Une représentation graphique est présentée sur la Figure 7.



FIGURE 7 – Représentation graphique des mouvements des groupes méthyles considérés dans la fonction de corrélation de Equation 2

Les paramètres des modèles ont été ajustés en utilisant la méthode de Monte-Carlo par chaîne de Markov,<sup>33</sup> afin d'obtenir les paramètres de la dynamique de ces sept chaînes latérales. Les tests ont été effectués successivement en utilisant quatre fonctions de densité spectrale correspondant à des modèles de complexité décroissante :

- $\mathcal{J}_5(\omega)$ , la fonction de densité spectrale à cinq paramètres ajustables.
- $\mathcal{J}_4(\omega)$ , où  $\tau_f$  est considéré comme négligeable.
- $\mathcal{J}_3(\omega)$ , où  $\tau_s$  est négligeable et  $S_s^2$  est égal à un (*ie* retour à une approche model-free pour le vecteur CC).
- $\mathcal{J}_2(\omega),$  où  $\tau_s$  et  $\tau_f$  sont négligeables et  $S^2_s$  est égal à un.

La pertinence statistique des différents modèles de fonction de densité spectrale a été évaluée par un test statistique en utilisant le critère d'information d'Akaike. Les résultats sont présentés dans le Tableau 1. La version étendue à cinq paramètres ( $\mathcal{J}_5(\omega)$ ) ainsi que celle réduite à deux paramètres ( $\mathcal{J}_2(\omega)$ ) n'ont jamais été sélectionnées. Pour l'Ile-44, les deux systèmes ont été choisis : cependant le temps caractéristique associé à la rotation du groupe méthyle semble peu probable. Les résidus 13 et 36 ont des temps caractéristiques du mouvement du CC proches de 2 ns.

Ces résultats ont été comparés avec ceux obtenus par des expériences de RMN en phase solide et de relaxation du deutérium en solution, ainsi qu'aux résultats de simulations de dynamique moléculaire obtenus par le groupe de Raphael Brüschweiler.

TABLE 1 – Paramètres obtenus après l'ajustement des paramètres des différents modèles. Dus à l'absence d'écart statistique entre la forme  $\mathcal{J}_3(\omega)$  et  $\mathcal{J}_4(\omega)$  pour l'isoleucine 44, les deux résultats ont été indiqués.

Résidus	${f S}_{ m f}^2$	$\mathbf{S}_{\mathrm{s}}^2$	$ au_{ m f}$ / ${f ns}$	$ au_{ m m}$ / ${ m ps}$
Ile 3	$0,\!627\pm0,\!018$	-	$0,\!247\pm0,\!037$	$3,\!61\pm0,\!42$
Ile 13	$0,\!658\pm0,\!027$	$0,\!482\pm0,\!083$	$2,\!456\pm1,\!043$	$19{,}09\pm2{,}05$
Ile 23	$0{,}427\pm0{,}014$	-	$0,\!277\pm0,\!048$	$17{,}04\pm0{,}98$
Ile 30	$0,\!663\pm0,\!022$	-	$0,\!345\pm0,\!075$	$5,72\pm0,52$
Ile 36	$0,\!709\pm0,\!034$	$0,\!486\pm0,\!074$	$1,716\pm0,808$	$13,\!03\pm2,\!00$
Ilo 44	$0{,}586\pm0{,}085$	$0,\!24\pm0,\!10$	$0,\!823\pm0,\!394$	$12{,}41\pm4{,}94$
110 44	$0,\!185\pm0,\!013$	-	$0{,}318\pm0{,}033$	$1,\!365\pm0,\!68$
Ile 61	$0,\!483\pm0,\!013$	-	$0{,}213\pm0{,}039$	$11,\!928\pm0,\!878$

D'autres formes de fonction de densité spectrale ont été envisagées afin de ne pas surévaluer le nombre de paramètres ajustables des fonctions de densité spectrales. Ces discussions sont décrites dans le chapitre 2.

### Spectroscopie RMN à deux champs

Il semble évident, à première vue, que l'accroissement des champs magnétiques de nos spectromètres est bénéfique, puisqu'il augmente la différence d'énergie entre les états de spin nucléaire, et ainsi la sensibilité et la résolution des spectres obtenus. Cependant dans certains cas, le système étudié nécessiterait qu'une partie de la séquence d'impulsion se déroule à des champs magnétiques plus faibles ou différents. Par exemple, la relaxation transverse du <sup>13</sup>C du carbonyle peut s'avérer extrêmement rapide et constitue un réel frein pour les expériences de RMN triple résonance qui impliquent ce noyau.<sup>34</sup> Cette relaxation rapide est due au grand déplacement chimique anisotrope de ce noyau. La vitesse de relaxation transverse du <sup>13</sup>C carbonyle dans une macromolécule est proportionnelle au carré du champ magnétique. Enfin, du point de vue technique, il existe une réelle difficulté à obtenir des champs radiofréquences intenses homogènes qui couvrent une large bande de fréquences (Figure 8). Certains noyaux tels que le (<sup>195</sup>Pt) possèdent une large gamme de déplacements chimiques, et souffrent de la limitation des champs rf à la plupart des champs magnétiques employés pour la RMN de routine (Figure 8).



FIGURE 8 – Simulation de la polarisation présente sur le plan transverse, après une impulsion 90° appliquée à la polarisation d'équilibre sur un 300 MHz (noir), un 600 MHz (orange), un 1,2 GHz (bleu). La valeur de la polarisation d'équilibre est normalisée à 1. La fréquence de la porteuse de l'impulsion est placée, dans les trois cas, à 100 ppm pour une irradiation sur le carbone-13.

Un nouveau prototype de spectromètre RMN a été développé par Bruker dans le cadre de notre collaboration. Partant d'un spectromètre opérant à 600 MHz utilisé pour la relaxométrie haute résolution, un second centre magnétique a été obtenu à 0,33 T en générant un plateau de champ magnétique dans le champ de fuite de l'aimant supraconducteur. Ce plateau a été obtenu grâce à cinq cylindres d'un alliage de fer et de nickel (l'ensemble est appelé ferroshims), combinés à des lamelles de mu-métal et à un ensemble de 20 bobines de shims à température ambiante dans un canon de shims (de type BOSS1). Le système pneumatique utilisé pour transférer l'échantillon entre les deux zones homogènes à 14,1 et 0,33 T est le même que celui utilisé dans la relaxométrie haute résolution, à ceci près que le guide de la navette a été remplacé par une pièce télescopique à ressort, en plastique, d'une trentaine de centimètres. De cette façon, le guide s'adapte parfaitement à l'espace entre les deux sondes. Les deux sondes sont conçues spécialement pour résister aux contraintes imposées par l'échantillon lors de son déplacement. La sonde haut champ a déjà été décrite et utilisée pour la relaxométrie haute résolution. La sonde bas champ, quant à elle, a été créée spécialement pour le système à deux champs, et possède trois canaux (proton, carbone et azote), ainsi qu'un gradient Z (Figure 9). Il est important de noter que le gradient Z du bas champ est obtenu grâce à un des trois canaux d'un amplificateur d'impulsions de gradients de champ à trois axes disponible sur le spectromètre à haut champ.



FIGURE 9 – Spectromètre à deux champs. En bleu/orange est représenté le système haut champ/bas champ. Les ferroshims et leur contrôle en température sont représentés en rouge. La régulation en température est gérée par deux BVT (Bruker Variable Temperature units) distinctes et est représentée en bleu.

Construire un tel prototype n'a pas été une mince affaire et de nombreux problèmes techniques ont dû être résolus. On notera par exemple l'impossibilité d'utiliser les systèmes habituels pour obtenir des impulsions radiofréquences pour les fréquences inférieures à 5 MHz (ce qui est le cas ici pour le carbone-13 et l'azote-15). Il a donc fallu adapter des systèmes de génération d'impulsion rf utilisés typiquement en spectrométrie de masse et des amplificateurs à basse fréquence. Par ailleurs, le gain d'homogénéité du second centre magnétique est considérable : la variation du champ magnétique à 0,33 T est de l'ordre de 200 000 ppm. Le système est maintenant fonctionnel pour développer une nouvelle spectroscopie à deux champs magnétiques.

#### Caractérisation et premières expériences sur le système à deux champs

**Caractéristiques du spectromètre à deux champs.** En polarisant l'échantillon RMN à haut champ (14,1 T), nous avons été en mesure d'acquérir un signal à une dimension, intense, pour une détection directe sur le proton à 0,33 T. Cela nous a donc permis de tester l'homogénéité du second plateau de champ magnétique, ainsi que les impulsions à bas champ. L'homogénéité du champ magnétique dans le second centre a été mesurée sur un échantillon de sucrose, où la largeur de raie à mi-hauteur était de 11 ppm. Ce résultat est bien sûr dépendant de la qualité des shims dans la zone de travail à 0,33 T. Il est certainement possible d'obtenir une homogénéité un peu plus importante mais qui ne changerait pas qualitativement les résultats des expériences réalisées. De plus, en l'absence de verrouillage en fréquence du bas champ (Lock), il serait inutile de rechercher une homogénéité plus élevée, celle-ci dépendant également de paramètres tels que la température de la pièce.

Nous avons en outre testé la qualité des impulsions basse fréquence sur une expérience de nutation, pour les noyaux de <sup>1</sup>H, <sup>13</sup>C et <sup>15</sup>N. Pour ce faire, l'échantillon est polarisé à 600 MHz, puis est transféré à 14,1 MHz. L'impulsion bas champ est alors effectuée et l'échantillon est transféré à haut champ pour l'acquisition du signal. Les résultats sont rapportés sur la Figure 10. Les puissances utilisées en watt sont respectivement 0,5 ; 100 ; 50 pour <sup>1</sup>H ; <sup>13</sup>C ; <sup>15</sup>N. La qualité des nutations pour le carbone et l'azote est remarquable, avec  $I_{900}/I_{180} = 80 \pm 2\%$  pour le proton,  $I_{900}/I_{180} = 98 \pm 2\%$  pour le carbone-13 et  $I_{900}/I_{180} = 96 \pm 15\%$  pour l'azote-15. Elle provient, entre autres, de la grande taille de la bobine bas champ.

Afin de vérifier les principes de base de la spectroscopie RMN à deux champs, quelques expériences usuelles ont été réalisées en l'absence de haute résolution pour le bas champ. La corrélation hétéronucléaire entre le carbone-13 à bas champ et l'hydrogène à haut champ a été réalisée grâce à une expérience de Two-Field Heteronuclear Single Quantum Coherence (2F-HSQC). Les expériences de relaxation longitudinale et transverse sur des petites molécules, avec contrôle des voies de relaxation croisées à bas champ ont été également réalisées pour démontrer le bon fonctionnement du spectromètre à deux champs.

**Une expérience insensible à l'inhomogénéité à bas champ.** Un réel avantage du bas champ est la qualité des impulsions radiofréquences à 0,33 T. En effet, sur une gamme de fréquence si restreinte, les impulsions peuvent être considérées comme dures sur l'intégralité de la fenêtre spectrale, tout du moins pour les atomes de <sup>13</sup>C et <sup>15</sup>N.

Une élégante application de ce concept peut être trouvée lors du transfert de cohérence durant une expérience de TOCSY.<sup>35</sup> Cette expérience RMN est réalisée par un transfert de cohérence d'un spin nucléaire à l'autre sous irradiation des spins avec un champ radiofréquence. L'effet de ce champ est d'annuler l'hamiltonien de déplacement chimique et d'obtenir un hamiltonien de couplage scalaire fort.



FIGURE 10 – Expérience de nutation basse fréquence sur <sup>1</sup>H, <sup>13</sup>C et <sup>15</sup>N. A) Séquence d'impulsions pour calibration des impulsions à bas champ. La durée  $\beta$  de l'impulsion rf a été modifiée itérativement. B) Calibrage de l'impulsion sur azote-15 (sur cet échantillon p<sub>90</sub> = 20 µs). C) Calibrage de l'impulsion sur le proton (sur cet échantillon p<sub>90</sub> = 10,5 µs). D) Calibrage de l'impulsion sur le carbone-13 (sur cet échantillon p<sub>90</sub> = 11 µs). Ces expériences ont été effectuées sur un échantillon 1 M de glycine enrichie en carbone-13 et azote-15 dans D<sub>2</sub>O.

De nombreux travaux de recherche ont porté sur la séquence de mélange, constituée d'impulsions composites afin d'améliorer le transfert de polarisation.<sup>36,37</sup> En utilisant la spectroscopie à deux champs, nous avons réalisé l'irradiation rf de mélange à bas champ où les impulsions peuvent être considérées comme très large bande. Il est ainsi possible de rendre le TOCSY efficace sur l'intégralité du spectre carbone-13. On peut donc transférer la polarisation sur un réseau s'étendant d'un groupe méthyle à un carbone-13 d'un groupement carbonyle, soit dans ce cas précis une différence de 154,7 ppm (Figure 11).

#### Haute résolution à bas champ

Pour pallier la faible homogénéité du champ, qui affecte le marquage du déplacement chimique à bas champ, deux séquences à deux dimensions ont été implémentées en utilisant les propriétés des cohérences à zéro quantum.<sup>38</sup>

**2F-INAZEQUATE :** La séquence *Two-Field - Incredible Natural Abundance Zero QUantum Transfer Experiment* (2F-INAZEQUATE) permet de corréler deux carbones dans une spectroscopie à deux



FIGURE 11 – Spectre de corrélation  ${}^{13}C{}^{-13}C$  TOCSY à deux champs, sur un échantillon à 0,1 M de leucine (bleu) et phénylalanine(rouge) uniformément marquées en carbone-13 dans D<sub>2</sub>O.

champs avec une haute résolution dans les deux dimensions. En effet, comme l'évolution du déplacement chimique se déroule à la différence des fréquences des deux spins corrélés, les effets des inhomogénéités du champ magnétique sont supprimés. Cette propriété peut être facilement observée dans l'équation suivante :

$$\omega_C^1 - \omega_C^2 = \gamma_C B_0 (\sigma^1 - \sigma^2 + \Delta_{Ino}^1 - \Delta_{Ino}^2) \tag{4}$$

où  $\omega_C^i$  est la fréquence du simple quantum du spin i,  $\gamma_C$  le rapport magnétogyrique du carbone,  $B_0$  le champ magnétique du spectromètre RMN,  $\sigma^1$  la constante d'écran du spin i suivant l'environnement chimique propre et enfin  $\Delta_{Ino}^i$  la constante d'écrantage due à l'inhomogénéité du champ magnétique. Considérant la très faible distance entre les noyaux au regard des distances de variation du champ magnétique, nous pouvons considérer que  $\Delta_{Ino}^1 = \Delta_{Ino}^2$ : la fréquence du zéro quantum n'est donc pas dépendante de l'homogénéité du champ magnétique. Dans ces conditions, la résolution obtenue pour la dimension carbone à bas champ est de 0,1 ppm.



FIGURE 12 – Spectres à deux champs et à deux dimensions à zéro quantum. Le spectre A) est le spectre du 2D-2F-INAZEQUATE sur un échantillon à 1 M de glycine enrichi en carbone-13 et azote-15 dans D<sub>2</sub>O. Le spectre 1D extrait à 41,29 ppm de la dimension directe est également représenté pour montrer la largeur du signal dans la dimension zéro quantum <sup>13</sup>C<sub>LF</sub>. B) Superposition des spectres 2F-HZQC (après transformée de cisaillement) et HSQC haut champ sur un mélange équimolaire à 0,5 M d'acétone et de tert-butanol dans D<sub>2</sub>O.

**2F-HZQC :** La version deux champs du *Heteronuclear Multiple Quantum Coherence* a également été implémentée avec une sélection de la cohérence à zéro quantum pour l'évolution du déplacement chimique à bas champ. Cette séquence, baptisée *Two Fields - Heteronuclear Zero Quantum Coherence* (2F-HZQC), inclut une mise à l'échelle de l'évolution sous le déplacement chimique du proton, de sorte que l'hamiltonien effectif lors de l'évolution à bas champ a pour forme :

$$\hat{\mathcal{H}}_{evol} = \frac{\gamma_C}{\gamma_H} \Omega_H \hat{\mathcal{H}}_z + \Omega_C \hat{\mathcal{C}}_z \tag{5}$$

Dans ces conditions, les inhomogénéités du champ magnétique sont compensées, en dépit d'un rapport magnétogyrique différent pour les deux atomes couplés, et l'homogénéité de la dimension carbone à bas champ a été mesurée à environ 0,8 ppm pour un mélange 0,5 M acétone / 0,5 M tert-butanol dans D<sub>2</sub>O.

Pour cette spectroscopie, le déplacement chimique usuel du carbone-13 peut être retrouvé dans la dimension indirecte en effectuant une transformée de cisaillement, en faisant l'hypothèse que les déplacements chimiques du proton sont identiques aux deux champs considérés. Les spectres peuvent donc être superposés au spectre usuel à simple quantum obtenu avec une expérience HSQC.

#### RMN à deux champs pour l'observation de signaux en coalescence

Les signaux de RMN d'un système en échange chimique sont affectés de manière différente en fonction de la comparaison entre le temps caractéristique de la RMN et celui de la réaction d'échange. Trois cas sont possibles. Si l'échange chimique est lent, chaque forme produira un spectre RMN distinct. En revanche, si l'échange est très rapide, l'évolution sous le déplacement chimique est rapidement moyennée et une unique fréquence peut être observée. Lorsque le régime est transitoire, la perte de cohérence des phases des polarisations transverses conduit à un élargissement important des signaux allant jusqu'à la perte totale du signal.(Figure 13)



FIGURE 13 – Évolution du signal RMN de deux espèces en échange chimique suivant la vitesse de l'échange ( $k_{exp}$ ) et du champ magnétique d'observation ( $B_0$ ).

Pour simplifier le problème, on peut envisager le cas simple du système en échange chimique entre deux états avec des populations identiques. La constante du système est alors  $k_{exp} = k_{-1} + k_1$  (où  $k_{-1}$  et  $k_1$  sont les vitesses de premier ordre, ou pseudo premier ordre, du sens direct et indirect de la réaction chimique). La différence de fréquence de précession des sites d'échange est :

$$\Delta\Omega = (\delta_A - \delta_B)\gamma_i B_0 \tag{6}$$

ou  $\delta_{A;B}$  est le déplacement chimique des noyaux i pour les environnements A et B;  $\gamma_i$  le rapport magnétogyrique du noyau i; et  $B_0$  le champ magnétique d'évolution du déplacement chimique. Le phénomène d'élargissement maximal à la coalescence se produit lorsque  $k_{\exp} = 5\Delta\Omega/\sqrt{2}$ . Ainsi, si le système peut être observé à un champ magnétique B<sub>0</sub> réduit, on attend une diminution importante de la largeur de raie du signal en échange chimique.

**Exemple des triazènes.** Les composés triazènes sont connus pour leurs propriétés d'échange chimique.<sup>39</sup> Lorsque l'azote terminal porte deux groupes méthyles, ces derniers sont équivalents dans un cas d'échange rapide et inéquivalents lors d'un échange lent. Lorsque l'azote mono-substitué est lié à un cycle aromatique, le·a chimiste peut à sa guise contrôler la vitesse d'échange de la fonction diazoamino. Ainsi, plusieurs composés triazènes ont été synthétisés, chacun avec deux groupements méthyles échangeables. Dans le premier cas, le groupe aromatique est substitué en para de la fonction diazoamino par un brome. Dans le second cas, qui nous intéressera particulièrement par la suite, le groupement en para est un groupement méthoxy. Les propriétés électroniques du groupement méthoxy rendent l'échange chimique de la fonction diazoamino plus rapide que dans le cas du brome. On observe ainsi, dans la deuxième dimension du spectre <sup>1</sup>H<sup>13</sup>C-HSQC entre 21°C et 31°C, la perte de signal due à l'élargissement extrême des pics sous un échange chimique de plus en plus rapide (Figure 14). Quelle que soit la vitesse d'échange des groupements méthyles, le 2F-HZQC nous permet d'échantillonner le carbone sur une gamme de fréquence plus faible, donc dans une gamme de fréquence où l'échange chimique est considéré comme rapide. La perte de cohérence par échange chimique, très mesurée, conduit à un spectre 2F-HZQC de qualité.



FIGURE 14 – Observation de la coalescence pour les signaux du carbone-13 des groupes méthyles en échange dans le composé triazène. Les groupements méthyles subissant une perte de cohérence due à l'échange chimique sont indiqués en orange. Le groupe méthyle de référence O-Me est indiqué en bleu.

### **Conclusion et perspectives**

Ce texte présente une partie importante des travaux réalisés durant les trois dernières années. Il décrit mon implication pour la production et la purification de protéines, avec marquage isotopique. Il retrace également mon étude de la dynamique des protéines, en s'intéressant fortement au type de modèle à considérer pour décrire convenablement les mouvements des groupes méthyles dans la gamme ps-ns qui contribuent à la relaxation. Enfin, il rapporte le développement de la spectroscopie RMN à deux champs. Ces deux derniers domaines auraient pu être approfondis largement et auraient même pu faire chacun l'objet d'une thèse. De nombreux prolongements sont, à partir de ces recherches, envisageables.

Je souhaite faire murir le programme REDKITE, en incluant une interface graphique afin de le rendre accessible à d'autres chercheur·euse·s, expert·e·s ou novices en relaxation des spins nucléaires. J'aimerais également effectuer les calculs de relaxation dans des bases différentes, qui seraient laissés au libre de choix de l'utilisateur·trice. En effet une fois les matrices obtenues, il peut être difficile d'effectuer un changement de base avec des objets de si grande taille. Ce calcul pourrait être parallélisé simplement puisque chaque terme de la matrice de relaxation est indépendant (hormis sa symétrie par transposition), réduisant de fait fortement le temps de calcul. Enfin, il serait souhaitable d'utiliser un logiciel libre pour réécrire ce programme.

Le programme ICARUS devra lui aussi être perfectionné, et je travaille actuellement avec Nicolas Bolik-Coulon (stagiaire de master 2), pour transférer ce programme sur РУТНОN et le rendre accessible à la communauté.

Dans l'analyse de la dynamique des chaînes latérales de protéines, il reste aussi à obtenir une procédure solide d'analyse et, sur l'ubiquitine, à élucider les déviations systématiques des vitesses de relaxation obtenues à bas champ sur certains résidus et à comparer ces résultats à la dynamique moléculaire.

Enfin, la spectroscopie à deux champs reste pour moi une source d'inspiration constante, avec de nombreuses idées à mettre en place, je l'espère avant la fin de ma thèse, en particulier l'acquisition d'une expérience de R<sub>1</sub> et R<sub>1,ρ</sub> à deux champs sur l'ubiquitine $-{^2H;^{12}C} - Ile - \delta_1 {^{13}C;^{1}H_3}$ . A terme, il serait souhaitable d'obtenir des sondes à haut champ et bas champ accordées différemment pour étudier d'autres noyaux, en particulier ceux qui possèdent une gamme de déplacement chimique très étendue (par exemple le platine-109), pour avoir accès à une spectroscopie non encore égalée. J'aimerais également évoquer la possibilité d'utiliser le gradient extrêmement important du champ de fuite du 600 MHz pour réaliser des expériences qui nécessitent des gradients intenses...

Après trois années de recherche sur le système de la RMN à champs multiples, je suis convaincu que la relaxométrie haute résolution et la spectroscopie à deux champs peuvent apporter énormément à la communauté RMN et au-delà, au chimiste, comme au biologiste structural. Ce sujet extrêmement vivant me paraît, à l'heure actuelle, sans fin et je suis très heureux d'avoir pu contribuer à sa naissance.

# **General introduction**

### Structures and dynamics of proteins

Proteins have many roles in living things : as messenger (hormones), as transporter,<sup>40</sup> as a tag,<sup>41</sup> or as a catalyst (enzymes<sup>42</sup>). Scientists have questioned the variability and accuracy of protein mechanisms for many decades. The support of biologists, physicists and chemists is essential in order to truly understand these macromolecules.

The variability of the protein functions and mechanisms has been explained for many years by the variability of their forms. The "structure/function" paradigm, also called the "lock and key" model, has been introduced by Fisher in 1894.<sup>43</sup> As an example, using this paradigm, the shape of the protein determined the protein-ligand interaction and so, determines the mechanism and the rate constant of an enzymatic reaction. The structure-function relationship has been particularly studied by high-resolution X-ray spectroscopy.<sup>44,45</sup> A striking example can be found with the proposition of a mechanism for the human polymerase- $\eta$  replication mechanism, determined with the crystal structure of a co-precipitation of the polymerase with DNA.<sup>46</sup>

However, proteins are soft materials that sample a huge number of conformations outside those visible in the crystal structure. In particular, the existence of intrinsically disordered proteins challenged the structure-function paradigm. These proteins do not have a particular 3D structure in their functional forms.<sup>47</sup> In that regard, the "lock and key" model has to be improved to include the temporal dimensionality. Unfortunately, it is not possible at the moment to watch atom motions directly, in order to determine with precision the protein dynamics.

The dominant motion that occurs in a well-folded single domain protein is the rotational diffusion of the entire domain, which is usually referred to as the overall or global motion. The study of the overall motions is informative on the hydrodynamics of a protein. In multi-domain proteins, motions of domains relative to each other are usually allowed and can have a wide amplitude. We refer to these as domain motions. Within a protein domain, loops and turns can wobble on fast nanosecond timescales or faster. Some conformational transitions involve a reorganisation of some interactions such as hydrogen bonds or salt bridges and the passage through a significant energy barrier. These transitions take place on timescales ranging from the hundreds of nanoseconds to seconds, and will not be treated. The transition between side-chain rotamers is often allowed on fast nanosecond and hundreds of picosecond time-scales. The fastest motions are vibrations and librations of the protein backbone and side-chain, which also lead to measurable contributions to the reorientation of groups of atoms both in the side chains and the backbone of a protein. Such motions are variable in the timescale and amplitude and depend on the local pattern of intramolecular interactions.

Many techniques can be used to describe dynamical processes, and an exhaustive list of all techniques are beyond the scope of this work. Nonetheless, I will focus on one particular techniques that allows the determination of protein motions with atomic resolution: nuclear magnetic resonance spectroscopy.

### Proteins by nuclear magnetic resonance

Nuclear magnetic resonance (NMR) is one of the key methods to assess protein structures and dynamics. Indeed, this spectroscopy permits determining precisely the nuclear spin environment and its evolution, in solid or liquid phases, with an atomic resolution. Combining both liquid- and solid-state NMR, a large number of proteins can be studied : virus capsid,<sup>48</sup> membrane proteins,<sup>40</sup> gels,<sup>49</sup> and even bacteria needle.<sup>50</sup>

Applications of NMR to biomolecules have to overcome several challenges. Because NMR spectroscopy is not a very sensitive technique, a relatively high amount of proteins must be produced for one sample. Additionally, the isotopic percentage of NMR observable isotopes for carbon and nitrogen require enriching the protein of interest, which drastically increases the cost of the sample. NMR spectra of proteins are generally crowded and complex to analyse due to the small chemical shift differences between protein residues. High resolution and high dimensionality of the NMR experiment is needed. These needs induce a long experimental time for the acquisition of the NMR spectrum, particularly since the polarization must be propagated in the spin system, or since the resolution of the spectra must be high. Therefore, the loss of polarization in the case of large macromolecules due to the absence of global tumbling can result in a large broadening of the signal.

For proteins larger than few tens of kilo Dalton, with conventional spectroscopy methods, NMR signals are generally not observable.<sup>51</sup> For the study of large proteins by liquid-state NMR, specific labelling can be introduced<sup>52–54</sup> to overcome this limitation.

After the first proton 1D NMR spectrum of a protein recorded in 1957,<sup>55</sup> much progress has been made in terms of NMR methodology<sup>56–58</sup> and hardware for the study of biomolecules.<sup>59</sup> The implementation of multidimensional heteronuclear NMR spectroscopy permits the assignment of complex proteins. In particular, and extensive use of HSQC<sup>60</sup> - TOCSY<sup>51</sup> - NOESY<sup>61</sup> and many other methods<sup>62</sup> can be found for the study of biomolecules. Combined with scalar-coupling constants and nuclear Overhauser effects, it becomes possible to determine the structure of proteins with NMR in liquid<sup>63</sup>- or solid<sup>64</sup>-states spectroscopy. Another beautiful NMR experiment called TROSY (transverse relaxation-optimized spectroscopy)<sup>65–67</sup> allowed a NMR spectroscopist to study large proteins in liquid-state NMR. The main idea of the TROSY experiment is benefiting from the interference between dipole-dipole coupling and the chemical shift anisotropy, in relaxation processes in a pair of spins <sup>1</sup>/<sub>2</sub>. One of the most successful implementations of this technique is the study of a 900 kDa chaperonin complex GroEL-GroES.<sup>61</sup>



Figure 15 – Development of NMR spectrometer since 1960, described with the successive use of several alloys. Superconducting magnets are constituted by several coils and the change of colour represents the change of the inner and strongest coil.<sup>68</sup>

Biological NMR also benefits from the improvement of NMR spectrometers. A tremendous gain in sensitivity/resolution has been obtained by increasing the magnetic field and by introducing cryocooled probe-head. Indeed the NMR signals increase quadratically with respect to the magnetic field, due to the Boltzmann polarization and Faraday induction consideration.<sup>16</sup> This development of a magnetic field is induced by the successive use of different alloys. After pushing the iron-core electromagnet to the limit of polarization (2.35 T in 1966), a further increase of the magnetic field could only come from low-temperature superconducting coils (Figure 15). Today, new projects are under development with the use of hot superconductors (discovered in 1987), and in 2016, seven 1.2 GHz magnets are under construction using Yttrium Barium Copper Oxide.<sup>16,68</sup>

### Dynamics by nuclear magnetic resonance

#### Correlation function and spectral density function

Relaxation can be defined as the irreversible evolution of a spin system toward a steady state, in most cases equilibrium. This evolution is produced by small fluctuations in the magnetic field induced by motions of the spin inside the lattice. In the case of biomolecules, relaxation is most often dominated by the fluctuations of strong internal interactions : dipolar interaction is the interaction of the spin with a second spin ; the chemical shift anisotropy describes interaction between the spin with the electronic cloud and finally the quadrupolar interaction describes the interaction of quadrupolar spin with the electric field produced by the electrons.

Most liquid-state NMR experiments are carried out in isotropic phases: for all conformations of a molecule, all orientations in the laboratory frame are equally probable. The transitions between these orientations are very fast: it takes a few picoseconds for water molecules to reorient in solution and
a few nanoseconds for proteins. First and foremost, this fast rotation leads to the averaging of all the above-mentioned orientation-dependent interactions. All the non-averaging parts contribute to the relaxation.

A quantitative description of the process presented above involves the introduction of two functions. First, the correlation function (C(t)) is used to describe the loss of memory of a system in time. Such correlation functions are represented in Figure 16a and b. (2) The Fourier transformation of the correlation function is the spectral density function, represented in Figure 16c and d. In this figure, several kinds of motion have been considered for the simulation of the correlation function and it spectral density for liquid- and solid-state experiments.

Slow fluctuations of the longitudinal magnetic field to transverse relaxation contribute to the value of the spectral density at zero frequency,  $\mathcal{J}(0)$ . Fast fluctuations of the transverse magnetic field contribute to the values  $\mathcal{J}(\omega_0)$ , where  $\omega_0$  is the Larmor frequency of the nucleus.



Figure 16 – Influence of the amplitudes and timescales of internal motions on the correlation functions C(t) (A and B) and spectral density functions  $\mathcal{J}(\omega_0)$  (C and D) found in solids (dashed lines) and liquids(solid line). (A and B) Influence of the amplitude of internal motions on the correlation function for (A) fast motions and (B) nanosecond motions with a correlation time similar to the one of overall tumbling (here the overall correlation time is  $\tau_c = 5$  ns). (C and D) Influence of the amplitude of internal motions on the spectral density function for (C) fast motions and (D) nanosecond motions with a correlation time similar to the one of overall tumbling. (C and D) The resonance frequencies of nitrogen-15,  $\omega_N$ , carbon-13,  $\omega_C$ , and proton,  $\omega_H$  are shown for magnetic fields of 9.4 T (400 MHz spectrometer) in green and 23.5 T (1 GHz spectrometer) in grey to illustrate which values of the spectral density function typically contribute to relaxation.

Spin relaxation processes are described completely with the relaxation matrix, which evaluates the evolution of all spin terms with respect to time. Diagonal elements represent the autorelaxation rates, whereas the non-diagonal elements are called cross relaxations. In the autorelaxation elements, two rates are particularly interesting : the relaxation of  $\hat{I}_z$  and  $\hat{I}_x$  corresponding respectively to the longitudinal (R<sub>1</sub>) and transverse (R<sub>2</sub>) relaxation rate of spin I. An evaluation of the contribution of the three main spin interactions to the R<sub>1</sub> and R<sub>2</sub> relaxation rates are displayed in Figure 17. In this figure, the relaxation rates of NH, carbonyl and methyl groups are considered for two correlation times ( $\tau_c = 4$  ns and 10 ns). Importantly, we can see that the relaxation of NH in protein back-bones can be extremely high in a low magnetic field. Additionally, we can see one limitation to work at ultra high field magnet by looking at the evolution of the relaxation rate above 10 T, inducing a tremendous relaxation.



- Total relaxation rate - CSA relaxation contribution - Dipolar relaxation contribution - Quadrupolar relaxation contribution

Figure 17 – Relaxation rates as a function of the magnetic field in a few representative groups: (A,E) the backbone amide <sup>15</sup>N; (B,F) backbone carbonyl <sup>13</sup>C; (C,G) methyl group <sup>13</sup>C; and (D,H) methyl group <sup>2</sup>H. Longitudinal (A-D) and transverse (E-H) relaxation rates are shown. (B) An inset provides a better view of contributions to the longitudinal relaxation rate of the backbone carbonyl An inset provides a better view of contributions to the longitudinal relaxation rate of the backbone carbonyl <sup>13</sup>C at high magnetic fields. Total relaxations are displayed in red, whereas the CSA, dipolar and quadrupolar contributions are shown, respectively, in green, yellow, and blue. These calculations were carried out for typical rigid groups in proteins with an overall correlation time  $\tau_c = 4$  ns (10 ns) for the plain (dashed) line.

#### High-field measurements of relaxation

High-field relaxation parameters contain information about the overall rotational diffusion and internal dynamics of biomolecules. Typically, extraction of this information follows two approaches. In the first case, the form of the spectral density function is assumed and so the analysis is straight-

forward. The most popular frameworks to derive the parameters of local dynamics from a set of <sup>15</sup>N relaxation data for backbone motions (or <sup>2</sup>H relaxation rates in methyl groups) are the model-free and extended model-free approaches.<sup>69,70</sup> Many models exist for the form of the correlation function, and will be discussed in chapter I. In general a model selection step based on statistical significance is performed to choose the appropriate functional form of the spectral density function.<sup>71</sup> Note that complex models of motions<sup>70</sup> are more likely to be statistically significant if a large number of experimental data points are used. Relaxation rates measured at multiple magnetic fields are often required to use the extended model-free description. The second approach relies on a minimal set of assumptions on the functional form of the spectral density function and the underlying model.

The spectral density mapping method<sup>72</sup> is based on the inversion of the set of equations for  $R_1$ ,  $R_2$ and NOE rates expressed as a linear combination of  $\mathcal{J}(\omega)$  at 0,  $\omega_H$ ,  $\omega_N$ , and  $\omega_H \pm \omega_N$  frequencies in the relaxation theory.<sup>22</sup> This method is general, irrespective of the overall diffusion properties and applicable to folded domain, multi-domain or disordered proteins. Its reduced version based on a limited set of relaxation rates<sup>73</sup> is straightforward to implement. However, the values of the spectral density function at a few frequencies represent a dataset difficult to interpret. The first approach, which provides most often local order parameters, is usually preferred when applicable. It is similarly applied to carbon-13 or deuterium relaxation in methyl groups. High-field relaxation samples a limited range of spectral density functions (Figure 16). The philosophy of high-field relaxation approaches is to obtain data of high precision and accuracy so that slight variations due to small amplitude motions can still be quantified. However, approaches with extensive sampling of the spectral density function should be favoured when motions on several orders of magnitude of correlation times are to be characterized.

# Multiple-field NMR spectroscopy

Relaxometry is the measurement of relaxation rates over a broad range of magnetic fields. The most popular approach to relaxometry is called fast field-cycling relaxometry.<sup>74</sup> In this approach, the initial polarization is carried out at a first magnetic field  $B_{pol}$ , which is then switched rapidly (within milliseconds) to a relaxation field  $B_{rel}$  (Figure 18.A). Finally, the magnetic field is switched to the observation field  $B_{obs}$ . Such fast field changes are possible with the use of electromagnets. However, all fields are below 2 T, with the exception of some hybrid systems where magnetic fields up to 3 T can be reached. Clearly, such limited magnetic field and magnetic field homogeneity preclude the measurement of high-resolution spectra of proteins. In general, the overall signal intensity is recorded as a function of relaxation delay. The site-specific nature of the measurements is lost.

High-resolution relaxometry is a technique that allows the NMR user to probe many points on the spectral density function with a single spectrometer, without loses of resolution. The principle is to use the stray-field profile of a conventional NMR magnet to perform the relaxation of the molecule in another magnetic field (Figure 18).

In short, high-resolution relaxometry is possible if the observation is performed in the high-field mag-



Figure 18 – A) Principle of relaxometry. B) Principle of high-resolution relaxometry: the magnetic field is changed by moving the sample from the magnetic center of a commercial magnet to a chosen position of the stray-field. The experiment is similar to (A) but the polarization and observation fields are identical.

net of an NMR spectrometer. The broad range of low magnetic fields  $B_{\rm rel}$  for relaxation is effortlessly obtained as the stray-field of the superconducting magnet. Transition from  $B_{\rm rel}$  and  $B_{obs}$  is achieved by displacing the sample (or the probe containing the sample) to a chosen position of the stray-field (Figure 18.B).<sup>17</sup> Many sorts of shuttling have been used : mechanical shuttling<sup>17,75</sup> or a pneumatic<sup>18</sup> one. In any case, this motion is slower than the induction times of electromagnets. Thus transitions between high and low fields can take 30 to 100 ms, which limits the range of accessible relaxation rates. The observation of the signal is performed using a liquid-state NMR probe on a solution-state sample. As mentioned above, this limits the range of accessible correlation times to values below or near the correlation time for rotational diffusion of the entire protein. So far, high-resolution relaxometry has been applied to two proteins.<sup>18,76</sup> Backbone nitrogen-15 relaxation rates have been measured at several high fields and at low fields down to 0.5 T. In these two studies, motions on low-nanosecond timescales (about 0.8 and 2 ns) have been identified in small subdomains: a  $\beta$ -hairpin of the SARS nucleocapsid protein<sup>76</sup> and the  $\beta_1$ – $\beta_2$  turn of human ubiquitin.<sup>18</sup>

# Labelling and choice of the biological system

Many proteins of interest have a molecular weight higher than 50 kDa, and liquid-state NMR is often complicated due to the slow global tumbling, and the quantity of <sup>1</sup>H-<sup>1</sup>H dipolar coupling in a macromolecule. In order to reduce the broadening of the NMR signal, one technique proposed is to "dilute" the number of spins in the proteins. For that, tremendous progress has been made in biological techniques to replace <sup>1</sup>H with <sup>2</sup>H. These techniques can be used to study backbones or protein side-chains independently.

**Study of protein backbones:** Multiple NMR studies of protein structure and dynamics have been performed on backbones,<sup>73,77–79</sup> looking at <sup>15</sup>N relaxation.

The study of high-molecular weight protein backbones can be studied by two techniques, starting from a growth in  $D_2O$ . First, NH groups can be re-protonated. However, in the case that a protein with an hydrophobic core is well defined, this procedure can be complex, with the need of unfolding

and refolding the protein to protonate all amines of the backbones.<sup>80,81</sup> It is also possible to label  $C_{\alpha}$  position with high cost and conserving a limitation on the size of the protein.<sup>82</sup>

**Study of protein side-chains:** Protein side-chain dynamics is one of the major components of molecular recognition.<sup>4-6</sup> Thus, it is important to know about protein side-chain dynamics. Stereo-array Isotope Labelling (SAIL) can be used to dilute spin by a factor two and study protein.<sup>83,84</sup> It is also possible to mark aromatic amino acids.<sup>85,86</sup>

Methyl groups present many advantages for their study in liquid-state NMR:

- They are well spread in different amino-acid, therefore in the entire protein.
- They can be labelled specifically and independently with a perfect control of metabolic pathways.
- Their relaxation time is reduced by the fast methyl rotation and high flexibility, due to the location at the end of side-chains averaging dipolar interactions.<sup>52, 87</sup>
- It is possible to combine several amino acids' specific labelling to obtain information on protein dynamics in many residues.<sup>6</sup>

Methyl groups have already been used to determine large protein structure<sup>54</sup> and dynamics.<sup>88</sup> However, dynamics of geometry of methyl groups are more complex than the one of NH groups studied for the backbones. The full description of the dynamics require many points on the spectral density function. Thus, there is a huge advantage to using high-resolution relaxometry.

# Thesis outline

Chapter 1 describes the theoretical frame to understand NMR relaxation. Starting from the fundamentals of Redfield theory, I will introduce interactions that contribute to spin relaxation. Then I will describe a program called REDKITE that uses this theory to determine relaxation matrices. The main results obtained for a specifically labelled methyl group with  ${}^{13}C^2H_2^1H$  will be outlined. Finally, I will discuss the form of spectral density function.

In the second chapter, I will use the output of REDKITE in order to determine protein side-chain dynamics of a model protein: Ubiquitin selectively labelled on methyl groups. This chapter also includes the protocol to express and purify proteins in D<sub>2</sub>O with a specific labelling on isoleucine methyl groups  $\delta_1$ -{ ${}^{13}C^2H_2^1H$ }.

Chapter 3 will describe advances made on the two-field system. After a specific description of the hardware that constitutes the two-field system, I will describe the basic NMR experiment performed to test the system. It includes relaxation experiments on small molecules ; HSQC experiment at 0.33 T using the polarization of 14.1 T, and 2F-HSQC that correlates the coherences of proton at high field

with the one of carbon-13 at low field. Using the advantage of the two-field pulses, a 2F-TOCSY has been measured. Finally I will describe the use of zero quantum coherences to obtain a high-resolution two-field experiment.

# Nuclear spin relaxation: the example of isotopically-labelled methyl groups

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# 1.1 Introduction

In many cases, NMR experiments start from a state close to equilibrium, for which the population of nuclear spin states follow a Boltzmann distribution. This equilibrium is generally perturbed, for instance, by the creation of coherences or by an inversion of polarisations (differences in the populations of spin states). The return to thermal equilibrium is called relaxation. Relaxation processes are induced by interactions within the spin system or between the spin system and its environment, called lattice. Those spin-lattice and spin-spin interactions fluctuate with time, inducing relaxation with a timescale of  $10^{-5}$  to  $10^{5}$  seconds, a long period of time by nuclear physicists' standards.

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Because relaxation is induced by interactions in the spin system, knowledge of relaxation rates gives access to structural information about distances between particles (through dipolar relaxation), relative orientation (through so called cross-correlated relaxation rates that are dependent of the relative orientations, see below), or the nature of molecular orbitals (through the chemical shift tensor, for instance). Relaxation is due to the fluctuations in time of these interactions so that, when structural parameters are known, offers a unique view on molecular motions. In this chapter, the Bloch-Redfield-Wangness (BRW)<sup>89,90</sup> theory will be introduced in order to get an accurate description of spin relaxation processes. This theory explains in an elegant manner how each interaction contributes to relaxation processes.

However this second-order time-dependent perturbation theory can become very complex as the dimension of matrices increases exponentially and the number of interactions to consider usually increases in a quadratic manner with the number of nuclear spins. This theory becomes even more complex if the asymmetry of motions is taken into account.<sup>91</sup>

In high-resolution relaxometry experiments, the spin system cannot be manipulated when the sample is out of the probe, during the transfers to and from the low-field position and during relaxation. Therefore, a clean spin term generated in the probe for relaxation will give rise, through cross-relaxation processes, to other spin terms. This will have the effect to alter the relaxation behaviour during the relaxation delay so that the relaxation decays measured will be different from a simple and desired exponential decay with a well-defined auto-relaxation rate.

In order to take in account this "polarisation leakage", a program called ICARUS<sup>18</sup> has been proposed to correct relaxation rates by simulating the evolution of the spin system during the experiment to predict the deviation of the measured rate from the true auto-relaxation rate. To do so, one needs to be able to predict the relaxation matrix. The BRW theory was used to develop a program (called REDKITE) to compute relaxation matrices for various spin systems.

The aim of this chapter is to define a theoretical framework for the computation of relaxation matrices in isotropic liquids, and for various spin systems. The general case of BRW theory will be presented, from the Liouville-von Neumann equation, to the description of the evolution of the density matrix as the product of the spectral density function and the double commutator of an irreducible tensor.<sup>92–94</sup>

Even though we intend to compute relaxation rates over a broad range of magnetic fields, we will stay in the range of magnetic fields where the Zeeman interaction is dominant. Thus, in order to compute the relaxation rates for a given spin system in the laboratory frame, we will transform the spin system in the interaction frame of the Zeeman interaction (called here "Zeeman frame"). This subsection includes the expressions of various spin terms in a Cartesian coordinate system, as well as the description of the Hamiltonian operators for relevant interactions: the dipole-dipole, the chemical shift anisotropy and the quadrupolar interactions.

This theory was implemented in REDKITE, and results obtained for the  $\{^{13}C^2H_2{}^{1}H\}$  spin system in a methyl group will be presented and discussed. Considering the size of the relaxation matrix of such a system, a reduced matrix was considered for the implementation of ICARUS.

Finally, I will also introduce models of spectral density functions, used to characterize dynamics of proteins, which will be useful for the interpretation of relaxation rates obtained with high-resolution relaxometry, corrected by a version of ICARUS adapted to methyl groups.

# 1.2 Fundamentals of BWR semi-classical relaxation theory

This description of the BRW relaxation theory below is based on many reviews<sup>95,96</sup> and books.<sup>7,93,94</sup> Firstly, the master equation governing spin relaxation will be established. Then this equation will be expressed, separating the spatial and spin parts.

#### 1.2.1 The Master Equation

**Second order perturbation theory:** The Schrödinger equation applied to the density matrix  $\hat{\sigma}(t)$  yields the Liouville-von Neumann equation, which describes the time evolution of the density matrix:

$$\frac{\mathrm{d}\hat{\sigma}(t)}{\mathrm{d}t} = -i[\hat{\mathcal{H}}(t), \hat{\sigma}(t)]. \tag{1.1}$$

The Hamiltonian of the system can be expressed as the sum of a stationary and a variable part:

$$\hat{\mathcal{H}}(t) = \hat{\mathcal{H}}_0 + \hat{\mathcal{H}}_1(t) \tag{1.2}$$

with  $\hat{\mathcal{H}}_0$  the time-independent part (in general, the Zeeman Hamiltonian) and  $\hat{\mathcal{H}}_1(t)$ , the timedependent part. This equation can be solved using a second-order time-dependent perturbation theory: the first step is to take into account the major time dependence *via* a transformation to the interaction frame of the Zeeman interaction. The tilde notation is introduced to distinguish the operator transformed in the Zeeman frame. Then:

$$\tilde{\hat{\sigma}}(t) = \exp\left(i\hat{\mathcal{H}}_0 t\right)\hat{\sigma}(t)\exp\left(-i\hat{\mathcal{H}}_0 t\right).$$
(1.3)

Looking at the density operator in the Zeeman frame, and considering the variable part of the Hamiltonian in the tilted frame  $\hat{\mathcal{H}}_1(t)$ , the Liouville-von Neumann equation can be written as:

$$\frac{\mathrm{d}\hat{\sigma}(t)}{\mathrm{d}t} = i[\tilde{\hat{\sigma}}(t), \tilde{\hat{\mathcal{H}}}_1(t)]. \tag{1.4}$$

Then, after integration:

$$\tilde{\hat{\sigma}}(t) = \tilde{\hat{\sigma}}(0) + i \int_{0}^{t} [\tilde{\hat{\sigma}}(t'), \tilde{\hat{\mathcal{H}}}_{1}(t')] \mathrm{d}t'.$$
(1.5)

Equation 1.5 can be used inside equation 1.4 and after integration:

$$\tilde{\hat{\sigma}}(t) = \tilde{\hat{\sigma}}(0) + i \int_{0}^{t} \left[ \left( \tilde{\hat{\sigma}}(0) + i \int_{0}^{t'} [\tilde{\hat{\sigma}}(t''), \tilde{\hat{\mathcal{H}}}_{1}(t'')] dt'' \right), \tilde{\hat{\mathcal{H}}}_{1}(t') \right] dt'$$
(1.6)

which can also be written as:

$$\tilde{\hat{\sigma}}(t) = \tilde{\hat{\sigma}}(0) + i \int_{0}^{t} \left[ \tilde{\hat{\sigma}}(0), \tilde{\hat{\mathcal{H}}}_{1}(t') \right] dt' + i^{2} \int_{0}^{t} \int_{0}^{t} \left[ [\tilde{\hat{\sigma}}(t''), \tilde{\hat{\mathcal{H}}}_{1}(t'')], \tilde{\hat{\mathcal{H}}}_{1}(t') \right] dt'' dt'.$$
(1.7)

Differentiating equation 1.7 yields:

$$\frac{\mathrm{d}\tilde{\hat{\sigma}}(t)}{\mathrm{d}t} = +i\left[\tilde{\hat{\sigma}}(0),\tilde{\hat{\mathcal{H}}}_{1}(t)\right] - \int_{0}^{t} \left[ [\tilde{\hat{\sigma}}(t'),\tilde{\hat{\mathcal{H}}}_{1}(t')],\tilde{\hat{\mathcal{H}}}_{1}(t) \right] \mathrm{d}t'.$$
(1.8)

By substituting the variable in the integral on the right-hand side of this equation, and considering that  $\tilde{\hat{\sigma}}(t')$  do not evolve a lot:

$$\frac{\mathrm{d}\hat{\tilde{\sigma}}(t)}{\mathrm{d}t} = +i\left[\hat{\tilde{\sigma}}(0), \hat{\tilde{\mathcal{H}}}_{1}(t)\right] - \int_{0}^{t} \left[ [\tilde{\tilde{\sigma}}(t'), \tilde{\hat{\mathcal{H}}}_{1}(t+\tau)], \tilde{\hat{\mathcal{H}}}_{1}(t) \right] \mathrm{d}\tau$$
(1.9)

**Approximations:** Equation 1.9 is exact, but it is necessary to perform some physical approximations in order to solve it:

- We take an ensemble average of all terms, denoted by the horizontal bar. The reason is that remote parts of a large system relax independently from each other. Since all spins have the same  $\tilde{\sigma}(0)$  and  $\tilde{\mathcal{H}}_1(t)$  has a vanishing average, the first term on equation 1.9 is equal to zero.
- The evolution of the spin system is negligible during the delay t because  $\|\hat{\mathcal{H}}_1(t)t_1\| \ll 1$
- There is no correlation between  $\tilde{\hat{\sigma}}(0)$  and  $\tilde{\hat{\mathcal{H}}}_1(t)$ .
- With the hypothesis  $t \gg \tau_c$ , the upper limit in the integral can be replaced by  $\infty$ .

Under these hypotheses, the Master equation can be rewritten as:

$$\frac{\overline{d\tilde{\sigma}(t)}}{dt} = -\int_{0}^{\infty} \overline{\left[\tilde{\hat{\mathcal{H}}}_{1}(t), [\tilde{\hat{\mathcal{H}}}_{1}(t+\tau), \tilde{\hat{\sigma}}(t)]\right]} d\tau$$
(1.10)

For the sake of simplicity, from this point on, the density operator and its matrix elements will refer to the average density operator.

#### 1.2.2 Irreducible tensor representation

The Master equation in this form is difficult to manipulate. The Hamiltonian  $\hat{\mathcal{H}}_1(t)$  can be written as the sum of products in which the spin operator and spatial parts are separated, to make the computation of the relaxation matrix more convenient. This set requires to change the basis of operators for the

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Hamiltonian. The perturbation Hamiltonian  $\hat{\mathcal{H}}_1(t)$  can be expressed with a set of irreducible tensors:

$$\hat{\mathcal{H}}_{1}(t) = \zeta \sum_{q=-l}^{l} (-1)^{q} V_{l,q}(t) \hat{T}_{l,-q}$$
(1.11)

where l is the rank of the tensor and  $\hat{T}_{l,-q}$  are rank-l irreducible tensor operators that depend only on the spin operators. The strength of the interaction is described by  $\zeta$ , and the spatial variables, which are time dependent, are described by the function  $V_{l,q}(t)$ .

The main advantage of working with irreducible tensor operators is their convenient evolution under rotation operations. Euler transformations are defined by three angles ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) that respectively describe:

- one rotation around the z axis  $(\hat{R}(\alpha))$
- a second rotation around the new y axis  $(\hat{R}(\beta))$
- a third rotation around the new z axis  $(\hat{R}(\gamma))$

A schematic representation of such a decomposition is shown in figure 1.1.



Figure 1.1 - Schematic representation of Euler rotations.

The Euler rotation can be represented by the operator  $\hat{R}(\alpha, \beta, \gamma)$  written as:

$$\hat{\hat{R}}(\alpha,\beta,\gamma)\hat{T}_{l,q} = \sum_{p=-l}^{l} \hat{T}_{l,p}\hat{\mathcal{D}}_{p,q}^{l}(\alpha,\beta,\gamma)$$
(1.12)

where  $\mathcal{D}_{p,q}^{l}(\alpha, \beta, \gamma)$  is the element of the order-l Wigner rotation matrix and l is the rank of the tensor At this point, the perturbation Hamiltonian  $\tilde{\mathcal{H}}_{1}(t)$  can be expressed as:

$$\hat{\mathcal{H}}_{1}(t) = \exp(i\hat{\mathcal{H}}_{0}t)\hat{\mathcal{H}}_{1}(t)\exp(-i\hat{\mathcal{H}}_{0}t)$$
$$= \zeta \sum_{q=-l}^{l} (-1)^{q} V_{l,q}(t)\exp(i\hat{\mathcal{H}}_{0}t)\hat{T}_{l,-q}\exp(-i\hat{\mathcal{H}}_{0}t).$$
(1.13)

Moreover it can be shown that:

$$\exp(i\hat{\mathcal{H}}_0 t)\hat{T}_{l,-q}\exp(-i\hat{\mathcal{H}}_0 t) = \sum_p \hat{A}_p^{(-q)}\exp(i\omega_p^{(-q)}t)$$
(1.14)

where  $(\hat{A}_p^{(-q)})$  is the set of irreducible tensors in the interaction basis and the frequencies  $\omega_p^{(-q)}$  are eigenvalues of the Hamiltonian  $\mathcal{H}_0$ . The following property is noticeably important:

$$\tilde{\hat{\mathcal{H}}}_{1}(t) = \zeta \sum_{q=-l}^{l} (-1)^{q} V_{l,q}(t) \hat{T}_{l,-q}$$
(1.15)

$$= \zeta \sum_{q=-l}^{l} (-1)^{q} V_{l,q}^{*}(t) \hat{T}_{l,-q}^{\dagger}$$
(1.16)

where the symbol (†) denotes the *hermitian conjugate* of the operator and (\*) denotes its complex conjugate.

This equation can be included in the Master equation 1.10 to obtain equation 1.17. The timeindependent part of this equation has been removed from the integral to obtain:

$$\frac{\mathrm{d}\tilde{\hat{\sigma}}(t)}{\mathrm{d}t} = \sum_{p,p',q,q'} (-1)^{q+q'} \exp\left(i(\omega_p^{(-q)} + \omega_{p'}^{(-q')})t\right) \left[\hat{A}_p^{(-q)}, [\hat{A}_{p'}^{\dagger(-q')}, \tilde{\hat{\sigma}}(t)]\right] \\
\times \zeta^2 \int_0^\infty \langle V_{l,q}(t) V_{l,q'}^*(t+\tau) \rangle \exp(i\omega_{p'}^{(-q')}\tau)\mathrm{d}\tau.$$
(1.17)

Finally, all non-secular terms, *i.e.* terms with an oscillating factor  $\exp\left(i(\omega_p^{(-q)} + \omega_{p'}^{(-q')})t\right)$ , are averaged to zero on a timescale much shorter than relaxation and can therefore be neglected. Only secular terms with  $\omega_p^{(-q)} + \omega_{p'}^{(-q')} = 0$  are kept,<sup>94</sup> yielding:

$$\frac{\mathrm{d}\tilde{\sigma}(t)}{\mathrm{d}t} = \sum_{p,q} \left[ \hat{A}_p^{(-q)}, \left[ \hat{A}_p^{\dagger(-q)}, \tilde{\sigma}(t) \right] \right] \\
\times \zeta^2 \int_0^\infty \langle V_{l,q}(t) V_{l,q}^*(t+\tau) \rangle \exp(i\omega_p^{(-q)}\tau) \mathrm{d}\tau.$$
(1.18)

#### 1.2.3 Correlation function and spectral density function

The term  $\langle V_{l,q}(t)V_{l,q'}^*(t+\tau) \rangle$  in equation 1.18 depends on motions that change the orientation of the principal axis of the Hamiltonian. It is relevant to introduce the correlation function  $C(\tau)$ . This function can be seen as the loss of memory of orientation of the system during the time  $\tau$ .

$$\mathcal{C}_{l,q,q'}(\tau) = \langle V_{l,q}(t) V_{l,q'}^*(t+\tau) \rangle$$
(1.19)

$$\mathcal{C}_{l,q,q'}(\tau) = \delta_{q,q'} \mathcal{C}(\tau) \tag{1.20}$$

In this equation  $\delta_{q,q'}$  is the Kronecker delta. Therefore, equation 1.18 can be written as:

$$\overline{\frac{\mathrm{d}\tilde{\sigma}(t)}{\mathrm{d}t}} = \sum_{p,q} \left[ \hat{A}_p^{(-q)}, [\hat{A}_p^{\dagger(-q)}, \tilde{\sigma}(t)] \right] \times \zeta^2 \int_0^\infty \delta_{q,q} \mathcal{C}_l(\tau) \exp(i\omega_p^{(-q)}\tau) \mathrm{d}\tau$$
(1.21)

$$=\sum_{p,q} \left[\hat{A}_p^{(-q)}, [\hat{A}_p^{\dagger(-q)}, \tilde{\hat{\sigma}}(t)]\right] \times \zeta^2 \int_0^\infty \mathcal{C}_l(\tau) \exp(i\omega_p^{(-q)}\tau) \mathrm{d}\tau$$
(1.22)

Using the Fourier transform of the correlation function, called the spectral density function ( $\mathcal{J}(\omega)$ ), we can obtain the final form of the Master equation:

$$\overline{\frac{\mathrm{d}\tilde{\hat{\sigma}}(t)}{\mathrm{d}t}} = -\frac{1}{2}\zeta^2 \sum_{q,p} \mathcal{J}\left(-\omega_p^{(-q)}\right) \left[\hat{A}_p^{(-q)}, [\hat{A}_{p'}^{\dagger(-q')}, \tilde{\hat{\sigma}}(t)]\right]$$
(1.23)

The index l has been removed from C(t) and  $\mathcal{J}(\omega)$  because we will only consider tensors of rank 2. The next step is to describe interactions in the irreducible tensor basis in order to determine  $\zeta$  and  $\hat{A}_p^{(-q)}$ .

It is important to notice that the previous description does not take in account the finite temperature condition, and the density operator evolves towards zero. To include this,  $\hat{\sigma}(t)$  have to be replaced in equation 1.10 by  $\hat{\sigma}(t) - \hat{\sigma}_{eq}$ .



# **1.3** Description of the basis and interaction

Figure 1.2 – Schematic representation of the main interactions contributing to relaxation in biomolecules. (a) Dipole-dipole interaction. (b) Chemical shift anisotropy, in this case with the main component pointing in the z direction (c) Quadrupolar coupling.

In order to ease the interpretation of the results, REDKITE was written using the Cartesian coordinate system. Description of this basis can be found in the next subsection. The three main interactions that occur in liquid NMR will be described: the dipolar coupling, which is the interaction between two nuclear magnetic dipoles or a nuclear magnetic dipole and an electron magnetic dipole, the chemical shift anisotropy, which describes the shielding of the magnetic field by the surrounding electrons, and the quadrupolar coupling, which is the interaction between the nuclear quadrupole and the electric field gradient of the electrons (note that the latter only occurs if the nucleus has a non-zero quadrupole, if  $I \ge 1$ ). A graphical representation can be found in figure 1.2

The general irreducible rank-2 tensors that will be used to decompose all the Hamiltonians are:<sup>97</sup>

$$\hat{A}_{0}^{(2)}(\vec{U},\vec{V}) = \frac{1}{2\sqrt{6}} (4U_{z}V_{z} - U_{+}V_{-} - U_{-}V_{+})$$

$$= \frac{1}{\sqrt{6}} (2U_{z}V_{z} - U_{x}V_{x} - U_{y}V_{y})$$
(1.24)

$$\hat{A}_{\pm 1}^{(2)}(\vec{U},\vec{V}) = \mp \frac{1}{2} (U_{\pm} V_z + U_z V_{\pm}))$$
(1.25)

$$\hat{A}_{\pm 2}^{(2)} = \frac{1}{2} (V_{\pm} U_{\pm}) \tag{1.26}$$

#### **1.3.1** Pauli Matrices for spin with $|m_s| \ge 1$

The cartesian representation of all spin terms was built with the Kronecker product of the Pauli matrices and the results are given in 1.27 (for spin 1/2) and 1.28 (for spin 1). Note that each term

has an index that denotes its number used in REDKITE and is defined in table 1.1.

$$E_{1/2} = \begin{pmatrix} 1 & 0 \\ 0 & 1 \end{pmatrix}; I_z = \frac{1}{2} \begin{pmatrix} 1 & 0 \\ 0 & -1 \end{pmatrix}; I_+ = \begin{pmatrix} 0 & 1 \\ 0 & 0 \end{pmatrix}; I_- = \begin{pmatrix} 0 & 0 \\ 1 & 0 \end{pmatrix}$$
(1.27)

$$E_1 = \begin{pmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{pmatrix}; S_z = \begin{pmatrix} 1 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & -1 \end{pmatrix}; S_+ = \begin{pmatrix} 0 & \sqrt{2} & 0 \\ 0 & 0 & \sqrt{2} \\ 0 & 0 & 0 \end{pmatrix};$$

$$S_{-} = \begin{pmatrix} 0 & 0 & 0 \\ \sqrt{2} & 0 & 0 \\ 0 & \sqrt{2} & 0 \end{pmatrix}; 3S_{z}^{2} - 2E_{1} = \begin{pmatrix} 1 & 0 & 0 \\ 0 & -2 & 0 \\ 0 & 0 & 1 \end{pmatrix}; S_{+}S_{z} + S_{z}S_{+} = \begin{pmatrix} 0 & \sqrt{2} & 0 \\ 0 & 0 & \sqrt{2} \\ 0 & 0 & 0 \end{pmatrix};$$
(1.28)  
$$S_{-}S_{z} + S_{z}S_{-} = \begin{pmatrix} 0 & 0 & 0 \\ \sqrt{2} & 0 & 0 \\ 0 & -\sqrt{2} & 0 \end{pmatrix}; S_{+}S_{+} = \begin{pmatrix} 0 & 0 & 2 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \end{pmatrix}; S_{-}S_{-} = \begin{pmatrix} 0 & 0 & 0 \\ 0 & 0 & 0 \\ 2 & 0 & 0 \end{pmatrix}$$

Operator name and coherence order	Operator	<b>RedKite convention</b>
Identity (spin $1/2$ ) $\{0\}$	$E_{1/2}$	$I_0$
Longitudinal polarisation (spin $^{1/2}$ {0}	$I_z$	$I_1$
Coherence (spin $1/2$ ) {+1}	$I_+$	$I_2$
Coherence (spin $1/2$ ) $\{-1\}$	I_	$I_3$
Identity (spin 1) $\{0\}$	$E_1$	$S_0$
Longitudinal polarisation (spin 1) $\{0\}$	$S_z$	$S_1$
Coherence (spin 1) $\{0\}$	$3S_{z}^{2} - 2E_{1}$	$S_4$
Coherence (spin 1) $\{+1\}$	$S_+$	$S_2$
Coherence (spin 1) $\{-1\}$	S_	$S_3$
Coherence (spin 1) $\{+1\}$	$S_+S_z\!+\!S_zS_+$	$S_5$
Coherence (spin 1) $\{+1\}$	$S_{-}S_{z}{+}S_{z}S_{-}$	$S_6$
Coherence (spin 1) $\{+2\}$	$S_+S_+$	$S_7$
Coherence (spin 1) $\{-2\}$	$S_{+}S_{+}, S_{-}S_{-}$	$S_8$

Table 1.1 – Basis used for the computation of relaxation matrices for spin 1/2 and 1.

#### 1.3.2 Dipolar interactions

The local magnetic field produced by the magnetic moment of a nuclear spin influences the nearest neighbour spins. This interaction, called dipolar coupling, has a spatial range of several angstroms. The dipolar coupling Hamiltonian between spin I and spin S can be described with equation 1.29:

$$\hat{\mathcal{H}}_{DD}^{I,S} = -\frac{\mu_0}{4\pi} \frac{\hbar \gamma_I \gamma_S}{r^3} \left[ 3 \left( \vec{I} \cdot \frac{\vec{r}}{r} \right) \left( \vec{S} \cdot \frac{\vec{r}}{r} \right) - \vec{I} \cdot \vec{S} \right]$$
(1.29)

 $\vec{r}$  is the internuclear vector and  $\gamma_i$  the gyromagnetic ratio of spin *i* (I or S in this case). In the laboratory frame, the dipolar Hamiltonian can be written using the rank-2 spherical harmonics and rank-2 tensor notation:

$$\hat{\mathcal{H}}_{D,ij} = \delta_{ij} \sum_{k=-2}^{2} \sum_{m=-2}^{2} \mathcal{D}_{m,k}^{(2)}(t) \hat{A}_{m}^{(2)}$$
(1.30)

where:

$$\delta_{ij} = \frac{\mu_0 \gamma_i \gamma_j \hbar}{4\pi r_{ij}^3}.$$
(1.31)

Tensors  $\hat{A}_m^{(2)}$  are described in table 1.2, using the equations 1.24 to 1.26 for two interacting spins I and S.

Operator	Value
$\hat{A}_{0}^{(2)}$	$-(1/\sqrt{6})[(2I_zS_z - (1/2)(I_+S+IS_+)]$
$\hat{A}_{1}^{(2)}$	$-(1/2)(I_+S_z+I_zS_+)$
$\hat{A}_{-1}^{(2)}$	$+(1/2)(I_{-}S_{z}+I_{z}S_{-})$
$\hat{A}_{2}^{(2)}$	$+(1/2)(I_+I_+)$
$\hat{A}_{-2}^{(2)}$	$+(1/2)(I_{-}I_{-})$

Table 1.2 – Rank-2 tensor components of the Hamiltonian for dipole-dipole interaction of spin I and S.

#### 1.3.3 Chemical Shift Anisotropy

Inside the static magnetic field  $B_0$ , the electrons of a molecule behave somehow as a conducting coil. Therefore, the nucleus is slightly shielded from the external magnetic field by the effect of the electrons. This well-known effect is called chemical shift (CS). The chemical shift Hamiltonian for spin I can be written as follows:

$$\hat{\mathcal{H}}_{CS,I} = \gamma \mathbf{\Omega} \vec{B}_0 \hat{I} \tag{1.32}$$

where  $\Omega$  is the chemical shielding tensor of spin I and  $\vec{B}_0$  is the external magnetic field. The chemical shielding tensor may be represented as the sum of an isotropic part and an anisotropic part. The corresponding CS Hamiltonian can similarly be represented as the sum of an isotropic (CSI) and an anisotropic (CSA) parts:  $\hat{\mathcal{H}}_{CS,I} = \hat{\mathcal{H}}_{CSI,I} + \hat{\mathcal{H}}_{CSA,I}$ . The isotropic part  $\hat{\mathcal{H}}_{CSA,I}$  is orientation-independent. Thus reorientational motions do not lead to fluctuations of the CSI, which does not contribute to relaxation processes and can be treated as part of the Zeeman interaction.

In the case of an isotropic liquid, the time-average value of the CSA is  $\langle \hat{\mathcal{H}}_{CSA,I} \rangle_t = 0$ . We will now treat the effect of the CSA on relaxation. The CSA Hamiltonian  $\hat{\mathcal{H}}_{CSA,I}$  has a simple expression in the principal axis system (PAS) of the chemical shift tensor in the Cartesian basis (Equation 1.33), and in the irreducible spherical components (Equation 1.36).

$$\Omega = \begin{pmatrix} \sigma_{xx}^{PAS} - \sigma_{iso} & 0 & 0\\ 0 & \sigma_{yy}^{PAS} - \sigma_{iso} & 0\\ 0 & 0 & \sigma_{zz}^{PAS} - \sigma_{iso} \end{pmatrix} = \frac{2\Delta\sigma}{3} \begin{pmatrix} -\frac{1-\eta}{2} & 0 & 0\\ 0 & -\frac{1+\eta}{2} & 0\\ 0 & 0 & 1 \end{pmatrix}$$
(1.33)

where  $\sigma_{iso}$  is the isotropic part of chemical shift tensor and

$$\frac{2(\Delta\sigma)}{3} = 2\frac{\sigma_{zz}^{PAS} - (\sigma_{xx}^{PAS} + \sigma_{yy}^{PAS})/2}{3}$$
(1.34)

and:

$$\eta = 3(\sigma_{xx}^{PAS} - \sigma_{yy}^{PAS})/(\Delta\sigma 2)$$
(1.35)

Irreducible spherical components are in the PAS, equal to

$$\sigma_{2,0}^{PAS} = \sqrt{\frac{2}{3}} \Delta \sigma$$

$$\sigma_{2,\pm 1}^{PAS} = 0$$

$$\sigma_{2,\pm 2}^{PAS} = \frac{1}{2} (\sigma_{xx}^{PAS} - \sigma_{yy}^{PAS})$$
(1.36)

We transform  $\hat{\mathcal{H}}_{CSA,I}$  into the laboratory frame

$$\hat{\mathcal{H}}_{CSA,I} = \zeta_{CSA,I} \sum_{k=-2}^{2} \sum_{m=-2}^{2} (-1)^{k} \mathcal{D}_{m,k}^{(2)}(t) \sigma_{2,m}^{PAS} \hat{A}_{-k}^{(2)}.$$
(1.37)

In this equation  $\zeta_{CSA,I}$  is the strength of the

$$\zeta_{CSA,I} = \frac{\Delta \sigma^2 \omega_I^2}{3} \tag{1.38}$$

and the Wigner matrices  $\mathcal{D}_{m,k}^{(2)}(t)$  are used to transform the irreducible tensor from the PAS to the laboratory frame. The elements of the shielding tensor  $\sigma_{2,m}^{PAS}$  are used. Finally,  $\hat{A}_{-k}^{(2)}$  are rank-2 tensors for chemical shift anisotropy interaction with respect to B<sub>0</sub> (table 1.3).

This expression can be simplified by assuming that the shielding tensor is axially symmetric, *i.e.* in the PAS, the chemical shift tensor is diagonal, with  $\sigma_{xx}^{PAS} = \sigma_{yy}^{PAS}$ . Then the only nonzero element of the chemical shift tensor expressed as an irreducible tensor representation is  $\sigma_{2,0}^{PAS}$  and the equation is reduced to:

$$\hat{\mathcal{H}}_{CSA,I} = \zeta_{CSA,I} \sum_{k=-2}^{2} (-1)^2 \mathcal{D}_{0,k}^{(2)} \hat{A}_{-k}^{(2)}$$
(1.39)

Taking the strength of the magnetic field (B<sub>0</sub>) out of  $\hat{A}^{(2)}_{-k}$  and adding the scalar chemical shift aniso-

Operator	Value
$\hat{A}_{0}^{(2)}(\hat{I},\hat{B})$	$\frac{1}{\sqrt{6}}(2\hat{I}_z\hat{B}_z-\hat{I}_x\hat{B}_x-\hat{I}_y\hat{B}_y)$
$\hat{A}_{0}^{(2)}$	$(2B_0\sqrt{6})I_z$
$\hat{A}_{\pm 1}^{(2)}(\hat{I},\hat{B})$	$\pm \frac{1}{2} (\hat{\mathbf{I}}_{\pm} \hat{\mathbf{B}}_z + \hat{\mathbf{I}}_z \hat{\mathbf{B}}_{\pm})$
$\hat{A}_{\pm 1}^{(2)}$	$-(B_0/2)I_+$
$\hat{A}^{(2)}_{\pm 2}(\hat{I},\hat{B})$	$\pm \frac{1}{2}(\hat{I}_{\pm}\hat{B}_{\pm})$
$\hat{A}_{\pm 2}^{(2)}$	0

Table 1.3 - Rank-2 tensor for chemical shift anisotropy interaction.

tropic, we obtain the following expression for  $\zeta_{CSA,I}$ :

$$\zeta_{CSA,I} = -\sqrt{\frac{2\pi}{15}} \gamma_I B_0 \Delta \sigma \tag{1.40}$$

where:

$$\Delta \sigma = \sigma_{zz}^{PAS} - \frac{\sigma_{xx}^{PAS} + \sigma_{yy}^{PAS}}{2} \tag{1.41}$$

#### 1.3.4 Quadrupolar interaction

In atoms with a spin number larger than 1/2, nuclei have a nuclear quadrupolar moment. Despite the fact that these nuclei represent more than 70 % of NMR active isotopes, they are not often studied in solution-state NMR because their quadrupolar moment interacts with the electric field gradient created by the electrons. This quadrupolar moment also induces relaxation and extensive peak broadening. The general form of the quadrupolar Hamiltonian is:

$$\hat{\mathcal{H}}_Q = \frac{eQ}{2m_s^I (2m_s^I - 1)} \vec{I} \mathbf{V} \vec{I}$$
(1.42)

where  $m_s^I$  is the quantum spin number of spin *I*, *Q* is the electric quadrupole moment, and **V** is the electric field gradient tensor. The Hamiltonian can be represented in the PAS of the electric field gradient *via* using the irreducible tensor representation:

$$\hat{\mathcal{H}}_Q = \frac{eQ}{2m_s^I(2m_s^I - 1)} \sum_{q=-2}^2 (-1)^q V_q^{(2)} \hat{T}_{-q}^{(2)}.$$
(1.43)

If we apply the rotation from the PAS frame to the laboratory frame:

$$\hat{\mathcal{H}}_Q = \frac{eQ}{2m_s^I(2m_s^I - 1)} \sum_{q=-2}^2 \sum_{p=-2}^2 (-1)^q \mathcal{D}_{p,q}^{(2)} V_p^{(2),PAS} \hat{T}_{-q}^{(2)}.$$
(1.44)

The irreducible tensors for the quadrupolar interaction in this representation are shown in table 1.4, and could be interpreted as the interaction of the spin I with itself, tilted by the electric field gradient tensor.

Operator	Value
$\hat{A}_{0}^{(2)}(\hat{I},\hat{I})$	$\frac{1}{\sqrt{6}}(2I_zI_z - I_xI_x - I_yI_y)$
$\hat{A}^{(2)}_{\pm 1}(\hat{I},\hat{I})$	$\mp \frac{1}{2}(\hat{I}_{\pm}\hat{I}_z + \hat{I}_z\hat{I}_{\pm})$
$\hat{A}^{(2)}_{\pm 2}(\hat{I},\hat{I})$	$\pm \frac{1}{2}(\hat{I}_{\pm}\hat{I}_{\pm})$

Table 1.4 - Rank-2 tensors for quadrupolar interaction.

The electric field can be described in terms of a component  $V_{zz}$  and its asymmetry  $\eta.$ 

$$V_{PAS}^{2,0} = \sqrt{6} V_{zz}; V_{PAS}^{2,\pm 1} = 0; V_{PAS}^{2,\pm 2} = \eta$$
(1.45)

From this point on, we consider that the quadrupolar coupling has a unique component along the z axis, without any anisotropy ( $\eta = 0$ ). This assumption may be incorrect for some systems.

# **1.4** Calculation of the relaxation matrix

In this section, the algorithm used to determine the relaxation matrix of a given spin system will be detailed with the formalism described previously. This formalism has already been used<sup>27</sup> for different systems, resulting in a MATHEMATICA<sup>98</sup> script that can be found on Ilya Kuprov's website.<sup>99</sup> Many cases are implemented: dipolar relaxation, chemical shift anisotropy or computation of CSA-CSA cross-correlation in a two-spin system. However, there is no case in which quadrupolar and dipolar interactions are considered jointly for the relaxation of a system of several nuclear spins. Moreover, this MATHEMATICA notebook does not take into account more than two spins and elements of the relaxation matrix have to be computed one by one.

In this context, I wanted to create a program with the following features:

- choice of the spin system, including up to six spins, which can be  ${}^{1}$ H,  ${}^{2}$ H, or  ${}^{13}$ C;
- computation of symbolic expressions for the elements of the relaxation matrix;
- computation of relaxation matrices with several forms of spectral density functions;
- including arbitrary orientations for the PAS of the interaction considered, in order to compute cross-correlated relaxation rates.

The program (REDKITE) was written for a MATHEMATICA environment, based on the original code previously mentioned,<sup>27</sup> and including the features mentioned above. The user can obtain individual relaxation rates, or a full relaxation matrix.

This program was used for the computation of the relaxation matrix in  $\{^{13}C^2H_2{}^1H\}$  methyl groups, and the results will be presented in the following subsection.

# **1.4.1** Relaxation rates of $\{{}^{13}C^2H_2{}^1H\}$ methyl groups

The interest for studying relaxation in methyl groups was discussed in the general introduction. In order to use high-resolution relaxometry, we decided to focus on a labelling scheme which provided slow enough relaxation of a carbon-13 longitudinal polarisation and weak cross-relaxation rates, in order to limit the deviations of measured relaxation rates to true relaxation rates , which led to methyl groups with the specific labelling  $\{^{13}C^2H_2^{1}H\}$ . The longitudinal relaxation rate for carbon-13 was expected to be small (for instance significantly smaller than the longitudinal relaxation rate for backbone nitrogen-15 nuclei). Indeed, the fast rotation of the methyl group around the CC bond reduces the contributions of the  $\{^{13}C^2H\}$  and  $\{^{13}C^{1}H\}$  dipolar couplings to relaxation. In addition, the low gyromagnetic ratio of deuterons ensures that two out of three dipolar interactions involving the carbon-13 nucleus are rather weak. To confirm this hypothesis, the longitudinal relaxation rate for carbon-13 was computed using REDKITE.

Because the principal axes of interactions do not necessarily have the same orientation in the molecular frame, such orientations must be considered. REDKITE was written to include angles in

the expressions of the spectral density function. In the example given below, the longitudinal and transverse relaxation rates are expressed with the spectral density function, taking in consideration the angle of the interaction with respect to the C-C bond. The spectral density function  $\mathcal{J}(\omega_J, \theta_{IS})$  will be expressed with  $\vec{I}$  (auto-relaxation) or  $\vec{I}, \vec{S}$  (cross-correlated relaxation), representing the relative orientation of those interactions. It is important to notice the differing notations for the spectral density function compared to equation 1.23:  $\omega_J$  and  $\theta_{IS}$  are, respectively, the frequency of spin J and the angle between the PAS of the interaction IS with respect to the C-C bond.

Taking the norm of the CSA Hamiltonian in equation 1.40 and the norm of the dipole-dipole interaction in equation 1.31, the longitudinal ( $R_1$ ) and transverse ( $R_2$ ) relaxation rates of the carbon in  ${^{13}C^2H_2^1H}$  methyl groups are:

$$R_{1} = \Delta_{C}^{2} (\omega_{C})^{2} \mathcal{J}(\omega_{C}, \theta_{CC}) + \frac{8}{12} \delta_{CD}^{2} (\mathcal{J}(\omega_{D} - \omega_{C}, \theta_{CD}) + 3\mathcal{J}(\omega_{C}, \theta_{CD}) + 6\mathcal{J}(\omega_{C} + \omega_{D}, \theta_{CD})) + \frac{1}{4} \delta_{CH}^{2} (\mathcal{J}(\omega_{H} - \omega_{C}, \theta_{CH}) + 3\mathcal{J}(\omega_{C}, \theta_{CH}) + 6\mathcal{J}(\omega_{C} + \omega_{H}, \theta_{CH}))$$

$$(1.46)$$

$$R_{2} = \frac{1}{6} \Delta_{C}^{2} (\omega_{C})^{2} (4\mathcal{J}(0,\theta_{CC}) + 3\mathcal{J}(\omega_{C},\theta_{CC})) + \frac{1}{3} \delta_{CD}^{2} (4\mathcal{J}(0,\theta_{CD}) + 3\mathcal{J}(\omega_{C},\theta_{CD}) + \mathcal{J}(\omega_{C} - \omega_{D},\theta_{CD}) + 6\mathcal{J}(\omega_{C} + \omega_{D},\theta_{CD})) + (1.47)$$

$$2 \frac{1}{4} \delta_{CH}^{2} (4\mathcal{J}(0,\theta_{CH}) + 3\mathcal{J}(\omega_{C},\theta_{CH}) + \mathcal{J}(\omega_{C} - \omega_{H},\theta_{CH}) + 6\mathcal{J}(\omega_{C} + \omega_{H},\theta_{CH}))$$

The longitudinal cross-relaxation rate determined by the NOE experiment<sup>51, 100, 101</sup> is taken to be equal to:

$$\sigma_{NOE} = \frac{\delta_{CH}}{2} (-\mathcal{J}(\omega_C - \omega_H, \theta_{CH}) + 6\mathcal{J}(\omega_C + \omega_H, \theta_{CH}))$$
(1.48)

We used:

$$r_{CH} = 1.115 \ 10^{-10} \text{ m}$$
  
 $r_{CD} = 1.110 \ 10^{-10} \text{ m}$   
 $\theta_{CD} = \theta_{CH} = 109.47 \frac{\pi}{180} \text{ rad}$   
 $\Delta_C = \frac{25}{\sqrt{3}} \ 10^{-6}$ 

The dimensions of the relaxation matrix for a  $\{{}^{13}C^2H_2{}^{1}H\}$  spin system is  $1296 \times 1296$ . In order

to reduce the computation time for ICARUS, we decided to neglect all terms that have a contribution lower than 5% of the auto-relaxation rate ( $C_z$ ) at all magnetic fields. Cross-relaxation rates for the  $C_z$  terms have been computed. Dependency of these rates with respect to the magnetic field is displayed on figure 1.3.



Figure 1.3 – Largest cross-relaxation rates for the longitudinal polarisation  $C_z$  in a  $\{^{13}C^2H_2^1H\}$  methyl group expressed as a percentage of the longitudinal relaxation rate  $R_1$  for carbon-13.

To perform this computation, a methyl-group spectral density function was used with typical geometric and dynamic parameters for a methyl group. The form of the spectral density function will be discussed in the next section. Only three relaxation rates have a value visible on the graph, and the cross-relaxation rate between  $C_z$  and  $D_z$  is less than 3 % of  $R_1$  of carbon-13 at all fields.

#### 1.4.2 Reduced matrix

In ICARUS, reduced relaxation matrices were computed only taking into consideration the terms  $\hat{C}_z$ ,  $\hat{H}_z$ ,  $\hat{C}_z\hat{H}_z\hat{D}_z^1$ ,  $\hat{C}_z\hat{H}_z\hat{D}_z^2$ . For example, the relaxation matrix was computed for 0.33 T and 14.1 T (Figure 1.4). The parameters used to compute the spectral density function are typical parameters for the methyl group at the  $\delta_1$  position of a rigid isoleucine residue.

In figure 1.4, it can be seen that the relaxation of  $\hat{C}_z$  is slow, and that the auto-relaxation rates of  $\hat{H}_z$ ,  $\hat{C}_z\hat{H}_z\hat{D}_z^1$ ,  $\hat{C}_z\hat{H}_z\hat{D}_z^2$  are high. Therefore, the correction that must be computed by ICARUS is expected to be smaller than the one computed previously for NH groups.<sup>102</sup>



Figure 1.4 – Relaxation matrices computed with typical parameters for  $^{13}\text{C}^2\text{H}_2{}^1\text{H}$  methyl group.  $\tau_c$ =5.11 ns,  $\tau_{\rm M}$ =4.5 ps,  $\tau_{\rm cc}$ =0.18 ns,  $S_{\rm cc}^2$ =0.61. The methyl group is assumed to have a perfect tetrahedral shape and the principal axis of the CSA tensor is collinear to the CC bond.

# **1.5** Forms of the spectral density function

So far, we have introduced important concepts about the relaxation of nuclear spins. We will now discuss further the nature of reorientational motions that give rise to relaxation *via* fluctuations of internal interactions.

Many types of motions occur in proteins. In this section, we will list the most important ones and present a few models used for the interpretation of relaxation rates in terms of motions. The dominant motion that occurs in a well-folded single domain protein is the rotational diffusion of the entire domain, which is usually referred to as the overall or global motion. The study of the overall motion gives information on the hydrodynamics of the protein. Loops and turns can wobble on short, nanosecond timescales that can be probed by relaxation measurements. The transition between sidechain rotamers is often allowed on short nanosecond and hundreds of picosecond timescales. The fastest motions are vibrations and librations of the protein's backbone and sidechain, which also lead to measurable contributions to the reorientation of groups of atoms both in the sidechains and the backbone of a protein. Such motions are variable in timescale and amplitude and depend on the local pattern of intramolecular interactions.

A core assumption of most analysis of relaxation in proteins is the decoupling of the overall motion and internal motions. Thus, the overall motion of a single-domain protein is described with the usual concepts of hydrodynamics for isotropic, axially symmetric or fully asymmetric rotational diffusion. In the case of isotropic rotational diffusion, the overall motion is characterized by a single correlation time  $\tau_c$ .



Figure 1.5 – Distribution of correlation times for a series of models of motions in proteins employed by NMR-based studies. In the (A) model-free (two correlation times) and extended model-free (three correlation times) approaches: the value and the relative weight of each correlation time can be fitted to experimental data. (B) A broad and continuous distribution of correlation times for local motions can be introduced. (C) SRLS approach for which local and overall motions with adjustable correlation times are coupled by a local potential. (D) A model-free reconstruction of the distribution of correlation times can be obtained by regularization, such as IMPACT: the values of the distribution of correlation times can be evaluated on a set of fixed positions, equally spaced on the logarithmic scale.

#### 1.5.1 Models for the understanding of protein dynamics

**Fractional Brownian Dynamics** Local motions can be described by physical models, such as diffusion in a cone<sup>103</sup> or anomalous diffusion in a rugged potential.<sup>104</sup> The latter is called Fractional Brownian Dynamics and makes use of the Mittag-Leffler function to describe the correlation function. This model has been applied to describe motions in methyl groups.<sup>105</sup>

**Model-free:** Other approaches voluntarily avoid the use of physical models of local motions and rely on effective parameters. The most popular approach for the interpretation of relaxation data in proteins falls in this category. The model-free approach<sup>69</sup> relies on two parameters: an order parameter  $S^2$ , which describes the amplitude of motions (full disorder for  $S^2 = 0$  and complete rigidity for  $S^2 = 1$ ) and an effective correlation time for these local motions. One should keep in mind that the "model-free" (MF) approach is indeed free of any precise physical model to describe the nature of the internal motion<sup>69, 106</sup> but not free of any model or hypothesis. It was further developed to include an additional motion in the "extended model-free" model,<sup>70</sup> or by taking the geometry of methyl groups<sup>32</sup> into account (Figure 1.5.A).

**Gaussian Axial Fluctuations:** Another similar approach is the Gaussian axial fluctuation (GAF)<sup>107</sup> model of internal motions, which was inspired by the analysis of motions in molecular dynamics simulations. The GAF model describes motions of the peptide plane in proteins with fluctuations of orientation angles around principal axes (the main axis is almost aligned with the  $C_{\alpha_{i,1}}-C_{\alpha_i}$  axis), with a Gaussian amplitude. This model assigns different amplitudes to motions around different axes but not different correlation times (or rotational diffusion coefficients) (Figure 1.5.B).

**SRLS:** The statistical independence of overall and local motions has been challenged in folded proteins, bringing significant differences in the quantitative description of loop and domain motions. The slowly relaxing local structure (SRLS) model describes local motions as diffusion in a potential imposed by the molecular environment.<sup>108</sup> Rotational diffusive motion of the local entity (peptide plane, loop or domain) and the overall macromolecule become coupled. Closer to *ab initio* computation, using the stochastic Liouville approach, no exponential form of the correlation function is assumed. This model is noticeably efficient when local motions are anisotropes<sup>109</sup> (Figure 1.5.C).

**IMPACT:** The general framework for the description of reorientational motions in proteins has to be rethought when there is no well-defined structure, as in intrinsically disordered proteins. In this case, the absence of rigid domains on the timescale of rotational diffusion prevents the definition of an overall diffusion tensor. So far, the analysis of relaxation rates in disordered proteins has focused on the description of local distributions of correlation times<sup>110–112</sup> (Figure 1.5.D).

**Conclusion:** A general description of these approaches can be found in figure 1.5. In the study of methyl groups, only the "model-free" was considered. The other methods are more difficult to imple-

ment and it is highly possible that motions in methyls groups are well-separated, allowing the use of an EMF approach.

#### 1.5.2 Model-Free model, applied on methyl groups

**The Model-Free Model:** In the classical form of the "model-free" approach, the correlation function is written as the product of the correlation functions of the isotropic rotation and of a faster motion:

$$\mathcal{C}^{MF}(t) = \mathcal{C}_0(t)\mathcal{C}_1(t) \tag{1.49}$$

where the isotropic rotational diffusion term is defined as:

$$\mathcal{C}_0(t) = e^{-t/\tau_c} \tag{1.50}$$

and the second motion term:

$$C_1(t) = S_f^2 + (1 - S_f^2)e^{-t/\tau_f}.$$
(1.51)

In these equations  $\tau_i$  is the correlation time of motion i,  $S_i^2$  is the generalized order parameter associated with restriction of motions. The Fourier transform  $\mathcal{J}(\omega)$  of this function is:

$$\mathcal{J}(\omega) = \frac{2}{5} \left( S_f^2 \frac{\tau_c}{1 + (\omega \tau_c)^2} + (1 - S_f^2) \frac{\tau_f^e}{1 + (\omega \tau_f^e)^2} \right)$$
(1.52)

In this equation,  $\tau_f^e$  is the effective correlation time, with:

$$\tau_f^{e-1} = \tau_f^{-1} + \tau_c^{-1} \tag{1.53}$$

**The Extended Model-Free Model:** The correlation function can be modified with the assumption that the local motion is too complex to be approximated by a mono-exponential decay. An empirical bi-exponential decay is used. This model has more parameters and needs a higher number of relaxation rates to be refined accurately. In this model, the correlation function is:

$$\mathcal{C}^{EMF}(t) = \mathcal{C}_0(t)\mathcal{C}_2(t) \tag{1.54}$$

where  $C_2(t)$  is a bi-exponential correlation function of the form:

$$C_2(t) = S^2 + (1 - S_f^2)e^{-t/\tau_f} + (S_f^2 - S)e^{-t/\tau_s}$$
(1.55)

with  $S^2 = S_f^2 S_s^2$ . The spectral density function resulting from this second correlation function in the case of an isotropic overall rotation is:

$$\mathcal{J}(\omega) = \frac{2}{5} \left( S^2 \frac{\tau_c}{1 + (\omega\tau_c)^2} + (1 - S_f^2) \frac{\tau_f^e}{1 + (\omega\tau_f^e)^2} + (S_f^2 - S^2) \frac{\tau_s^e}{1 + (\omega\tau_s^e)^2} \right)$$
(1.56)

**Methyl group model:** The correlation function to describe motions in methyl groups has to be modified to introduce the correlation function for the fast rotation of the methyl group around the CC axis. This correlation function depends on the angle between the principal axis of the interaction and the axis of rotation (the CC bond). This motion is assumed to be completely independent from the motions of the CC bond. The correlation function is therefore broken down as the product of a global tumbling term ( $C_0(t)$ ), a methyl rotation term ( $C_m(t)$ ) and term describing a motion of the CC bond with one or two correlation times (( $C_1(t)$ ) or ( $C_2(t)$ )).

It must be noted that the order parameter for the rotation of the methyl group depends on  $\theta_{\vec{i},\vec{j}}$ , which is the orientation of interactions defined by the vectors  $\vec{i}$  and  $\vec{j}$  with respect to the CC bond. In the case of cross-correlated correlation functions, this correlation function depends on the orientations of the principal axes of both interactions with the axis defined by the CC bond and between each other. The correlation function can be written as:

$$\mathcal{C}_{\text{Methyl}}^{MF}(t,\theta_{\vec{\imath},\vec{\jmath}}) = \mathcal{C}_0(t)\mathcal{C}_m(t,\theta_{\vec{\imath},\vec{\jmath}})\mathcal{C}_{1/2}(t)$$
(1.57)

where  $C_0(t)$ ,  $C_1(t)$  and  $C_2(t)$  are the correlation functions defined previously. The correlation function of the methyl group rotation is defined by  $C_m(t, \theta_{\vec{\imath}, \vec{\jmath}})$ :

$$C_m(t,\theta_{\vec{i},\vec{j}}) = S_m^2(\theta_{\vec{i},\vec{j}}) + (\mathcal{P}_2(\cos\theta_{i,j}) - S_m^2(\theta_{\vec{i},\vec{j}}))e^{-t/\tau_m}$$
(1.58)

In this equation,  $\tau_m$  is the correlation time for the rotation of the methyl group around the CC axis.  $\mathcal{P}_2(\cos \theta_{i,j})$  is the second-order Legendre polynomial, and  $\theta_{i,j}$  is the angle between vector  $\vec{i}$  and  $\vec{j}$ .  $S_m^2(\theta_{\vec{i},\vec{j}})$  is the order parameter of the methyl group rotation. If we assumed a tetrahedral geometry for the methyl group, the value of this order parameter can be calculated. In the Lipari-Szabo approach, the order parameter of a motion is the limit of the internal correlation function<sup>91</sup>:

$$S_{i,j}^{2} = \frac{4\pi}{5} \sum_{q} \langle \mathcal{D}_{2,q}(\theta_{i}^{MOF}(t)) \rangle \langle \mathcal{D}_{2,q}^{*}(\theta_{j}^{MOF}(t)) \rangle$$
(1.59)

where MOF is the molecular frame. Note that this equation is valid under the fast internal motion limit. In this case, the order parameter of methyl rotation is:

$$S_m^2(\theta_{\vec{\imath},\vec{j}}) = \mathcal{P}_2[\cos(\theta_{\vec{\imath},\vec{CC}})] \times \mathcal{P}_2[\cos(\theta_{\vec{\imath},\vec{CC}})]$$
(1.60)

where  $\theta_{\vec{i},\vec{CC}}$  is the angle between the CC bond of methyl groups and the interaction oriented along the vector  $\vec{i}$  (and  $\vec{j}$  for cross-correlated relaxation). In the case of auto-relaxation, using the properties of Legendre polynomial, we found that the order parameter for CH-oriented interaction is equal to 1/9, and the order parameter for CSA to 1. The five-parameter correlation function can be written as:

$$\mathcal{C}_{5}(t) = e^{t/\tau_{c}} \left( S^{2} + (1 - S_{f}^{2})e^{t/\tau_{f}} + (S_{f}^{2} - S_{s}^{2})e^{t/\tau_{s}} \right) \left( S_{m}^{2}(\theta) + (P_{2}(\cos\theta) - S_{m}^{2}(\theta)e^{t/\tau_{m}} \right)$$
(1.61)

with associated motions represented in Figure 1.6.



Figure 1.6 - Representation of the motions occurring in methyl groups.

**Forms of spectral density function:** For the rest of this study, we decided to perform a model selection based on the number of experimental data and the number of parameters that have to be fitted. Therefore, the extended model-free methyl (EMFM) correlation function (*i.e.*  $C(t) = C_0(t)C_m(t)C_2(t)$ ) is simplified with the following assumptions, for different choice of parameters:

- The first spectral density function is a five-parameter function called  $\mathcal{J}^{\mathrm{EMFM}}$ .
- The second spectral density function is a four-parameter function assuming  $\tau_f$  cannot be fitted, by simplification of  $\mathcal{J}^{\rm EMFM}$ .
- A three-parameter function is  $\mathcal{J}^{\text{MFM}} = \mathcal{C}_0(t)\mathcal{C}_m(t)\mathcal{C}_1(t)$
- A two-parameter function is assumed by simplification of  $\mathcal{J}^{MFM}$  assuming that  $\tau_{CC}$  is too fast.

The spectral density function used for the fitting of methyl relaxation will be described below, the angle dependencies of  $\mathcal{J}_i$  and  $S_m^2$  being omitted. The five parameter spectral density function is given by:

$$\mathcal{J}_{5}(\omega) = \frac{2}{5} \left( S_{m}^{2} S^{2} \frac{\tau_{c}}{1 + (\omega\tau_{c})^{2}} + S^{2} (P_{2}(\cos\theta) - S_{m}^{2}) \frac{\tau_{m}'}{1 + (\omega\tau_{m}')^{2}} + S_{m} (1 - S_{f}) \frac{\tau_{f}'}{1 + (\omega\tau_{f}')^{2}} + S_{m}^{2} S_{f}^{2} (1 - S_{s}^{2}) \frac{\tau_{s}'}{1 + (\omega\tau_{s}')^{2}} + (P_{2}(\cos\theta) - S_{m}^{2})(1 - S_{f}^{2}) \frac{\tau_{f,m}'}{1 + (\omega\tau_{f,m}')^{2}} + (P_{2}(\cos\theta) - S_{m}^{2})(1 - S_{s}^{2}) \frac{\tau_{s,m}'}{1 + (\omega\tau_{s,m}')^{2}} \right)$$

$$(1.62)$$

with  $S^2 = S_f^2 S_s^2$ ,  $\tau_i'^{-1} = \tau_i^{-1} + \tau_c^{-1}$  and  $\tau_{i,m}'^{-1} = \tau_i^{-1} + \tau_c^{-1} + \tau_m^{-1}$ .

# 1.6 Conclusion

In this chapter I introduced the basis of relaxation theory, and used it in a newly written program called RedKite. This program is user-friendly and can compute, for a system made of spin 1/2 and 1, any relaxation element of the relaxation matrix in a Cartesian product operator basis.

This program was used to compute cross-relaxation rates for the longitudinal polarisation  $\hat{C}_z$ , which are of high importance in high-resolution relaxometry, and a reduced relaxation matrix was used for inclusion in the program ICARUS. This program was adapted to MATHEMATICA, and to the study of relaxation processes in methyl groups.

Several models of spectral density function were explored. Starting from the model-free correlation function, we proposed a MF methyl and EMF methyl spectral density function for the analysis of high-resolution relaxometry experiments applied on methyl groups.

In the next chapter, a full description of high-resolution relaxometry experiments will be performed. Those experiments were used on a  $\{{}^{2}H^{12}C^{15}N\}$ -ubiquitin specifically labelled on isoleucine- $\delta_{1}$  methyl groups with  $\{{}^{13}C^{2}H_{2}{}^{1}H\}$ .

# Protein sidechain dynamics from high-resolution relaxometry

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# 2.1 Studies of protein sidechains motions

The study of protein structures is important for the understanding of biological mechanisms. Since the second half of the 20th century, scientists developed many methods to study those structures, using various techniques: mainly X-ray diffraction and NMR, joined more recently by cryo-electron microscopy. More recently, structural considerations have been combined with the characterization of motions for a better understanding of protein function. The understanding of biological processes at an atomic resolution requires the addition of time as a fourth dimension over a braod range of timescales, from picoseconds to seconds. Getting complete insight into the full range of physical parameters of motions requires the use of diverse experimental techniques, generally complemented by molecular dynamics (MD) simulation.

Many experimental approaches allow for a quantitative study of the dynamics of proteins, such as infrared spectroscopy.<sup>113</sup> Atomic resolution information about protein motions can be obtained by X-ray diffraction, particularly at room temperature, which can identify weakly populated states and

motions.<sup>114–116</sup> NMR is certainly a method of choice for the determination of protein dynamics at atomic resolution and is exquisitely sensitive to dynamical processes, from the pico- to the millisecond.

Dipolar couplings can be measured by solid-state NMR  $(ssNMR)^{117-119}$  to characterize motions. The sample in the solid phase does not tumble and motions slower than the correlation time for overall rotational diffusion  $\tau_c$  found in solution can be detected. Additionally, ssNMR allows for the study of high molecular-weight proteins. Relaxation rates of nuclear spins can also be measured in the solid state and provide information on the correlation times of the motions identified. However, a motion recorded in ssNMR can be different from the one found in a cell, in particular for proteins in crystals, where intermolecular contacts present in solid may alter dynamical properties.

Liquid-state nuclear magnetic resonance can be used to characterize protein motions, in particular through the measurement of relaxation rates.<sup>70,109</sup> The physical parameters of motions can be exploited to determine thermodynamic parameters.<sup>120</sup> These measurements only identify motions faster than the correlation time for overall diffusion.

Residual dipolar couplings (RDC)<sup>121–124</sup> can also be used to sample supra- $\tau_c$  motions, such as the interconversion between sidechain rotamers, as well as fast libration-type motions about an equilibrium position.<sup>125</sup> The range of timescales observed is typically from a few ns to about 10 ms.<sup>126, 127</sup> However, this technique is very challenging, because it relies on multiple measurements in several alignment media. The conformational space of biomolecules of interest can be affected by such a change.



Figure 2.1 – Schematic representation of the exploration of the conformational space of a protein. The type of motion explored by each nuclear spin relaxation-based method is indicated. A) On the energy graph, a stopwatch indicates that a method provides the timescale of a given dynamic process. Methods shown on the population graph B) can access populations of highenergy states.

It is important to keep track of what a given technique can measure and which motions are likely to be identified using a specific approach. In the simple case of a "single-well energy profile", the slower the motion, the higher the energy reached during the process and the less populated the state explored is. For instance, longitudinal relaxation rates measured on proteins in isotropic liquids provide order parameters, which are closely related to an average of accessible orientations, with decreasing weights as motions become slower than overall tumbling. The analysis of these relaxation rates will provide a solid estimate of the width of the well of the ground state on the energy surface, similarly to the analysis of residual dipolar couplings (Figure 2.1). However, both measures won't be sensitive to the presence of weakly populated states.

For both solid- and liquid-state NMR, the sampling of dynamical process studied through spin relaxation is determined by the indirect sampling of the spectral density function (see chapter 1). The approach is illustrated in figure 2.2 for backbone nitrogen-15 relaxation. It is similarly applied to carbon-13 or deuterium relaxation in methyl groups. High-field relaxation measurements sample spectral density functions over a limited range of frequencies. The philosophy of high-field relaxation approaches is to obtain highly accurate data so that even slight variations due to small amplitude motions can be measured. However, approaches with extensive sampling of the spectral density function should be favoured when motions on several orders of magnitude of correlation times are to be characterized.

An extensive sampling of the spectral density function is possible by changing the magnetic field during which relaxation occurs to perform high-resolution relaxometry.<sup>17, 18</sup> This approach employs a high-field magnet. During the experiment, the sample travels inside the stray field gradient to a known position where nuclear spins relax. The sample is then transferred inside the superconducting coil and high-resolution probe for the rest of the sequence. Such an approach allows for the measurement of longitudinal relaxation rates for different magnetic fields, without any resolution loss.

Many proteins of interest have a molecular weight higher than 50 kDa, and liquid-state NMR is often complicated due to slow global tumbling, and the prevalence of relaxation under <sup>1</sup>H-<sup>1</sup>H dipolar couplings in a protein. In order to reduce the broadening of the NMR signal, one technique proposed is to "dilute" the number of spins in the proteins. For that, tremendous progress has been made in recombinant protein expression to replace <sup>1</sup>H with <sup>2</sup>H. These techniques can be used to study backbones or protein sidechains independently.

Multiple NMR studies of structural dynamics of the protein backbone have been performed,  $^{71,77-79,117,128-130}$  using  $^{15}$ N relaxation.

The backbone dynamics of high-molecular weight proteins can be studied by two techniques, starting from a growth in D<sub>2</sub>O. First, NH groups can be re-protonated. However, in the case that a protein with a stable hydrophobic core, this procedure can be difficult, with the need of unfolding and refolding the protein to protonate all amide sites of the backbones.<sup>80,81</sup> It is also possible to label  $C_{\alpha}$  position with high cost and conserving a limitation on the size of the protein.<sup>82,131</sup>

Protein sidechain dynamics is one of the key factors in molecular recognition.<sup>4–6</sup> Thus, it is important to explore protein sidechain dynamics. In general such approaches require specific isotope labelling. Stereo-array Isotope Labelling (SAIL) can be used to dilute spin by a factor of about two and study protein.<sup>83,84</sup> It is also possible to label aromatic amino acids.<sup>85,86</sup>



Figure 2.2 – High-field relaxation measurement analysis. A) Principle of the <sup>15</sup>N R<sub>1</sub>, <sup>15</sup>N R<sub>2</sub> and <sup>15</sup>N-<sup>1</sup>H NOE measurements. B) <sup>15</sup>N R<sub>1</sub>, <sup>15</sup>N R<sub>2</sub> and <sup>15</sup>N-1H NOE (Ubiquitin, 14.1 T).<sup>18</sup> C) Spectral density mapping to derive values of the spectral density function for a few characteristic frequencies (J(0), J( $\omega_N$ ) and J(0.87 $\omega_H$ )). D) Order parameters obtained from the fit to the spectral density mapping results C) or from a direct fit to measured relaxation rates B) using the model-free approach.

Methyl groups present many advantages for their study in liquid-state NMR:

- They are well spread in different amino-acid, therefore in the entire protein.
- They can be labelled specifically and independently with a good control of metabolic pathways.
- Carbon-13 nuclei have favourable relaxation properties thanks to the fast rotation of the methyl group, which reduces the effect of dipolar interactions.<sup>52,87</sup>
- It is possible to combine specific labelling of several amino acids to obtain information on protein dynamics in many residues<sup>6</sup> (Figure 2.3).

Methyl groups have already been used to determine large protein structure<sup>54</sup> and dynamics.<sup>88</sup> However, dynamics of methyl groups are generally more complex than the one of NH groups studied for the



Figure 2.3 – Chemical shifts of methyl groups assembled by amino acids. Figure adapted from Plevin and Boisbouvier<sup>132</sup>

backbones.<sup>117</sup> The full description of the dynamics requires many points on the spectral density function. Thus, studies of sidechain motions from nuclear spin relaxation in methyl groups are expected to greatly benefit from high-resolution relaxometry.

New methodological approaches require a well-known system. So we decided to work on a standard protein called ubiquitin. This protein is well-known for the (poly)ubiquitination present in many biological processes. Ubiquitin has a rich conformational space<sup>133</sup> that allows for binding with many partners.<sup>134</sup> Extensive motions were found for the turn  $\beta_1$ - $\beta_2$  highly studied in ssNMR, liquid state NMR and high-resolution relaxometry.<sup>18,135-137</sup> Ubiquitin sidechain motions have also been studied by many groups, relying on many techniques: RDC,<sup>125,134</sup> solid state NMR,<sup>118</sup> deuterium relaxation measurement<sup>32,138,139</sup> and finally MD simulation.<sup>140</sup> However, we believe that high-resolution relaxometry can be used in sidechain dynamics to obtain an accurate description of the pico- and nanosecond timescales in protein dynamics.

The work presented here illustrates the first attempt to understand side chain motions, by studying methyl groups using high-resolution relaxometry. Because we wanted to characterize the spectral density function over a large range of frequencies (at magnetic fields from 0.33 to 14.1 T), we decided to work with the  $\{^{13}C^{1}H^{2}H_{2}\}$ -methyl group, which relaxes slowly compared to other spin systems (chapter 1).
# 2.2 Measurement of relaxation rates over a broad range of magnetic fields

A U-[<sup>2</sup>H, <sup>15</sup>N], Ile- $\delta_1$ [<sup>13</sup>C<sup>1</sup>H<sup>2</sup>H<sub>2</sub>]-ubiquitin sample was prepared at a concentration of 1.2 mM first in H<sub>2</sub>O and then transferred in D<sub>2</sub>O. <sup>1</sup>H-<sup>13</sup>C and <sup>1</sup>H-<sup>15</sup>N HSQC spectra are displayed in figure 2.4, respectively in D<sub>2</sub>O and H<sub>2</sub>O. These spectra were assigned using the chemical shift published in BMRB (4769).



Figure 2.4 – U-[<sup>2</sup>H, <sup>15</sup>N], Ile- $\delta_1$ [<sup>13</sup>C<sup>1</sup>H<sup>2</sup>H<sub>2</sub>]-ubiquitin spectra at 600 MHz, 25°C, 1.2 mM, pH = 4.5. A) <sup>15</sup>N-<sup>1</sup>H HSQC spectrum in H<sub>2</sub>O. B) <sup>13</sup>C-<sup>1</sup>H HSQC spectrum in D<sub>2</sub>O

#### 2.2.1 High-field relaxation experiments

High-field relaxation rate constants were measured using conventional high-field spectrometers operating at 400, 600 and 800 MHz frequencies. Pulse sequences needed to acquire relaxation rates are described in the following section, including longitudinal, transverse relaxation rates, as well as cross relaxation. Relaxation experiments were performed at 298 K.

**Longitudinal relaxation rate constant.** R<sub>1</sub> on <sup>13</sup>C of Ile- $\delta_1$  was recorded on U-[<sup>2</sup>H, <sup>15</sup>N], Ile- $\delta_1$ [<sup>13</sup>C<sup>1</sup>H<sup>2</sup>H<sub>2</sub>]-ubiquitin using the pulse sequence described in figure 2.5. Firstly, the polarization is transferred from proton to carbon-13 using a refocused INEPT.<sup>141</sup> Then, the spin term  $\hat{C}_z$  relaxes while a series of 180° pulses are applied on <sup>1</sup>H and <sup>13</sup>C channels, in order to suppress unwanted cross-correlated relaxations. After that, the chemical shift of <sup>13</sup>C is encoded for a duration t<sub>1</sub>, and the polarization is transferred back to protons using a reverse INEPT. Note that for t<sub>1</sub> = 0, the delay  $\delta$  was chosen to obtain an echo for carbon-13 polarization. Finally the proton magnetization is detected under carbon-13 decoupling. Sign discrimination is performed in the indirect dimension using the States-TPPI scheme.



Figure 2.5 – Pulse sequence used for measuring <sup>13</sup>C longitudinal relaxation rates. All narrow (filled) and (wide-open rectangles represent 90° and 180° pulses. Pulses are along the x-axis of the rotating frame unless otherwise mentioned).  $\tau_a = 1/4J_{CH}$  where  $J_{CH}=135$  Hz.  $\Delta$  is set to perform an echo for the first increments. The phase cycle is :  $\phi_1 = 2\{y\}$ ,  $2\{-y\}$ ;  $\phi_2 = \{x\}$ ,  $\{-x\}$ ;  $\phi_3=4\{y\}$ ,  $4\{-y\}$ ;  $\phi_4 = 4\{x\}$ ,  $4\{-x\}$ ;  $\phi_{acq} = \{x\}$ ,  $2\{-x\}$ ,  $\{x\}$ ,  $\{-x\}$ ,  $2\{x\}$ ,  $\{-x\}$ . The durations and amplitudes of pulsed field gradients over x, y and z orientations were :  $G_1 = [0, 11.5, 8.5]$  G.cm<sup>-1</sup> during 1 ms;  $G_2 = [0, 0, 3.5]$  G.cm<sup>-1</sup> during 0.9 ms;  $G_3 = [14.5, 0, 23.5]$  G.cm<sup>-1</sup> during 2 ms;  $G_4 = [0, 0, 11.5]$  G.cm<sup>-1</sup> during 0.9 ms;  $G_5 = [5.5, 5.5, 9.5]$  G.cm<sup>-1</sup> during 0.9 ms;  $G_6 = [0, 0, 6.5]$  G.cm<sup>-1</sup> during 0.9 ms;  $G_7 = [0, 0, 14.5]$  G.cm<sup>-1</sup> during 1.5 ms;  $G_8 = [33.5, 33.5]$  G.cm<sup>-1</sup> during 0.7 ms. Frequency sign discrimination in the indirect dimension was performed with the States-TPPI scheme.

**Transverse relaxation rate.** R<sub>2</sub> CPMG pulse sequence (section 2.5.3.4) was acquired first to determine transverse relaxation rates. Those rates could not be fitted simultaneously as R<sub>1</sub> experiments, probably due to scalar relaxation of the second kind due to the coupling with the two deuterium nuclei.<sup>22,51</sup> An R<sub>1ρ</sub> experiment was thus recorded. In this experiment, a continuous-wave RF field is applied. In the rotating frame, the magnetization is locked along an effective field which is the sum of the RF field and the frequency offset (Figure 2.6). The R<sub>1ρ</sub> relaxation rate depends on longitudinal and transverse relaxation rates, and on the tilt angle  $\theta$  between the effective field and the z axis.<sup>22</sup> When exchange occurs, the R<sub>1ρ</sub> rate also depends on the amplitude of the effective field ( $\omega_e$ ).<sup>22,142</sup>

Figure 2.6 shows a series of magnetization trajectories during a  $R_{1\rho}$  experiment in the case of slow chemical exchange. The magnetization is initially aligned with the effective field corresponding to the ground, most populated state A. When exchange occurs, the offset component of the field is altered by the difference of chemical shifts between the two states. The tilt angle of the effective field  $\theta$  changes and the magnetization is no longer aligned with the effective field. Thus, the magnetization leads to a nutation around this new effective field. A series of exchange steps back and forth makes the magnetization drift away stochastically from the initial effective field. When an ensemble average is considered, the magnetization of the whole sample, aligned with the effective field, is progressively lost: chemical exchange leads to  $R_{1\rho}$  relaxation. If the effective field is increased, through an increase of the offset, the RF field amplitude or both the change of the effective field and the tilt angle due to the jump between the two exchanging states becomes smaller (Figure 2.6.B).Thus, increasing the effective



Figure 2.6 – Evolution of the magnetization during an  $R_{1\rho}$  experiment. The effective magnetic fields are represented as solid arrows and their directions are identified by dashed lines. A) A weak RF field is applied at the resonance frequency with the major state A. Jumps between states lead to large variations of the rotation axis and a divergent trajectory of the magnetization: the global magnetization along the effective field of the major state will decay faster. B) Upon increasing the amplitude of the effective field, the change in orientation between the two exchanging states is reduced. C) The decay of the magnetization aligned with the effective field becomes negligible when the effective field is large enough.

field progressively quenches  $R_{1\rho}$  relaxation due to the exchange process. In the limit of a large effective field, the relaxation rate tends towards the population-weighted average of the  $R_{1\rho}$  relaxation rates in all exchanging states. The parameters of the exchange process can be extracted from the relaxation dispersion graph showing  $R_{1\rho}$  as a function of the effective field. Due to the larger effective field amplitude achievable in  $R_{1\rho}$  experiments, this technique is applicable to the study of millisecond<sup>143</sup> down to microsecond exchange processes.<sup>144</sup> The pulse sequence used for  $R_{1\rho}$  experiments is displayed in figure 2.7.

**Steady-state** <sup>13</sup>C-<sup>1</sup>H **nuclear Overhauser effect.** Cross-relaxation rate between carbon-13 and neighbouring proton can be obtained using the <sup>13</sup>C-<sup>1</sup>H nuclear Overhauser effect (NOE). This experiment is done with two interleaved spectra, with or without effective proton saturation.<sup>101,145</sup> The NOE values are obtained from ratio of carbon-13 polarization measured under saturation and with no saturation of proton resonances.

For the unsaturated experiment (Figure 2.8), a recovery time  $T_{NOE}$  is followed by a very selective water flip-back pulse on water (4 ms). For the saturated experiment, a "train" of 180° pulses is applied on the proton channel. A gradient  $G_1$  is applied at the end of the saturation sequence to suppress the transverse component of the proton polarization.



Figure 2.7 – Pulse sequence for the measurement of  $R_{1\rho}$  relaxation rates. Pulse displayed in orange are off resonance by 50 kHz. The heat compensation period delay  $\tau_H$  is set so that the total spin-lock period remains the same for all delays  $\delta$ .  $\tau_a = 1/4J_{CH}$  where  $J_{CH}=135$  Hz. Pulse 1 and 2 are adiabatic half passage pulse.  $\delta_1$  is set to perform an echo for the first increment in the indirect dimension. The durations and amplitudes of pulsed field gradients over x, y and z orientations were :  $G_1 = [0, 11.5, 8.5]$  G.cm<sup>-1</sup> during 1 ms;  $G_2 = [0, 0, 3.5]$  G.cm<sup>-1</sup> during 0.9 ms;  $G_3 = [14.5, 0, 23.5]$  G.cm<sup>-1</sup> during 2 ms;  $G_4 = [0, 0, 11.5]$  G.cm<sup>-1</sup> during 0.9 ms;  $G_5 = [8.5, 0, 5.5]$  G.cm<sup>-1</sup> during 0.9 ms;  $G_6 = [5.5, 5.5, 9.5]$  G.cm<sup>-1</sup> during 0.9 ms;  $G_7 = [0, 0, 6.5]$  G.cm<sup>-1</sup> during 0.9 ms;  $G_8 = [0, 0, 14.5]$  G.cm<sup>-1</sup> during 1.5 ms;  $G_9 = [33.5, 33.5, 33.5]$  G.cm<sup>-1</sup> during 0.7 ms. The phase cycle is :  $\phi_1 = 2\{y\}, 2\{-y\}; \phi_2 = \{x\}, \{-x\}; \phi_3=4\{y\}, 4\{-y\}; \phi_4 = 4\{x\}, 4\{-x\}; \phi_{acq} = \{x\}, 2\{-x\}, \{x\}, \{-x\}, 2\{x\}, \{-x\}.$ 



Figure 2.8 – Pulse sequence for recording steady-state  ${}^{13}C{}^{-1}H$  nuclear Overhauser effects. Pulses displayed in orange are centred on methyl groups.  $T_{NOE}$  has been taken respectively to 12 s, 16 s, 20 s for 400, 600, 800 MHz experiment.  $\tau_a = 1/4J_{CH}$  where  $J_{CH}=135$  Hz.  $\Delta$  is set to perform an echo for the first increments. Pulse and strength of the gradient are described with respect to 600 MHz experiments. Selective pulse on the Ref box is a Sinc pulse of 4 ms. The two other shape pulse are also SinC of 890 µs. Proton composite-pulse is applied during  $t_1$  with a GARP scheme (70 µs), as well are on carbon-13 during acquisition (80 µs). The durations and amplitudes of pulsed field gradients over x, y and z orientations were:  $G_1 = [14.5, 0, 10.5]$  G.cm<sup>-1</sup> during 1 ms;  $G_2 = [0, 11.5, 8.5]$  G.cm<sup>-1</sup> during 1.0 ms;  $G_3 = [0, 0, 6.5]$  G.cm<sup>-1</sup> during 0.9 ms;  $G_4 = [0, 0, 14.5]$  G.cm<sup>-1</sup> during 1.5 ms;  $G_5 = [24.5, 24.5, 24.5]$  G.cm<sup>-1</sup> during 0.7 ms. The phase cycle is :  $\phi_1 = \{y\}, \{-y\}; \phi_2 = 4\{y\}, 4\{-y\}; \phi_3 = 4\{x\}, 4\{-x\}; \phi_4 = 2\{-x\}, 2\{x\}; \phi_5 = 2\{x\}, 2\{-x\}; \phi_{acq} = \{x\}, 2\{-x\}, \{x\}, \{-x\}, 2\{x\}, \{-x\}.$ Frequency sign discrimination in the indirect dimension was performed with the States-TPPI scheme.

 $\tau_c$  determination. In addition to  $R_1$ ,  $R_2$  and cross-relaxation experiments on <sup>13</sup>C for several magnetic fields,  $R_1$  and cross-relaxation experiments were also performed on <sup>15</sup>N, after the transfer of an ubiquitin sample in H<sub>2</sub>O with the same concentration. The correlation time for overall rotational diffusion  $\tau_c$  was determined using ROTDIF<sup>30</sup> to analyse nitrogen-15 relaxation rates. Because the viscosities of D<sub>2</sub>O and H<sub>2</sub>O are different,  $\tau_c$  in D<sub>2</sub>O was derived using the Stokes-Einstein diffusion equation. The correlation time  $\tau_c$  for the ubiquitin sample in D<sub>2</sub>O was found to be  $\tau_c = 5.11$  ns at 298 K.

#### 2.2.2 High-resolution relaxometry

Relaxation rates obtained with conventional high-field spectrometers ( $R_1$ ,  $R_2$ , heteronuclear NOE ratio) have been combined with  $R_1$  rates obtained by high-resolution relaxometry. A pneumatic-shuttle system was installed on a conventional 600 MHz magnet. To control the strength of the magnetic field in which the relaxation occurred, the stray-field had been measured previously (Figure 2.9).<sup>18</sup> Due to the installation of a ferroshim system at 0.33 T (see chapter 3), the stray-field slope was modified and values obtained by simulation were used (work of Pierre-Alain Bovier, Bruker Biospin). Details on the shuttling processes are given in the subsection 2.5.3.1. Importantly, all experiments were recorded at 298 K following the protocol described in subsection 2.5.3.2.



Figure 2.9 – High-resolution relaxometry system installed on a conventional spectrometer. The inner part of the shuttle guide (in pink) can be set up on a top of the magnet to control the magnetic field in which the relaxation occurs.

The pulse sequence was adapted from a high-field  $R_1$  experiment, with a few differences due to the quality of the high-resolution relaxometry probe and the shuttling conditions. Firstly, no pulse can be performed in the stray field of the magnet. Then, the sign discrimination in the indirect dimension is done with an Echo-AntiEcho scheme.<sup>51</sup> Finally, we use a sensitivity-enhanced experiment<sup>146</sup> (Figure 2.10).



Figure 2.10 – Pulse sequence used for measuring <sup>13</sup>C longitudinal relaxation rate using highresolution relaxometry. All narrow (filled) and wide-open rectangles represent 90° (180°). Pulses are along the x-axis of the rotating frame unless otherwise mentioned.  $\tau_a = 1/4 J_{CH}$ where  $J_{CH}$ -135 Hz.  $\tau'_a = \tau_a$ -900 µs.  $\tau_1$  is the delay corresponding to half of the  $t_1$  delay incremented for the indirect dimension. The phase cycle is:  $\phi_1 = 4\{y\}, 4\{-y\}; \phi_2 = \{x\}, \{-x\}; \phi_3 = 8\{y\}, 8\{-y\}; \phi_4 = 16\{y\}, 16\{-y\}; \phi_5 = 2\{x\}, 2\{-x\}; \phi_6 = 2\{-y\}, 2\{y\}; \phi_{acq} = \{x\}, 2\{-x\}, \{x\}, \{-x\}, 2\{x\}, \{-x\}, \{x\}, 2\{y\}, \{x\}, \{y\}, 2\{x\}, \{y\}, \{x\}, 2\{y\}, \{x\}, \{y\}, 2\{x\}, \{y\}.$  The amplitude profile of the pulsed field gradient had a sine-bell shape frequency and peak amplitudes of  $G_{1,z} = 7\%$ ,  $G_{2,z} = 17\%$ ,  $G_{3,z} = 11\%$ ,  $G_{EA,z} = 20\%$ ,  $G_{5,z} = 10.05\%$  for a duration of 900 µs. Sign discrimination in the indirect dimension was performed with the Echo-AntiEcho scheme.

A relaxation profile was obtained by measuring relaxation times with high-resolution relaxometry for 18 magnetic field strength values, ranging from 0.33 to 3.5 T. These results were combined with those of the conventional  $R_1$  experiment and displayed in figure 2.11. This sample was damaged before we could measure field strength values between 3 and 9 T.

In addition, two plateaus were observed for some residues, with different intensities. This particular behaviour required special care and another ubiquitin batch is actually being prepared in order to confirm (or not) both the behaviour at the lowest fields and the bump near 3 T.



Figure 2.11 – Dispersion profile for sample 2 measured for 21 magnetic field strength values, from 0.27 to 18.8 T. Points at 9.4, 14.1 and 18.8 T were obtained using a conventional high-field NMR set up.

# 2.3 Results

#### 2.3.1 ICARUS

Relaxation matrices for the { ${}^{13}C^{1}H^{2}H_{2}$ }-methyl group were introduced in chapter 1. The basis of our subspace was spanned by the operators  $\hat{C}_{z}$ ,  $2\hat{C}_{z}\hat{H}_{z}$  and  $\hat{C}_{z}\hat{H}_{z}\hat{D}_{z}^{-1}$ ,  $\hat{C}_{z}\hat{H}_{z}\hat{D}_{z}^{-2}$  (three-spin orders with deuterons 1 & 2, see chapter 1). Then, the simplest form of the spectral density function  $\mathcal{J}^{MFM}(\omega)$  was assumed, and high-resolution relaxometry experiments were corrected with a new version of ICARUS, written in MATHEMATICA.



Figure 2.12 – Schematic version of the ICARUS process. High-field relaxation rates are fitted using  $\mathcal{J}(\omega)$  suited to the spin system and the relaxation rates obtained by REDKITE. Then  $\mathcal{J}(\omega)$  is used to simulate the whole experiment. Deviations of the rates given by the relaxation decays and the true rates are used to define a correction factor. This correction factor is applied to experimental rates to carry out a new round of analysis of relaxation rates. This process is repeated until we obtain stable corrections and parameters for local dynamics.

ICARUS can be decomposed in several steps (Figure 2.12). A first fit on high-field dataset using

the simplest model outputs parameters that are used to compute the theoretical propagator for each experiment. For each residue, the true and "experimental" relaxation rates of the simulation are compared to obtain a correction ratio, which is applied to the experimental rates. These new rates are then fitted, and the previously obtained spectral density function is used once again to compute the propagator of the full trajectory. Then this procedure is reiterated. For the  $\{^{13}C^{1}H^{2}H_{2}\}$ -methyl group relaxation, the correction factor was small (below 5 % for the weakest field), and a single iteration was enough to reach a stable correction and set of fitted parameters.

The procedure to fit the spectral density function in ICARUS is performed with  $\chi^2$  minimization, where  $\chi^2$  is equal to:

$$\chi^{2} = \sum_{i=1}^{nexp} \frac{(R_{1,i}^{\text{Calc}} - R_{1,i}^{\text{Expt}})^{2}}{(R_{1,i}^{\text{Error}})^{2}} + \sum_{i=1}^{nexp} \frac{(R_{2,i}^{\text{Calc}} - R_{2,i}^{\text{Expt}})^{2}}{(R_{2,i}^{\text{Error}})^{2}} + \sum_{i=1}^{nexp} \frac{(\sigma_{i}^{\text{Calc}} - \sigma_{i}^{\text{Expt}})^{2}}{(\sigma_{i}^{\text{Error}})^{2}}.$$
 (2.1)

where  $R_1^{Calc}$ ,  $R_2^{Calc}$  and  $\sigma^{Calc}$  are extracted from the expression described in chapter 1. The correlation function used in ICARUS was a 3-parameter spectral density function (described in chapter 1):

$$C_3(t) = e^{t/\tau_c} \left( S^2 + (1 - S_f^2) e^{t/\tau_f} \right) \left( S_m^2(\theta) + (1 - S_m^2(\theta) e^{t/\tau_m} \right)$$
(2.2)

#### 2.3.1.1 Results of the MCMC fit

Once the corrected relaxation rates are obtained, the spectral density function for each model  $\mathcal{J}_i(\omega)$  (i=2..5) is fitted using the combination of the high-field relaxation dataset and the data from high-resolution relaxometry. We employ Markov chain Monte Carlo (MCMC), which yields the distribution of the microdynamics parameters. The Akaike information criterion (AIC)<sup>147–149</sup> is used to select the most statistically relevant model for  $\mathcal{J}_i(\omega)$ .

The MCMC algorithm converged rapidly and results for all residues are displayed in figure 2.13. Remarkably, we identify correlation times for motions of the CC bond vector in the nanosecond range for three residues (Ile-13, 36 and 44).



Figure 2.13 – Order parameters computed with the MCM approach on the corrected data obtained on U-[<sup>2</sup>H, <sup>15</sup>N], Ile- $\delta_1$ [<sup>13</sup>C<sup>1</sup>H<sup>2</sup>H<sub>2</sub>]-ubiquitin, using high-resolution relaxometry.

Figure 2.14 shows the quality of the results on the example of residue Ile-23.  $R_1$  and  $R_2$  are well reproduced. Each parameter obtained by MCM is well defined. The correlation between parameters is also represented.

**Residue 44:** This residue is probably one of the most interesting. The AIC could not distinguish  $\mathcal{J}_3(\omega)$  from  $\mathcal{J}_4(\omega)$  for it (Figure 2.15). For the three-parameter spectral density function, the correlation time for methyl rotation is probably too fast, and the four parameters spectral density function has been chosen. Dipolar cross-relaxation rates are well reproduced, but  $\mathcal{J}(0)$  is likely underestimated (R<sub>2</sub> rates are not well reproduced). We cannot exclude that the high value of R<sub>2</sub> comes from microsecond dynamics. In the longitudinal relaxation profile, the R<sub>1</sub> rates for the weakest fields show signs of the contribution of a larger correlation time. This could be due to the presence of a small population of ubiquitin dimers with a more rigid side chain for Ile-44, contributing to longitudinal relaxation rates for the weakest field strengths. However, the absence of a similar pattern for all residues does not support this hypothesis.



Figure 2.15 – Comparison between the fit using either an extended model-free (4 parameters) or model-free (3 parameters) for Ile-44. Plain (dashed) lines correspond to the EMF (MF) spectral density function.



Figure 2.14 – MCMC results for Ile-23. R<sub>1</sub> rate (red),  $\sigma_{\rm NOE}$  and R<sub>2</sub> are well reproduced with the 3-parameter spectral density function. The MCMC trajectory is in cyan, the histogram of data taken in the MCMC trajectory in orange. Correlations between parameters are shown in grey.

## 2.3.2 Comparison with measurements and simulations of methyl group motions

#### 2.3.2.1 Comparison with solid-state NMR measurements and <sup>2</sup>H relaxation



Figure 2.16 – Comparison of the generalized order parameters  $S^2$  (where  $S^2 = S_{CC}^2$  or  $S^2 = S_{CC,f}^2$  $S_{CC,s}^2$  depending on the model used to analyse data) for the motions of the CC bond for the seven isoleucine- $\delta_1$  methyl groups (purple), the order parameter in solid extracted from dipolar coupling measurements in ssNMR (orange, Schanda et *al.*) and the order parameter obtained in the analysis of deuterium relaxation rates in solution (orange, Wand et *al.*)

Data acquired on deuterium relaxation<sup>150</sup> and solid-state dipolar coupling measurement<sup>118</sup> were compared with our results obtained by high-resolution relaxometry (Figure 2.16).

In the relaxation profile performed on deuterium,<sup>150</sup> the range of frequency used to probe the spectral density function is lower, from 92 and 230 MHz. This early contribution does not treat separately the rotation of the methyl group and the motions of the CC-bond. Thus, high frequency motions are probably not analysed in an optimal fashion. More importantly, the availability of a limited number of relaxation rates and the absence of sampling of the spectral density function at low (but non zero frequency) makes that slow motions with correlation times comparable to the overall rotation diffusion correlation time  $\tau_c$ -are not identified. Indeed, a simple "Model Free" spectral density function has been used for the interpretation of data with a single correlation time to describe motions ranging from slow ns rotamer transitions of the side chain to the fast ps rotation of the methyl group. Figure 2.17 represents the sampling of the spectral density function from high-resolution relaxometry combined with high-field relaxation measurements. Such a dataset gives access to the spectral density function over a broad range of frequencies. Additionally, it is possible to see the effects of CC motions on hundreds of ps, as well as methyl rotation. Interestingly, parameters for the motions of the CC axis have to be precisely determined in order to have an accurate evaluation of the correlation time for methyl rotation. Indeed, the inflexion of the Lorentzian function for this motion falls in a range of frequencies inaccessible to NMR.



Figure 2.17 – Spectral density functions using our model (orange) in comparison to the on used for the analysis of deuterium relaxation at two magnetic fields (blue). Parameters used are taken for Ile-23 in both cases with a  $\tau_c$  of 5.1 ns Blue arrows indicate the points of the spectral density function sampled in the course of deuterium relaxation measurements. A continuous orange shade underlines the range accessed by high-resolution relaxometry combined with high-field measurements of carbon-13 relaxation.

The comparison with order of parameters obtained by ssNMR is less straightforward. On the one hand, some modes of motions can be constrained in the crystal. For instance, some residues can be involved in intermolecular interactions of the crystal. In this case  $S_{CC}^2$  in solid is expected to be higher than the one obtained by high-resolution relaxometry in solution state. On the other hand, the motions that contribute to the averaging of dipolar interactions in solid range from picoseconds to hundreds of microseconds. Thus, solution-state relaxation is blind to rotamer transitions much slower than 1 ns, which can be readily identified by solid-state NMR measurements. In this case, order parameters obtained in solid-state NMR are expected to be lower than those measured in solution-state NMR. Figure 2.16 shows that order parameters obtained in the analysis of relaxometry data are either equal to or higher than those obtained by solid-state NMR. We make the hypothesis that most of the motions throughout the hydrophobic core of ubiquitin are not affected by the presence of the crystal lattice. In addition, the side chain of isoleucine 44, exposed to the surface does not seem to be involved in intermolecular contacts in the crystal. In this frame, the comparison of relaxometry in solution and solid-state measurements shows that, for most residues few additional orientations are explored by the protein side chains in the window 10 ns to  $100 \,\mu s$ . This does not exclude rare conformational transitions but indicates that such rare conformational transitions lead to orientations that are only very transiently explored and do not contribute significantly to averaged properties. By contrast, the

order parameters obtained for residues Ile3 and Ile30 illustrate the fact that conformational transitions that occur on timescales slower than a few nanosecond lead to populations of conformations that are sufficient to alter average properties, such as order parameters.

#### 2.3.2.2 Comparison with molecular dynamics simulation

We initiated a collaboration with Raphael Brüschweiler in order to analyse the results of highresolution relaxometry, with the assistance of molecular dynamics simulations, in order to assign to particular molecular processes the motions identified by experiments. Two MD trajectories of 500 ns and 1  $\mu$ s were analysed. Parameters obtained in a MF approach on the 500 ns MD trajectory are displayed in table 2.1. In addition, the sidechain Ramachandran representation is also shown on figure 2.18, in order to represent the conformational space of each isoleucine sidechain.

Table 2.1 – Data obtained by MD simulation for a trajectory of 500 ns (in black) compared with our order parameters and timescales (red).

Residue	$\mathbf{S}^2$	$\mathbf{S}_{\mathrm{f}}^2$	$S_{ m s}^2$	$ au_{ m e}$ (ps)	$\chi^2$
Ile 3	0.73 - <mark>0.63</mark>	0.91 - <mark>0.63</mark>	0.80 - 1.0	15.15 - <mark>247</mark>	0.04
Ile 13	0.74 - 0.32	0.79 - <mark>0.66</mark>	0.94 - <mark>0.48</mark>	647.78 - <mark>2460</mark>	0.07
Ile 23	0.75 - <mark>0.43</mark>	0.82 - <mark>0.43</mark>	0.91 - <mark>1.0</mark>	459.07 - <mark>277</mark>	0.09
Ile 30	0.87 - <mark>0.66</mark>	0.92 - <mark>0.66</mark>	0.95 - <mark>1.0</mark>	788.37 - <mark>345</mark>	0.02
Ile 36	0.43 - <mark>0.34</mark>	0.76 - <mark>0.7</mark> 1	0.56 - <mark>0.49</mark>	644.42 - <mark>1720</mark>	1.20
Ile 44	0.23 - 0.15	0.66 - <mark>0.5</mark> 9	0.35 - 0.26	1239.70 - <mark>823</mark>	0.32
Ile 61	0.51 - 0.48	0.86 - <mark>0.48</mark>	0.59 - 1.0	1063.80 - <mark>0.13</mark>	0.15

Most order parameters S<sup>2</sup> obtained by MD (500 ns) are in good agreement with the ones obtained by high-resolution relaxometry, except for Ile-13 and Ile-23. For Ile-13, as shown by recent simulations, a timescale of 500 ns does not yield realistic results for rotameric transition.

Figure 2.18 shows that Ile-3/23/30 populate only a single rotameric state of  $\chi_1$  over the course of the molecular dynamics trajectory. This conclusion is different from the work published in 2009,<sup>125</sup> in which dynamical ensemble (1xqq, 2nr2, 2k39) lean toward a single rotameric state of  $\chi_1$  for Ile-3, Ile-36, Ile-44 and Ile-61.

In the MD simulation, Ile-13/36/44/61 cover multiple  $\chi_1$  dihedral angle states. These transitions between two to three  $\chi_1$  rotamers at the ns timescale should contribute to the motion identified with correlation times of the order of 1 ns obtained from both MD and experiments.



Figure 2.18 –  $\chi_1/\chi_2$  configuration for the seven isoleucines, identified along the 1  $\mu s$  MD trajectory.

# 2.4 Concluding remarks

Using high-resolution relaxometry combined with high-field relaxation experiments, an unprecedented relaxation dataset (from 18.8 T down to 0.24 T) was obtained for the relaxation of carbon-13 nuclei from Ile- $\delta_1$  methyl groups in the protein U-[<sup>2</sup>H, <sup>15</sup>N], Ile- $\delta_1$ [<sup>13</sup>C<sup>1</sup>H<sup>2</sup>H<sub>2</sub>]-ubiquitin. A new version of the program ICARUS was written for MATHEMATICA, suited to the correction of low-field relaxation rates measured in methyl groups. Using the combination of REDKITE and ICARUS, relaxation rates obtained by high-resolution relaxometry were corrected and analysed. The main features of the relaxation dispersion profiles are well reproduced in our analysis with some deviations. Some residues show a low-field profile that is similar to what was observed in concentrated protein solutions, with a so-called "long tail" of the correlation function.<sup>151</sup>

These corrected rates were fitted using Monte Carlo Markov Chain analysis, and compared with molecular dynamics simulations, as well as with the measurement of dipolar couplings in solid-state NMR and the analysis of <sup>2</sup>H relaxation. The discussion of the differences observed between various experimental techniques offers insights in the nature of the motions identified. Molecular dynamics simulations point to slower  $\chi_1$  dynamics, which likely correspond to the motions identified in the ns range .

This high-resolution relaxometry allows us to explore complex models of the spectral density function. Correlation functions describing the motion of methyl groups, including global tumbling, methyl group rotation and motion(s) of the CC bond were studied. Future work will explore whether the motions of the CC bond can be better modelled as diffusion in a rough potential.<sup>104,105</sup> We are still in the process of fitting the data to include a Mittag-Leffler correlation function to describe the motions of the CC bond.

# 2.5 Materials and Methods

The experimental procedures for protein expression and purification are described here, alongside a discussion of the properties of the different samples used in this study. Some NMR-related details are also provided in a second part, with the description of shuttling processes, as well as at temperature calibration protocol.

#### 2.5.1 Sample preparation

DH5 $\alpha$  and BL21(DE3)pLysS-competent cells were used respectively for plasmid production and for protein expression. DH5 $\alpha$  indeed has a recA mutation that ensures a high insert stability. Some endonucleases are also removed to avoid losing the inserted plasmids. BL21 cells lack some proteases that can consume the expressed protein. Moreover, the DE3 strain was used with a T7 RNA polymerase in order to induce cell growth.pLysS has an additional mutation carrying the gene encoding T7 lysozyme, which reduces the background expression level of target genes controlled by the T7 promoter. This mutation is important for selective labelling.

#### 2.5.1.1 Expression of proteins in several medias

Two protocols were used for the production of U-[<sup>2</sup>H, <sup>15</sup>N], Ile- $\delta_1$ [<sup>13</sup>C<sup>1</sup>H<sup>2</sup>H<sub>2</sub>]-ubiquitin. The first protocol is the one presented by Tugarinov et al.,<sup>52</sup> while the second one was presented by Pr. Boisbouvier's group.<sup>152</sup> For further specific labelling, I recommend the use of the second protocol, as it adapts the bacteria in D<sub>2</sub>O more slowly. Moreover the dilution of cells in the first protocol is probably too high, and growth can be quite slow, which is often impractical.

We used appropriate antibiotics (ampicillin and chloramphenicol) for each culture.

#### First protocol for protein expression: Final volume 500 mL.

1- Start with a 2.5 mL culture of LB rich growth medium from a freshly transformed colony of BL21(DE3)pLysS. For ubiquitin, we usually start from glycerol stocks. Let it grow in a shaking incubator at  $37^{\circ}$  C until the cells reach an OD<sub>600</sub> of 0.7-0.8.

2- Spin the cells down (1.000 g at room temperature, 10 min) and resuspend cells in 10 mL sterile M9/H<sub>2</sub>O medium (prepared with unlabelled glucose and unlabelled NH<sub>4</sub>Cl), in a 50 mL flask, in order to start with an OD<sub>600</sub> of 0.1. Let the cell grow at 37°C under agitation, until a OD<sub>600</sub> of 0.6 is reached.

**3-** After spinning down the cells under the same condition as (2), resuspend in 50 mL M9(D<sub>2</sub>O) -250 mL flask- with a starting OD<sub>600</sub> of 0.1. This M9(D<sub>2</sub>O) should be prepared with D-[<sup>2</sup>H, <sup>12</sup>C]glucose and [<sup>15</sup>N]NH<sub>4</sub>Cl.

4- After incubation until  $OD_{600}$  reaches 0.4, the culture have to be diluted to 100 mL M9(D<sub>2</sub>O) prepared under the same conditions as step (3). The growth has to be stopped at  $OD_{600}$  0.4-0.5.

5- Add 250 mL M9(D<sub>2</sub>O) and let the cells grow until  $OD_{600}$  reaches 0.25

6- Add 35 mg of 2-keto-3-d<sub>2</sub>-4- $^{13}$ C,d<sub>1</sub>-butyrate and continue incubating during 1 h to reach an OD<sub>600</sub>

around 0.3-0.4

7- Induce with IPTG as usual and let growth continue for 6 to 8 h (not more, to avoid scrambling).

In this protocol, there is no cell adaptation to  $D_2O$ .



#### Second protocol for protein expression: Final volume 250 mL.

Figure 2.19 – Schematic representation of the 4-day protocol for producing  $\{^{12}C, ^{2}H, ^{15}N\}$ ubiquitin with isoleucine- $\delta_1$  methyl group labelled selectively  $\{^{13}C^2H_2{}^{1}H\}$ . This protocol includes the adaptation of *E.coli* in D<sub>2</sub>O.

**1-** Starting from freshly transformed agar plate (or glycerol stocks for ubiquitin), grow several clones in 5 mL LB H<sub>2</sub>O medium in a 50 mL flask for 7 h approximately.

**2-** Transfer 50  $\mu$ L of this culture in 5 mL M9(H<sub>2</sub>O) medium for an overnight growth at 37° C under 200 rpm shaking.

**3-** In the morning, the OD<sub>600</sub> should be around 2.3-2.5. Inoculate 10 mL of 50% D<sub>2</sub>O M9 medium with the right amount of cell in order to start with a OD<sub>600</sub> of 0.25 (centrifuge at 4.000 g at RT).

4- By the end of the day, the  $OD_{600}$  should be around 2.3. Transfer the right amount of cell to start with  $OD_{600}$  of 0.25 in a 50 mL preculture and centrifuge under the same conditions as 3. Then transfer the cell in 50 mL M9(D<sub>2</sub>O) prepared with D-[<sup>2</sup>H, <sup>12</sup>C]glucose and [<sup>15</sup>N]NH<sub>4</sub>Cl, for an overnight growth (37° C, 200 rpm).

5- In the morning, the  $OD_{600}$  should be around 1.5-2. Calculate the required volume to inoculate 175 mL of the culture at  $OD_{600}$  0.25. Centrifuge this volume under the same conditions as previously, and transfer bacteria in 175 mL of M9(D<sub>2</sub>O) (without centrifugation).

**6-** When the OD<sub>600</sub> reaches 0.7-0.8, transfer the 175 mL in a 1 L flask. Add in 75 mL M9(D<sub>2</sub>O) of the isoleucine precursor (15 mg of 2-hydroxy-2-(1'-[D<sub>2</sub>],2'-[<sup>13</sup>C])ethyl-3-keto-4[D<sub>3</sub>]butanoic acid) and add to the culture until the OD<sub>600</sub> reaches 0.7.

7- Incubate 20 min at 37° C and 200 rpm, then induce with IPTG (1mM final concentration).

8- Incubate overnight in shaker at 20° C, 200 rpm.

#### 2.5.1.2 Purification of ubiquitin

The Lysis procedure is shared by all three following protocols and is summed up here. The *Lysis buffer* is a 50 mM tris-hydroximethyl (pH 7.6) solution, with 0.4 mg/mL of lysozyme added just before the lysis.

After the growth, spin down the media at 30.000 g and  $4^{\circ}$  C for 30 minutes. Add to the cell pellet 30 mL of Lysis buffer with an anti-protease cocktail and leave this solution for 20 min at room temperature. Then proceed to 10 cycles of sonication (50 % intensity, 10 s pulse, 50 s break) on ice. Spin down the cell at 8.000 g and  $4^{\circ}$  C for 20 min.

#### Perchloric acid purification:

**Proteins precipitation:** 1- Transfer the supernatant into a small beaker cooled down with ice. Stir thoroughly and add dropwise 5 % by volume of 70 % perchloric acid. Keep stirring for 10 min once all the acid was added.

**2-** Centrifuge at 8000 g and 4° C for 10 min. Decant the clear supernatant into a beaker, put on ice again and stir.

**3-** Bring the pH to near-neutral by adding, dropwise, a 5 M NaOH solution, checking the pH with pH paper. The pH has to be between 4 and 8.

4- Transfer the neutralized supernatant into a 3.5 kD MWCO dialysis bag.

**Dialysis and gel filtration:** 5- Dialyze at 7° C against 4 L miliQ-water for 2 h. Change the water and dialyze for two more hours against miliQ-water. Then dialyze for 2 h in the dialysis buffer. *Dialysis buffer:* 50 mM ammonium acetate, 150 mM NaCl, pH 5.

**6-** After the dialysis step, the ubiquitin obtained is not completely pure. An additional step of gel filtration in the same buffer was added to obtain a purer sample.

7- The final step of the purification is a gel filtration FPLC, in the same buffer with a HiLoad 16/600 Superdex 75 pg column at 1 mL per minute. The ubiquitin obtained is tested by mass spectroscopy and NMR.

**Multi-step chromatography procedure.** This protocol has been optimised by Lei Bruschweiler (Ohio state University). **1-** Take the expression Lysis supernatant, check the pH and adjust to 7.0. Heat up to 85° C for 5 minutes then put on ice to cool it. Centrifuge the solution at 10.000 g for 30 minutes at 4° C.

**2-** Take the supernatant and treat with concentrated acetic acid until the pH drops to a value between 4.5 and 5.0. The solution will turn milky white, since many of the bacterial proteins will fall out of solution. Centrifuge the solution at 10.000 g for 10 minutes at 4° C. Then adjust the pH with sodium hydroxide to pH 5.1. This prevents other proteins from precipitating during the ion exchange step.

3- Setup the cation exchange column. Use a GE Healthcare SP HP 5 mL column.

4- Binding buffer: Ammonium acetate 20 mM pH 5.1

Elution buffer: Ammonium acetate 500 mM pH 5.1

Prepare the buffers. A good buffer to use for ubiquitin purification at pH 5.1 is ammonium acetate, since the buffer is volatile and mass spectrometry can be performed directly on the fractions. When you adjust the pH, be sure to always use either ammonium hydroxide ( $NH_4OH$ ) or acetic acid, since the goal is to not have any non-volatile metal ions in the solutions.

**5-** Pre-equilibrate the cation exchange column by first running 2 column volumes of high salt (elution buffer), followed by extensive washing by 5 column volumes of low salt (binding buffer).

**6-** Load the protein sample into the column. Save the flow through. Wash the column with 2 to 3 column volumes of binding buffer. Then elute the proteins from the column with a linear gradient

(10 column volumes at 1 mL/min flow rate) of increasing concentration of ammonium acetate (elution buffer).

7- Analyze your fractions at the main peak of the gradient (determined typically by absorbance at 280 nm) by mass spectrometry or gel electrophoresis. Pool the pure fractions together, dialyze against pure water, and lyophilise (or freeze-dry) the protein solution to powder.

8- Gel filtration buffer: 50 mM ammonium acetate, 150 mM NaCl, pH 5.

Concentrate the fractions that correspond to ubiquitin and replace the buffer with the gel filtration buffer. The final volume should be around 2.5 mL in order to optimize the injection inside the 5 mL loop. The protein should get out of the column at 80 mL after starting injection inside the Superdex column.

#### 2.5.2 Sample description

Two samples of U-[<sup>2</sup>H, <sup>15</sup>N], Ile- $\delta_1$ [<sup>13</sup>C<sup>1</sup>H<sup>2</sup>H<sub>2</sub>]-ubiquitin were expressed and purified. They were produced following two different protocols and will be called sample 1 and sample 2 for the rest of the chapter. A plasmid provided by Kaushik Dutta (New York Structural Biology Center, USA) was amplified and transformed into E.Coli (Bl21(DE3)p-LysS) cells. The first ubiquitin sample was expressed (subsection 2.5.1.1) and purified using a perchloric acid precipitation protocol (subsection 2.5.1.2), and relaxation experiments were carried out on a solution in H<sub>2</sub>O with 7 %D<sub>2</sub>O at 1.2 mM. The second sample was expressed (subsection 2.5.1.1) and purified by a multi-step chromatography protocol (subsection 2.5.1.2). Relaxation experiments were carried out in solutions in in D<sub>2</sub>O at 1.2 mM so as to prevent any problem of suppression of the proton signal of the solvent.

#### 2.5.2.1 Second sample

Although sample 1 provided a perfect  ${}^{13}C{}^{-1}H$  HSQC spectrum, unexpected features of its  ${}^{15}N{}^{-1}H$  HSQC made us doubt the integrity of the protein. A second sample was prepared and the relaxation data presented in this chapter were all recorded on this second sample. Note that the analysis of relaxation rates measured at several magnetic fields were in excellent agreement in the two samples. In order to avoid any alteration of ubiquitin, the second sample was prepared without perchloric acid precipitation. The  ${}^{15}N{}^{-1}H$  HSQC spectrum (in H<sub>2</sub>O) is shown in figure 2.20.A. The problems seen of sample 1 were not observed in sample 2. The sample was transferred to D<sub>2</sub>O in order to measure the  ${}^{13}C{}^{-1}H$  HSQC spectrum (Figure 2.20.B) and record a series of carbon-13 relaxation rates.



Figure 2.20 – A)  ${}^{15}$ N- ${}^{1}$ H HSQC / B)  ${}^{13}$ C- ${}^{1}$ H HQCS spectrum on a sample 2 obtained with the new purification protocol. The NH spectrum was obtained with 1.2 mM sample in H<sub>2</sub>O. The  ${}^{13}$ C- ${}^{1}$ H HSQC spectrum was acquired in a fully deuterated solvent.

NOESY experiments were performed on sample 2. No cross-peaks were observed, which seems to prove that the precursor (NMR-Bio) and the expression protocol lead to an excellent selective labelling on isoleucine.

#### 2.5.2.2 Comparison and conclusion

Samples 1 and 2 were compared using relaxation on methyl groups (sequences of high-field relaxation rates are given in subsection 2.5.3.4). Results are displayed on figure 2.21. Even though the concentration is the same for both sample, the global tumbling is not, because of the difference in viscosity (*c.f.* Stoke-Einstein equation). NOE ratio and R<sub>1</sub> rates depend on relatively high-frequency motions and are not noticeably affected by  $\tau_c$ . R<sub>2</sub> depends directly on  $\mathcal{J}(0)$  and a difference up to 10 % can be observed between samples 1 and 2. Additionally, R<sub>2</sub> was measured with CPMG methods for sample 1 and R<sub>1</sub> $\rho$  for sample 2. Both measurement methods are not sensitive to the same chemical exchange timescales, and can also change the observed rate.



Figure 2.21 – Comparison between  $R_1$ ,  $R_2$  and NOE for both samples. Importantly, the error bars for  $R_1$  rates are much smaller (this may be due in part to the absence of water signal).  $R_2$  rates were measured with a CPMG experiment for sample 1, and with  $R_{1,\rho}$  for sample 2.

#### 2.5.3 Materials and methods (NMR)

#### 2.5.3.1 Sample shuttle

Dataset corrections using ICARUS require the knowledge of the trajectory of the sample. Therefore, shuttling processes have to be monitored and analysed carefully. The characteristic duration of the transfer between the high-field and the low-field positions of the shuttle is dependent on the pressure (and the quantity of dust inside the shuttle guide, when applicable). Additionally, some changes of the firmware in the shuttle controller were performed during the acquisition of the series of relaxation rates of ubiquitine to improve the stability of the system.

The shuttle transfer durations for two high-resolution relaxometry experiments at 0.35 T on sample 2 are shown on figure 2.22. Because data are corrected by ICARUS for each experiment, the shuttle time difference for both experiments are minor. The effects of a minor distribution centered around 245 ms on the shuttling events after the modifications of the firmware of the shuttle controller are negligible, considering the small correction on  $R_1$  rates that is required. Therefore, we have used the average transfer delays to calculate relaxation rate corrections.



Figure 2.22 – Details on shuttling processes before and after the change of firmware to the controller for ubiquitin relaxation experiments (sample 2) for a given position of the shuttle guide and a field strength of 0.35 T.

#### 2.5.3.2 Temperature calibration

Sample 1 was prepared with a deuterated acetic acid buffer (50 mM). The temperature was calibrated using the deuterium chemical shift difference between acetic acid and water signals, and then compared with a standard methanol sample. The temperatures obtained with both samples are in good agreement (difference of 0.35 K). Because of the shuttle conditions, "fake" R<sub>1</sub> shuttle experiment were performed with the acquisition of deuterium spectra. For each field, the temperature was calibrated for the shortest relaxation delay. The difference between the shortest and the longest delays does not exceed 0.2 K. The temperature was calibrated for each position of the shuttle guide, or every two experiments in case the experiments have to be repeated multiple times.

For the deuterated sample, a second shuttle tube was prepared with only an identical acetic acid buffer in order to preserve the actual protein sample.

#### 2.5.3.3 Alternate pulse sequence for transverse relaxation measurements

#### **2.5.3.4** $R_2$ CPMG and $R_1$ , $\rho$ experiments

Transverse relaxation rates were measured with two methods. The first one is a  $R_{2,CPMG}$  experiment in which the scalar coupling between carbon-13 and proton, as well as the effects of supra-ms chemical exchange, are suppressed using a train of  $\pi$ -pulses. As for the HF  $R_1$  experiments, the effects of cross-relaxation due to the cross-correlation of CSA and dipole-dipole interactions are suppressed by a 180° pulse on <sup>1</sup>H



Figure 2.23 – Pulse sequence used for measuring <sup>13</sup>C transverse relaxation rates under a CPMG echo train. All narrow (filled) and wide (empty) rectangles are 90° and 180° pulses. Pulses are along the x-axis of the rotating frame unless otherwise mentioned. The phase cycle is:  $\phi_1 = 2\{-x\}, 2\{-y\}; \phi_2 = \{x\}, \{y\}; \phi_3 = 4\{-x\}, 4\{-y\}; \phi_4 = 4\{x\}, 4\{y\}; \phi_{acq} = \{x\}, 2\{y\}, \{x\}, \{y\}, 2\{x\}, \{y\}$ . The amplitude profile of the pulsed field gradient had a sine-bell shape. Frequency sign discrimination in the indirect dimension was performed with the States-TTPI scheme.

# **Two-field NMR spectroscopy**

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This chapter includes figures and descriptions from manuscripts under writing or submitted: about twofield broadband carbon-13 NMR, two-field-zero-quantum NMR and two-field-HZQC experiments. These manuscripts have been combined in order to obtained consistent chapter on two-field NMR.

# 3.1 Introduction

Nuclear Magnetic Resonance is known as a rather insensitive technique in comparison to other spectroscopies, in particular when low-gyromagnetic ratio nuclei with low natural abundance are considered (like <sup>13</sup>C or <sup>15</sup>N). To increase the signal-to-noise ratio of NMR experiments, many hardware developments have been achieved. Over the last four decades, a steady increase of available magnetic fields (from 9.4 to 23.5 T) has moved forward the sensitivity limits of NMR. Nowadays, new alloys should give access to even higher magnetic fields, with the high-temperature superconductors such as Yttrium Barium Copper Oxide (YBCO), assuring a steady increase of available magnetic field in the forthcoming decade. As of today, seven NMR spectrometers operating at 1.2 GHz (*i.e.* 28.3 T) have been ordered. Additionally, in the last twenty years, the noise of NMR experiments has been reduced with the use of cryocooled probe-heads and the signal of many experiments has been increased with the use of hyperpolarization techniques.

The ultra-high-field NMR technology has a cost, not only a financial cost, but also in terms of quality of the radiofrequency pulses needed, or of nuclear properties that become less favourable at higher magnetic field ( $B_0$ ). Indeed, the wide range of resonance frequencies for nuclei such as carbon-13 or fluorine-19 at the highest fields makes it challenging to apply broadband rf pulses. Many NMR experiments suffer from this limitation. In terms of nuclear properties, the transverse relaxation rates due to chemical shift anisotropy increase as the square of the magnetic field. This increase may become dramatic for some nuclei, such as the sp<sup>2</sup> carbonyl carbon in large proteins (Figure 17). Line broadening due to chemical exchange processes may also become extreme at high-magnetic fields.

Magnetic-field dependent properties can be probed by a broad range of NMR techniques that explore two or more fields in a single experiment, as in fast field-cycling relaxometry,<sup>74</sup> zero-field NMR,<sup>153,154</sup> dynamic nuclear polarisation<sup>155–158</sup> and other methods.<sup>159</sup> The change of magnetic field can be achieved in two different manners. The first option is to rapidly switch the electric current in an electromagnet as in field-cycling relaxometry where polarisation, relaxation and detection take place at different magnetic fields.<sup>74,160</sup> Fruitful developments have been performed in order to measure field-dependent NMR relaxation providing information on molecular motions, such as water dynamics in solution,<sup>161,162</sup> porous media,<sup>163,164</sup> or motions in polymers.<sup>165,166</sup> The second approach consists in moving physically the sample in the fringe field of a high-field magnet ( $B_0^{\rm HF}$ ) to reach a low-field position ( $B_0^{\rm LF}$ ). In this last strategy the sample is shuttled between different spatial positions either by moving the entire NMR probe,<sup>159,167</sup> or the sample with a mechanical<sup>17,75</sup> or pneumatic system.<sup>18,168,169</sup> However, to our knowledge no experiment has been proposed that allows one to correlate chemical shifts at two different fields.

Development of two-field NMR spectroscopy is motivated by the will of combining the highresolution and high-sensitivity of high-field NMR, with the better properties obtained at lower magnetic fields. For this purpose, a prototype of a two-field spectrometer has been built. This prototype includes two magnetic centres with vastly different homogenous magnetic fields (14.1 T and 0.33 T) coupled by a sample shuttle device. The high magnetic-field centre is a commercial superconducting NMR magnet while the second homogenous magnetic field is an engineered plateau

superconducting NMR magnet while the second homogenous magnetic field is an engineered plateau installed in the stray field of the superconducting magnet. Both magnetic centres are equipped with triple-resonance probes with z-axis pulsed field gradients. A six-channel NMR console is designed to generate radiofrequency pulses. Fast sample transport/shuttling between the two magnetic centres is achieved by a pneumatic shuttle apparatus. Note that the subscripts LF and HF will be used to distinguish channels dedicated to the low-field centre at 0.33 T and the high-field centre at 14.1 T, respectively.

The construction of this unique device will be described in the first section, as well as an introduction of pulse sequences where radiofrequency pulses and evolutions under chemical shifts take place at either of the two fields. In particular, we show that efficient isotropic mixing and proper control of relaxation pathways can be achieved at low field. However, the residual magnetic field inhomogeneities at 0.33 T prevent the development of high-resolution single-quantum NMR spectroscopy at low field (LF). The second part describes the use of the zero-quantum coherences<sup>38</sup> to obtain chemical shift correlation spectra at two fields with high resolution in homonuclear (2F-INAZEQUATE) and heteronuclear spin systems (2F-HZQC). The use of the 2F-HZQC sequence leads to dramatic improvements of spectra in some systems under chemical exchange. When the signals of exchanging sites are at coalescence at high field, dramatic line-broadening may render these signals invisible. Such signals can be recovered by probing the chemical shift at low field in the indirect dimension of a two-field experiment.

# 3.2 Development of two-field NMR spectroscopy

#### 3.2.1 Two-field NMR spectrometer

The two-field spectrometer has been designed in the frame of a strong collaboration with Bruker Biospin as a series of accessories to a 600 MHz spectrometer, such as the one installed at ENS, which was already equipped with a pneumatic sample shuttle for high-resolution relaxometry. The following modifications have been achieved:

- Modification of the shuttle guide and sample detection.
- Installation of a second working volume at 0.33 T.
- Generation of radiofrequency pulses at low field.
- Control of the temperature.

These modifications will be discussed in the following part describing the hardware of the two-field NMR spectrometer.

#### 3.2.1.1 Shuttle device

**Sample shuttle:** The sample is assembled the same way that the one used for high-resolution relaxometry. A quartz container was chosen, made by synthetic amorphous quartz glass with favourable magnetic properties. Three pieces of quartz are assembled to create one active volume ( $60 \mu$ L) and a "bubble catcher" with a volume of >10  $\mu$ L. The sample is sealed on the upper part using UV-glue and super-glue. Two additional parts are added on both side of the glass container, which are made out of high performance polyimide polymers to resist the strong shocks caused by sudden stops. The plastic part had to be slightly modified to correct an error in the design of the low-field probe (the coils were 15 mm off the expected position for the sample). An additional 1.5 cm long extension has been added in the top of the shuttling sample to position it inside the rf coils.

**Pneumatic system for shuttling:** In order to move inside the stray field gradient of the 600 MHz, the shuttling device used for high-resolution relaxometry has been adapted. As previously, the motion of the sample is controlled by generating positive or negative relative pressure from the top or the bottom of the bore. The system has been designed to apply a maximum pressure of 5 bar. However, due to the new long sample shuttle, we decided to be conservative and not use more than 600 mbar. Two additional specifications have to be added:

- The system has to be slightly over-pressured in order to minimise the quantity of dusty room air that enters inside the spectrometer.
- Pressure settings to go to low-field should be stronger than the one used to go back to high field.

Pressure setting used for the two-field system are written in table 3.1. The shuttle controller used in the two-field system is the same as the one of high-resolution relaxometry.

Set for two-field system			Cleaning of the system	
Pressure HF Pressure LF		Pı	essure HF	Pressure L
From HF to LF			From LF to HF	
350 -300			700	700
From L				
-250 300				
Sample at LF				
200	-100			
Sample				
-40	100			

Table 3.1 – Pressure setting for two-field NMR shuttling in mbar.

**Sample detection:** The system used for monitoring the position of the sample shuttle consists of two commercial optical sensors (*Digital Fiber Sensor FS-N10*). In the high-field (resp. low-field) probe, the path for light crosses the position of the bottom (resp. top) polyimide cap. The sample is detected when the light transmitted decreases below an arbitrary threshold. It is important to notice that the presence of dust can perturb sample detection in both the high- and low-field probes.

**Monitoring of the shuttle delay:** All the shuttle delays are monitored during the experiment, and it is recommended to save the shuttle report (a file with all delays for shuttle transfer) after each experiment. These delays have been analysed in the following graph (Figure 3.1), for a  $R_1$  relaxation experiment.

The analysis of 6584 shuttling events shows two populations of events for both transfers from high- to low-field centres and back. The reason of this behaviour has not been determined at the moment. The difference between transfer delays is relatively small (less than 10 %), and only marginally impacts the quality of experiments.



Figure 3.1 – Analysis on the shuttle transfer delays on the two-field system, during two experiments for longitudinal ( $R_1$ ) relaxation measurements at 0.33 T. Theses graphs summarize 6584 transfers between the two magnetic centres. The average shuttle transfer delay to go from the high- to the low-field centre is 106.8 ms, average shuttle transfer delay is 101.6 ms for the opposite transfer.

#### 3.2.1.2 Second magnetic centre

The first requirement to obtain a second working volume is to reach a magnetic-field homogeneity sufficient to apply radio frequency pulses that cover the full working volume. The target homogeneity in the design of the two-field system was 10 ppm, which is sufficient to apply band-selective pulses on the carbon-13 channel in triple-resonance experiments on proteins or nucleic acids. The variation of the magnetic field along the length of the sample (20 mm) was measured to be 67 mT, or  $2.03 \times 10^5$  ppm before the installation of any additional device.

The magnetic field of the superconducting magnet is very homogeneous in a large volume that corresponds approximately to the region inside the coil. After a transition about 25 cm above the centre of the magnet, the magnetic field decays rapidly along its symmetry axis. This variation is exponential to a good approximation (Figure 3.3.B). A first ferroshim system has been proposed<sup>170</sup> to generate a plateau of magnetic field at 0.33 T, with a homogeneity of a few thousands of ppm. This original ferroshim consists in the combination of three rings of a soft magnetic material, here Fe<sub>52</sub>Ni<sub>48</sub> (Nifemax®). Ferroshims placed in the magnetic field become polarized and provide a magnetic field opposed to  $\vec{B}_0$  along its symmetry axis. This system was designed for a liquid-state Overhauser DNP system,<sup>155,168</sup> with a magnetic centre for electron paramagnetic resonance (EPR) at 0.33 T.

To enhance the homogeneity of the second working volume, a new ferroshim design has been proposed by Bruker, using five rings of FeNi<sub>48</sub> (Figure 3.2). This new design reduced magnetic field inhomogeneities to a few hundreds of ppm. The magnetic field homogeneity has been further improved by the use of strips of  $\mu$ -metal called shim foils, which are commonly used in magnetic resonance imaging (MRI) systems, and a set of twenty room-temperature electric shim coils (system BOSS1). The shimming of the low-field working volume is performed while monitoring the shape of a proton spectrum at low field. An auxiliary Bruker Smart Magnet System (BSMS) has been installed in order to control the homogeneity of the second magnetic centre.



Figure 3.2 – Picture of five ferroshims

The resonance frequencies at 0.33 T  $^{1}H_{LF}$ ,  $^{13}C_{LF}$  and  $^{15}N_{LF}$  are shown in (Table 3.2).

The magnetic field created by the ferroshim is weak and fairly localised. Therefore the magnetic field of the high-field centre and its homogeneity are not affected by this installation.

#### 3.2.1.3 Probes

**Description of the probes:** The high-field triple resonance probe used for this configuration has been described previously.<sup>18</sup> This probe is very similar to high-resolution probes, with two saddle coils. The outer coil is doubly tuned for <sup>1</sup>H and <sup>2</sup>H. The inner coil is tuned for <sup>13</sup>C and <sup>15</sup>N. A shielded z-gradient is also implemented. Both high- and low-field probes are specially designed to operate in spite



Figure 3.3 – A. Ferroshims designed to compensate the inhomogeneity of the magnetic field positioned with respect to the distance from the magnetic centre at 14.1 T. B. Magnetic field profile along the axis of the bore of a 14.1 T superconducting magnet between the centre of the magnet to 0.6 m above this centre: without ferroshims (blue, measured) and with ferroshims (red, simulated). C. Magnetic field profile close to the low-field magnetic centre at 0.33 T.

Table 3.2 – Exact resonance f	requencies of	each nucleus	at 0.33 T after	the installation	of the
five-ring ferroshim inside the	bore.				

Field	Nucleus	SFO1
	$^{1}\mathrm{H}$	600.082402
High field	<sup>13</sup> C	150.896485
	$^{15}$ N	60.807490
	$^{1}\mathrm{H}$	14.009940
Low field	<sup>13</sup> C	3.5203030
	$^{15}$ N	1.419561

of the vibrations caused by the landing of the sample. The triple-resonance low-field probe (Figure 3.4) consists of two coils: the inner coil has three windings and saddle coil geometry. It is optimised for the proton frequency (14.1 MHz). The outer coil has seven windings and saddle coil geometry. Its resonance frequencies are optimised for both carbon-13 (3.52 MHz) and nitrogen-15 (1.42 MHz) frequencies. In addition, this probe is equipped with a coil for z-axis pulsed field gradients (maximum intensity 50 G/cm). The shape of the probe is slightly different from conventional probes. The probe body is longer, in order to reach the working volume at 0.33 T and its tip has the shape of a truncated cone, in order to fit inside the ferroshim system.

No deuterium frequency lock was installed at 0.33 T. The main reason is that the sample is not expected to stay in this working volume for long delays. Thus, this limits the amount of time for



Figure 3.4 – Details on low-field probe. The coil for pulsed field gradients is placed around the radiofrequency coils, which are represented in blue. A spring is used to absorb the energy of the sample at landing. Matching and tuning capacitors are presented in orange.

detection and correction of the magnetic field. In addition, deuterium detection at low field is expected to be quite insensitive. Finally, <sup>15</sup>N, <sup>2</sup>H, and <sup>13</sup>C resonance frequencies are close to each other at such a low field and designing a circuit with all resonance frequencies would have been challenging. A frequency lock system based on proton detection at low field will be implemented if necessary in the near future.

**Troubleshooting the probe:** Working with prototypes requires more hands on operations than working on commercial systems. We have spent a lot of time cleaning the paths of both probes and of the shuttle guide to remove dust that impaired the optical detection of the position of the sample. This problem was severe when the magnet was positioned in a dusty room, next to the work zone during a building renovation. The high-field probe can also be damaged after a high number of shuttling processes ( $\approx 10^5$  transfers). The insert, the tube made of amorphous quartz, which isolates the inner coil from the sample shuttle, cracks. To solve this issue, we were trained to disassemble the probe body, then un-solder the gradient coil and change the quartz insert.

#### 3.2.1.4 Low-field spectrometer

Current commercially available state-of-the-art Avance IIIHD NMR spectrometers can operate down to a frequency of 6 MHz. A low-frequency signal-generating unit (SGU) used for mass spectroscopy has been used in order to generate radiofrequency pulses with defined frequencies, phases and amplitude profiles below 6 MHz. Radio-frequency pulses are amplified by two low-frequency 200 W amplifiers (1020L Power Amplifier from Electronics & Innovation). These amplifiers are used on the carbon-13 channel ( $^{13}C_{LF}$ ) and the nitrogen-15 channel at low field ( $^{15}N_{LF}$ ).

The linearity of amplifiers is important for convenient set up of experiments and is essential if we want to use shape pulses, where the amplitude profile has to be conserved over more than an order of magnitude of amplification. The linearity of 1020L Amplifiers has been tested. The initial linearity was poor. A correction table (Cortab) was built for the rf amplitude, similarly to the correction table available on all high-frequency amplifiers used in Bruker spectrometers. The linearity of the amplifiers after correction is shown in figure 3.5.



Figure 3.5 – Linearity of the amplifiers after a Cortab correction measuring the output power as function of the nominal power for carbon-13 and nitrogen-15 channel.

The system allows to perform an experiment in which one uses up to six channels: such as the combination of three channels at both high and low field (e.g  ${}^{1}H_{LF/HF}$ ,  ${}^{13}C_{LF/HF}$ ,  ${}^{15}N_{LF/HF}$ ) or four channels at high field (e.g.  ${}^{1}H_{HF}$ ,  ${}^{13}C_{HF}$ ,  ${}^{2}H_{HF}$ ,  ${}^{15}N_{HF}$ ) and two channels at low field (e.g.  ${}^{1}H_{LF}$ ,  ${}^{13}C_{LF}$  or  ${}^{1}H_{LF}$ ,  ${}^{15}N_{LF}$ ). The general scheme of the system is shown in figure 3.6. Finally, a conventional gradient amplifier for three-axis gradients is used (GREAT3/10), with one channel used for z-axis gradients at low field and one for z-axis gradients at high field (note that the z axis is aligned with the direction of the static magnetic field).



Figure 3.6 – Schematic representation of all connections in the two-field NMR spectrometer. The red connections are modifications of a conventional spectrometer. The new pieces of equipment added to a conventional NMR spectrometer, in order to complete the two-field NMR spectrometer, are shown with red contours.

#### 3.2.1.5 Temperature control

The temperature of the low-field probe is regulated with a Variable Temperature Unit (VTU). A resistance controlled by the VTU warms up nitrogen gas cooled in a BCU2. As in the high-field probe, the gas used for temperature regulation is not the gas used to push the shuttle. The gas used for temperature regulation passes around the glass insert of the probe. Importantly, the magnetic properties of the FeNi alloy used in the ferroshims are dependent of the temperature.<sup>171</sup> A second channel of the VTU is employed to stabilise the temperature of the ferroshim (at 300.2 K). The nitrogen gas used for the temperature of the ferroshim is at room temperature.

#### 3.2.1.6 Temperature stability

**Stability with respect to the temperature of the Dewar:** The magnetic field and the temperature of the ferroshim have been measured during an overnight experiment and is displayed in figure 3.7. We can see a strong correlation between the temperature of the room and the magnetic field produced. A variation of 0.1K change the magnetic field of  $\sim$ 100 Hz.



Figure 3.7 – Magnetic field (in Hz) during an overnight measurement, compared with the temperature of the room during the same night.

The temperature of the ferroshims is monitored by a thermocouple and regulated by the second BVT. However, the temperature of the Dewar is also important for the stability of the magnetic field in the second magnetic centre. Indeed, changes in the temperature of the room induce slight (>100  $\mu$ m) changes of the position of the superconducting coil with respect to the Dewar. The high-field centre is insensitive to this motion. However, the ferroshims are fixed to the Dewar and the slight change of position of the ferroshims with respect to the superconducting coil leads to a significant shift of the magnetic field in the low-field centre and a degradation of its homogeneity. The sensitivity to the temperature of the Dewar has been measured in three conditions: in a regulated room, just after nitrogen filling and during an overnight experiment. These results can be found in figure 3.7.A/B/C. The evolution of a proton signal are shown in (Figure 3.8.D). This effect has to be taken in account when long experiments have to be performed.



Figure 3.8 – Pseudo 2D experiments performed for monitoring the evolution of the magnetic field in the low-field centre. (A) Experiment performed after nitrogen filling. The room temperature was constant at 25.7° C (at the top of the bore). The temperature of the ferroshims was measured at the beginning and the end of the experiment at 303 K. (B) Experiment performed in a room with poor temperature regulation, where most experiments presented below were recorded. (C) Overnight experiments in a properly temperature-regulated room. (D) 1D spectra extracted from the pseudo 2D of figure 3.7 every ten minutes illustrate the deterioration of the magnetic field homogeneity.

**Temperature of the sample:** The temperature regulation of the sample inside the two-field system is very complex. Ferroshims have to be maintained at a given temperature (300.2 K). The shuttle gas is at room temperature ( $\sim$ 294 K). The low-field and high-field probes can regulate the temperature of the sample in an indirect manner (the nitrogen gas used for regulation is not in direct contact with the sample shuttle). The temperatures of the sample in the high-field and low-field centres have to be adjusted for every experiment. This process is tedious as the low-field and high-field temperature regulation systems are not independent. For that reason, the temperature calibration can be extremely fastidious to perform, especially when the pulse sequence under use can heat the sample.

In this context, the temperature is adjusted for each experiment by running the pulse sequence of choice during 10 minutes, and then immediately measure and adjust the temperatures at the high-field and low-field positions iteratively until both temperatures reach the desired value.

As an example, for an experiment of high-resolution relaxometry designed for the measurement of  $R_1$  at 0.33 T, the high-field probe is set to 298.1 K (flow 300 Liters per Hour), the low-field probe is set at 292.0 K and the final sample temperature measured was 298.0 K. The temperature of the sample was obtained from a 1D deuterium spectrum performed on 50 mM acetic acid.

#### 3.2.1.7 Additional information about the two-field NMR spectrometer

**Matching and tuning at low field:** The only channel that can be tuned in a straightforward manner at low field is the proton channel. In our hands, the resonance frequency of the proton channel barely depends on the sample. Carbon-13 and nitrogen-15 channels were tuned and matched at the installation of the system and are also very stable.
**Softwares:** A new version of Bruker TopSpin has been developed to control two BSMS, six channels and two probes at the same time. The shuttle controller executes a program defined by a stand-alone software, installed on a second computer, which is the one used previously for high-resolution relaxometry.

**Conclusion** A general figure (figure 3.9) represents the elements described above integrated in the two-field NMR spectrometer.



Figure 3.9 – Schematic representation of the two-field NMR spectrometer. Ferroshims positioned in the bore of the magnet provide a plateau at  $B_0^{\rm LF} = 0.33$  T. In addition to a triple-resonance probe at the high-field centre  $B_0^{\rm HF} = 14.1$  T, a second probe is introduced from the top of the bore and positioned at the plateau  $B_0^{\rm LF}$ . The sample is transferred between the two magnetic centres by a pneumatic shuttle system.

#### 3.2.2 First experiments in the low-field center

#### 3.2.2.1 Quality of rf pulses at low field

Homogeneity of the low-field magnetic centre. Obtaining a sufficiently homogeneous plateau in the stray field of a superconducting magnet is a challenge. The variation of the magnetic field along the 20 mm active length of the sample was determined from measurements of the stray field to be 67 mT at 0.33 T, that is  $2.03.10^5$  ppm before compensation, figure 3.11.B shows a typical proton spectrum recorded for a sample of sucrose in D<sub>2</sub>O at 0.33 T using both ferroshims and electric shims. The sensitivity of the experiment was enhanced by polarization at 14.1 T. The full width at half height is 10 ppm, which corresponds to a dramatic improvement of the homogeneity by a factor  $10^4$ . Such homogeneity was

achieved reproducibly on several samples with adjustment of the shim currents limited to the main shim coils, with z,  $z^2$ , and  $z^3$ , geometries.

**Radiofrequency pulses in the low-field centre.** Nutation experiments have been performed at 0.33 T for all channels (*i.e.*, <sup>1</sup>H, <sup>13</sup>C, <sup>15</sup>N) in order to characterize the amplitudes and homogeneities of all radio-frequency fields in the low-field centre (Figure 3.10). The sensitivity was enhanced by allowing the polarization to build up in high field and by observing the signals in the high field. The sample is transferred to the low field where radiofrequency pulses with variable durations are applied. The transverse components of the magnetization are suppressed by gradients during the transfer from low to high field. The remaining longitudinal polarization after the radiofrequency pulse at low field is detected at high field after a 90° pulse.

Table 3.3 – Results of nutation experiments at 0.33 T with polarization and acquisition at high-field

Channel	Power (W)	90° $\sim$ pulse (µs)	$\omega_1/2\pi~({ m kHz})$	$I_{900}/_{180}$
$^{1}\mathbf{H}$	0.5	10.2	24.5	$80\pm2\%$
$^{13}$ C	100	11.1	22.5	$98\pm2\%$
$^{15}$ N	50	20	12.5	$96\pm12\%$

The amplitudes of the rf pulses at low field are comparable or superior to those obtained with the high-field probe. Typical pulse specifications at low field are summarised in table 3.3. Thus, rf pulses with exceptional broadband capabilities can be readily obtained at low field. In addition, the homogeneity of rf pulses at low field is excellent, with  $I_{900}^H/I_{180}^H = 80 \pm 2\%$  for proton,  $I_{900}^C/I_{180}^C = 98 \pm 2\%$  for carbon-13 and  $I_{900}^N/I_{180}^N = 96 \pm 12\%$  for nitrogen-15.



Figure 3.10 – Two-field nutation experiments designed to characterise the rf fields on the three channels at low field. (A) Pulse sequence for nutation experiments at low field. The nutation angle  $\beta$  was varied in a linear fashion. (B) Nutation on the proton channel at 14.1 MHz with  $\omega_1^H/2\pi = 24.5$  kHz; (C) Nutation on the carbon-13 channel at 3.52 MHz with  $\omega_1^C/2\pi = 22.5$  kHz (D) Nutation on the nitrogen-15 channel at 1.42 MHz with  $\omega_1^N/2\pi = 12.5$  kHz. All experiments have been performed with 1 M  $^{13}$ C- $^{15}$ N labelled glycine in D<sub>2</sub>O.

Shaped pulses can be used on all low-field channels. And the same nutation experiment can be performed by replacing the  $\beta$  pulse by a shape pulse.

#### 3.2.2.2 Resolution in the low-field centre

**Resolution tested by 1D NMR:** Several experiments have been recorded in order to test rf pulses and detection at low field. A  ${}^{1}H_{LF}$  1D-spectrum recorded on a 1 M sucrose sample in 90% H<sub>2</sub>O (Figure 3.11) provides a measurement of the magnetic field homogeneity of the magnetic field in the second working volume. In this experiment, the sample is polarized at high field, and transferred to the



Figure 3.11 – <sup>1</sup>H NMR spectrum of 1 M sucrose sample in 90 % H<sub>2</sub>O 10 % D<sub>2</sub>O.

low field centre, where a 90° pulse is applied prior to detection. As described previously, this <sup>1</sup>H sig-

nal is used to shim the low-field centre. A full width at half height of 10 to 12 ppm is routinely obtained.

 ${}^{1}\mathbf{H}_{\mathrm{LF}}$ - ${}^{13}\mathbf{C}_{\mathrm{LF}}$  **HSQC spectra at 0.33 T:** In order to confirm the origin of the broad lines at low field, we have run a heteronuclear two-dimension correlation, where we expected that resonance frequencies due to magnetic field inhomogeneities were fully correlated. A heteronuclear single-quantum coherence experiment (HSQC)<sup>60</sup> at 0.33 T was performed.



Figure 3.12 – (A) Pulse sequence for an HSQC experiment at low field. This experiment includes a recovery delay at high-field to polarise the sample. Narrow (filled) and wide (open) rectangles represent 90° and 180° pulses, respectively. The stabilisation delay  $\tau_s = 100$  ms serves to settle the convection currents induced by the sample landing and vibrations of the sample.  $\delta$  is defined to obtain an echo when  $t_1 = 0$  s. The transfer of polarisation is performed with  $\tau_a = 1/4J_{CH}$ . The phase cycles were:  $\phi_1 = 8\{x\}, 8\{-x\}; \phi_2 = \{x\}, \{-x\}; \phi_3 = 2\{x\}, 2\{-x\}; \phi_4 = 4\{x\}, 4\{-x\}; \phi_{acq} = \{x\}, 2\{-x\}, \{x\}, \{y\}, 2\{-y\}, \{y\}.$ 

Signal enhancement was obtained by polarising the sample at 14.1 T. As expected, a strong correlation of resonance frequencies due to  $B_0$  field inhomogeneities appears along the "diagonal" (Figure 3.12). Note that the chemical shift labels on the axis of the spectrum are not correct. This is due to a bug in an early  $\beta$  version of Topspin that has been solved after this spectrum was recorded.

 ${}^{1}\mathbf{H}_{\mathrm{HF}}$ - ${}^{13}\mathbf{C}_{\mathrm{LF}}$  **2F-HSQC spectra:** So far, with the exception of simple nutation experiments, we have only presented experiments where the polarization takes place at high field and the pulse sequence, including detection, at another field. The two-field NMR spectrometer enables us to design experiments where transfers between the high- and low-field centres take place during the sequence. The first two-dimensional two-field experiment presented here is a two-field heteronuclear single-quantum coherence (2F-HSQC) experiment. The original HSQC experiment was modified to provide a correlation between  ${}^{13}$ C chemical shifts at low field, in the indirect dimension, and proton chemical shifts at high field in the direct dimension. The pulse sequence is shown in figure 3.13. After the recovery delay at high field, the proton polarisation is transferred to the carbon-13 nucleus by an INEPT. The INEPT is interrupted before the first <sup>13</sup>C 90° pulse so that a two-spin order  $2C_zH_z$  is generated prior to the transfer of the sample shuttle to the low-field centre. An anti-phase <sup>13</sup>C coherence is obtained and allowed to evolve under the <sup>13</sup>C chemical shift at low field. Polarisation is stored as a two-spin order  $2C_zH_z$  for the transfer of the sample to the high-field centre. A proton signal is detected under <sup>13</sup>C composite pulse decoupling after a reverse INEPT. The 2F-HSQC spectrum in figure 3.13 was obtained with a 1 M sample of <sup>13</sup>C and <sup>15</sup>N labelled glycine in D<sub>2</sub>O. The linewidth in the indirect  $\omega_1$  dimension is dominated by field inhomogeneities, as expected from the low-field <sup>1</sup>H spectrum (Figure 3.11). Nevertheless, the resolution was sufficient to observe the line splitting due to the large one-bond scalar coupling <sup>1</sup>J<sub>CC</sub> = 53.5 Hz between <sup>13</sup>C<sub> $\alpha$ </sub> and carboxyl <sup>13</sup>C' in glycine. This coupling constant corresponds to 15.2 ppm at 0.33 T.



Figure 3.13 – (A) <sup>1</sup>H<sub>HF</sub>-<sup>13</sup>C<sub>LF</sub> HSQC spectrum of 1 M {<sup>13</sup>C-<sup>15</sup>N}glycine in D<sub>2</sub>O. (B) Pulse sequence for the 2F-HSQC experiment. Proton chemical shifts recorded at high field are correlated with carbon-13 chemical shifts labelled at low field. Narrow (filled) and wide (open) rectangles represent 90° and 180° pulses, respectively. The stabilisation delay  $\tau_s = 100 \text{ ms}$  is used to settle both convection currents induced by the sample landing and vibrations of the sample.  $\delta$  is defined to obtain an echo in the first increment in the indirect dimension. The transfer of polarisation is performed with  $\tau_a = 1/4J_{CH}$ . The transfer delays are  $\tau_{H-L} = 80 \text{ ms}$  to transfer the sample to the low-field centre and  $\tau_{L-H} = 100 \text{ ms}$  to go back to the high-field centre. Pulses are applied along the x-axis of the rotating frame, unless otherwise indicated, the phase cycles are:  $\phi_1 = 8\{x\}, 8\{-x\}; \phi_2 = \{x\}, \{-x\}; \phi_3 = 2\{x\}, 2\{-x\}; \phi_4 = 4\{x\}, 4\{-x\}; \phi_5 = 8\{x\}, 8\{-x\}; \phi_{acq} = \{x\}, 2\{-x\}, \{x\}, \{y\}, 2\{-y\}, \{y\}$ .

#### 3.2.3 Total carbon-13 correlation with the two-field TOCSY experiment

#### 3.2.3.1 Principle of two-field TOCSY

Two-field single-quantum correlations are impaired by the low resolution in the indirect dimension at low field. However, the more favourable properties found at low field may be used to design two-field correlation experiments where all chemical shift evolutions take place at high field. Here, we will use the broadband character of carbon-13 pulses at low field. The TOtal Correlation SpectroscopY (TOCSY)<sup>35</sup> experiment is routinely used to assist the assignment of proton spectra of moderately complex molecules. Carbon-13 correlations with TOCSY are key experiments used for the assignment of carbon-13 signals in uniformly labelled biomolecules. TOCSY experiment reveals through-bond connections within networks of scalar-coupled spins, including between nuclei that are too far from each other to be directly coupled. The main use is to identify <sup>1</sup>H spins or <sup>13</sup>C spins belonging to the same scalar coupling network in a single experiment. Therefore it is used to identify nuclei that belong to the same molecule in a complex mixture or to the same subunit in a polymer (protein, polysaccharide, etc.). This information is essential to assignment strategies of both unlabelled proteins<sup>59</sup> and fully <sup>13</sup>C, <sup>15</sup>N labelled proteins.<sup>51</sup> Pulse sequences developed for the investigation of isotopically enriched proteins became the most popular approach used in NMR studies of proteins because of its simplicity and robustness.<sup>172</sup> Experiments based on the TOCSY transfer are standardly used as a second step in the process of the resonance assignment after a sequential assignment<sup>173</sup> of nuclei of the protein backbone (amide <sup>1</sup>H-<sup>15</sup>N spin pair as well as carbonyl and alpha <sup>13</sup>C), and first carbon of the side-chains ( $C_{\beta}$ ). The resonances of the other carbons and protons in the protein aliphatic side-chains are assigned to the individual amino acids using experiments based on the TOCSY transfer, like HCC(CO)NH- TOCSY<sup>174</sup> and CC(CO)NH-TOCSY<sup>175</sup> experiments.

TOCSY transfer is based on a so-called isotropic mixing. The isotropic mixing irradiation ensures that the effective time-independent part of the Hamiltonian is reduced to the full scalar-coupling part and evolutions due to chemical shifts are suppressed. Then, the magnetization is not transferred only between the directly coupled nuclei but subsequently throughout an entire network of coupled spins. TOCSY is limited for spin systems that display a broad range of carbon-13 chemical shifts. A strong broadband irradiation has to be performed during a sufficient long period in order to record a good TOCSY experiment. The effort for the development of the pulse sequence for isotropic mixing over a wide range of frequencies with a minimal power requirements has resulted in the most popular types of sequences: DIPSI<sup>176</sup> and FLOPSY.<sup>177</sup> However, technical limitations of the maximum irradiation power applied for a relatively long time do not allow to perform the magnetization transfer between all types of carbon-13 nuclei on commonly used NMR spectrometers even if such optimised pulse sequences are used. Therefore, the TOCSY experiment is usually performed for a given type of carbons (e.g. aliphatic side chains in proteins) and correlations between carbon-13 nuclei with large differences of chemical shifts are not observed. This problem of TOCSY becomes even more acute at very high magnetic fields. Here, we present a new TOCSY experiment, where isotropic mixing takes place in the low-field magnetic centre of a two-field NMR spectrometer. This approach overcomes the limitations and makes possible, in principle, the TOCSY transfer between all carbon-13 nuclei irrespective of their chemical shifts, while preserving the resolution of the high magnetic fields used in biomolecular studies (B<sub>0</sub>  $\geq 11.75$  T).

#### 3.2.3.2 Pulse sequence

The two-field system allows us to manipulate the spin system at two fields and combine the advantage of sensitivity and frequency resolution at 14.1 T and a narrow carbon-13 frequency dispersion at 0.33 T. In this condition, isotropic mixing at low field becomes very efficient over the full range of carbon-13 chemical shifts

The scheme of the experiment is shown in figure 3.14. The carbon polarisation is enhanced by nuclear Overhauser enhancement under proton irradiation. The carbon chemical shift is labelled during thet<sub>1</sub> delay in the segment  $90^{\circ}-t_1-90^{\circ}$  before the isotropic mixing period. The carbon-13 polarization is stored along the z axis before the sample is transferred to the low field position. Note that the long and strong gradient experienced by the sample in the stray field of the magnet during the transfer ensures that the carbon longitudinal magnetization is filtered. The isotropic mixing irradiation is applied during the period  $t_{mix}$  at the low-field position after which the sample is transferred back to the high-field position. The applied gradient ensures that the symmetrical gradients during the sample transfers to the low field position and back do not work as a gradient echo. Finally, a 90° pulse is applied and the carbon observable magnetization is detected. In order to compare this experiment to a conventional TOCSY, a reference standard experiment was recorded. The pulse sequence was modified so that the sample stays at the high-field position for isotropic mixing. An additional gradient preceding the  $t_{mix}$  period is required to reproduce the shuttling conditions after the initial  $90^{\circ}-t_1-90^{\circ}$ .



Figure 3.14 – Pulse sequence for the two-field TOCSY experiment. Rectangular 90° pulses are denoted as filled boxes. Proton and nitrogen-15 decoupling schemes during both direct (t<sub>2</sub>) and indirect (t<sub>1</sub>) evolution delays are depicted by open rectangular boxes. FLOPSY isotropic mixing is depicted as an open wide rectangular box. The phase cycles are  $\phi_1 = \{x\}, \{-x\}; \phi_2 = 4\{x\}, 4\{-x\}; \phi_3 = 2\{x\}, 2\{-x\}, \phi_{aq} = \{x\}, 2\{-x\}, \{x\}, \{-x\}, 2\{x\}, \{-x\}\}$ . The smoothed rectangle-shaped pulsed field gradient is represented by a filled rectangle. The sign discrimination in the indirect dimension is achieved by an alternation of  $\phi_1$  phase in the States-TPPI manner.

#### 3.2.3.3 Spectrum description

Uniformly <sup>13</sup>C labelled leucine and phenylalanine have been prepared in D<sub>2</sub>O at a 0.1 M concentration each. The NMR experiments were performed at 25°C. The FLOPSY-16 isotropic mixing<sup>177</sup> has been used in the experiments with amplitudes of the irradiation  $\omega_1/2\pi = 1.5$  kHz and  $\omega_1/2\pi = 5.55$  kHz in experiments with TOCSY transfer performed at the low field position (0.33 T) and the high field (14.1 T) position, respectively. The carrier frequency of the isotropic mixing irradiation was placed at 100 ppm and the FLOPSY16 isotropic mixing lasts  $\tau_{mix,LF} = 31.28$  ms and  $\tau_{mix,HF} = 16.96$  ms in the experiment with TOCSY transfer performed at the low-field and high-field positions, respectively. The carbon polarisation enhancement was achieved by 1,3 s proton composite pulse decoupling applied with the amplitude  $\omega_1/2\pi = 714$  Hz. For this experiment, the durations of the transfers were 105 ms from HF to LF and 80 ms from LF to HF.



Figure 3.15 – Spectrum obtained by the two-field TOCSY experiment (A) and the conventional experiment with isotropic mixing at high field (B). The peaks corresponding to the leucine (blue) and phenylalanine (red) molecules are distinguished by their colours. The signal splitting due to the homonuclear carbon-carbon coupling was suppressed during the processing to simplify the spectrum. All possible correlations between aliphatic carbons (21-56 ppm), aromatic carbons (128–135 ppm) and carbonyls (174–176 ppm) were detected in the two-field TOCSY experiment (A), while only the cross-peaks arising due to effects of strong coupling in the aromatic ring of phenylalanine were detected in the second experiment (B). (C) 1D spectrum extracted from the 2F-TOCSY experiment extracted at 40 ppm (127.6 ppm) for the red (blue) spectrum.

The results of the 2F-TOCSY experiment are shown in figure 3.15. As expected, the isotropic mixing performed with FLOPSY irradiation at 0.33 T is very effective. The off-diagonal peaks between all pairs of carbon-13 nuclei belonging to the same spin system (to the same molecule in our case) were identified. Even the correlation peaks between methyl group and carbonyl carbon-13 nuclei (which represent the most challenging pairs separated by up to 155 ppm) were clearly observed. By contrast, the conventional TOCSY experiment performed at the high field ( $B_0 = 14.1 \text{ T}$ ) provides few correlations. Only the correlation peaks between aromatic carbons were clearly detected because their resonance frequencies were close to the carrier frequency of the isotropic mixing irradiation. Off-diagonal peaks representing the correlations between carbonyls and alpha carbon-13 nuclei were almost at the noise level (Figure 3.15.C) and they appear with a dispersive phase. In addition, no long-distance correlations between aromatic and aliphatic carbons, aromatic carbons and carbonyl, or aliphatic carbons and carbonyl were detected in the spectrum.

Radiofrequency amplitudes for isotropic mixing at the technical limit of our probe were not sufficient to create conditions for efficient TOCSY transfer over the full carbon spectral width. Simulated profiles for the efficiency of the FLOPSY-16 isotropic mixing show that an rf amplitude of 30 kHz would be necessary to cover the 200 ppm of the carbon-13 spectrum at 14.1 T. The rf amplitude increases to 60 kHz on a 1.2 GHz spectrometer. The corresponding power levels are difficult to achieve, let alone sustain for 20 ms on a solution-state probe .

The delays flanking the isotropic mixing period are supposed to be as short as possible to avoid a significant contribution of NOE. Despite the fact that <sup>13</sup>C-<sup>13</sup>C longitudinal cross-relaxation rate is expected to be small due to relatively small <sup>13</sup>C gyromagnetic ratio, a control experiment has been performed to verify that a fairly long time necessary for the sampling shuttling do not result in an unwanted cross-peak intensity contribution originating from NOE instead of through bond transfer. This experiment follows the scheme shown in figure 3.14, but the TOCSY irradiation period was omitted. The results of this experiment is shown in figure 3.16. The lack of off-diagonal peaks even between adjacent carbonyl and aliphatic carbons confirms that the corresponding cross peaks in figure 3.15 did not arise because of NOE transfer. The detected cross-peaks within the aliphatic carbon network can be explained by the effect of a strong coupling between carbons which resonance frequencies are at the low field (0.33 T) close enough (*i.e.* the difference between the resonance frequencies is comparable to the spin-spin scalar coupling). In principle, this experiment would be efficient and as broadband as the 2F-TOCSY experiment if the magnetic field was low enough to ensure that all one-bond carbon-13/carbon-13 scalar couplings are strong couplings. This would require the use of a low-field centre at a magnetic field below 50 mT. Polarization losses due to a longer transfer of the shuttle through regions of lower magnetic field would preclude the application of 2F-TOCSY to macromolecules. In addition, the control of the evolution of the spin system would be limited. Indeed, the change of the magnetic field from 14.1 T to 50 mT would likely lead to the generation of non-desired coherences during the passage through level anti-crossings.<sup>178</sup>



Figure 3.16 – Control spectrum for the 2F-TOCSY experiment, were the pulse sequence described in figure 3.14 is applied without isotropic mixing at low field.

The sensitivity of the proposed experiment is limited by elevated longitudinal auto-relaxation rates at the low field position in macromolecules and during both sample transfers. Therefore, the length of the isotropic mixing irradiation is constrained to prevent a complete polarisation loss due to the rapid relaxation.

#### 3.2.3.4 Conclusion

A new TOCSY experiment is introduced which extends applications of two-field NMR spectroscopy. The presented experiment uses the isotropic mixing at low field while high resolution is preserved by detection of both direct and indirect dimensions at the high-field position. The small differences between carbon-13 resonance frequencies at low field ensure that the isotropic mixing is effective even for carbon-13 nuclei with very different chemical shifts, which is difficult to achieve using the standard TOCSY experiment on high-field spectrometers due to the technical limitations of maximum applied irradiation power. The applicability of the experiment was demonstrated using a solution of two uniformly carbon-13 labelled amino acids (leucine and phenylalanine) where correlations between all carbons were observed. Further applications of this work can be found for the study of carbon-13 labelled macromolecules, such as proteins or nucleic acids.

# 3.3 High-resolution two-field NMR spectroscopy

#### 3.3.1 2F-INAZEQUATE

**Introduction:** As described previously, the homogeneity of the two-field system is not sufficient to produce a high-resolution two-dimension two-field NMR spectrum. To do so, the sequence requires the suppression of heterogenous component of the signal to reduce the line width of the low-field dimension. This can be obtained in a correlation experiment between single-quantum coherences at high field and homonuclear zero-quantum coherences at low field. Zero- and multiple-quantum coherences have been used for many years to obtain high-resolution spectra in inhomogeneous magnetic fields.<sup>38, 179</sup> Indeed the frequency of the zero quantum coherence between spin I and S evolve at the difference of the two frequency of spin I and S, as described in equation 3.1.

$$\Omega_{\rm ZQ} = \omega_I - \omega_S \tag{3.1}$$

In this equation, we can decompose the Larmor frequency of spin I and S ( $\omega_I$ ;  $\omega_S$ ) by introducing three components of magnetic field. The homogeneous part of magnetic field is described by B<sub>0</sub>. Then, the chemical shift specific for each nuclear spin *i* will be described by B<sub>i</sub>. The third contribution is the heterogeneous component of the magnetic field and, due to the proximity of the two spins forming the zero quantum, is identical for both spins and equal to B<sub>ino</sub>.

$$\Omega_{\rm ZQ} = \gamma_I (B_0 + B_I + B_{\rm ino}) - \omega_S (B_0 + B_s + B_{\rm ino})$$

$$(3.2)$$

If I and S are both carbon-13 nuclei, the heterogeneous part is suppressed and the eigenfrequency of the zero-quantum coherence is:

$$\Omega_{\rm ZQ} = \gamma_{I,S} (\mathbf{B}_I - \mathbf{B}_s). \tag{3.3}$$

In the following paragraphs, we modified the refocused Incredible Natural Abundance Double QUAntum Transfer Experiment (INADEQUATE) experiment,<sup>180181</sup> to obtain the two-field Incredible Natural Abundance ZEro QUAntum Transfer Experiment 2F-INAZEQUATE experiment. The 2F-INADEQUATE has also been elaborated to compare the line shape of the 2F-INAZEQUATE and 2F-INADEQUATE.

**Pulse sequence:** The 2F-INAZEQUATE and the gradient-selected refocused INADEQUATE experiments (Figure 3.17) are based on the same principles, so only their differences are discussed. Most pulses are applied at low field, in order to attenuate off-resonance effects of <sup>13</sup>C pulses. The <sup>13</sup>C polarization is enhanced during the recovery delay by a steady-state nuclear Overhauser effect<sup>182,183</sup> obtained by saturating the proton polarisation at high field. The sample is then transferred to the low field where a zero-quantum coherence is excited before the evolution interval t<sub>1</sub>. Scalar couplings between <sup>13</sup>C and <sup>15</sup>N are refocused with a single 180° pulse on the N<sub>LF</sub> channel. The evolution under scalar couplings between <sup>13</sup>C and <sup>1</sup>H is suppressed by composite pulse decoupling on the proton channel. Bipolar pulsed field gradients are applied at low field after the evolution period t<sub>1</sub> where chemical shifts evolve

to select zero-quantum coherences as well as populations. The delay  $2\tau_a$  is used to convert the polarization into in-phase single-quantum coherences  $\hat{C}_y$  and selectively refocus the desired components with a bipolar gradient pair G<sub>2</sub>. In the zero-quantum version of the experiment, G<sub>2</sub> = 0; while in the double-quantum version of the experiment, G<sub>2</sub> = 2G<sub>1</sub>. A 90° pulse on <sup>13</sup>C follows to bring the desired polarization along the z-axis for storage during the transfer to high field. At high field, a single 90° pulse is followed by <sup>13</sup>C signal detection. The efficiency of the excitation of zero-quantum coherences relies on the imbalance between the longitudinal <sup>13</sup>C polarization of the two nuclei before the first 90° pulse. Here, the difference in NOE enhancements between the <sup>13</sup>C nuclei in the explored molecule was sufficient to ensure the efficient excitation of a zero-quantum coherence. Other approaches, such as the application of selective inversion pulses, may be employed to amplify such an imbalance.



Figure 3.17 – Pulse sequence for 2F-INAZEQUATE. Narrow black and wide-open rectangles represent 90° and 180° pulses, respectively. Pulses are applied along the x-axis of the rotating frame unless otherwise indicated. The delays are  $\tau_a = 1/4J_{CC} = 4.7 \text{ ms}$ ,  $\delta$  and  $\delta'$  are chosen to obtain an echo for  $t_1 = 0$  s. Composite pulse decoupling was performed with a WALTZ-16 scheme<sup>184</sup> with rf amplitudes of 1 kHz on both HF and LF proton channels. Gradient pulses were applied along the z-axis and had duration of 1 ms and maximum amplitudes  $G_1 = 0.4 \text{ G/cm}$  and  $G_2 = 0 \text{ G/cm}$ . In the 2F-INADEQUATE variant,  $G_2 = 0.8 \text{ G/cm}$ . The phase cycle was  $\phi_1 = \{x\}, \{-x\}; \phi_2 = \{x\}; \phi_3 = 2\{-x\}, 2\{x\}; \phi_4 = 4\{-x\}, 4\{x\}; \phi_{acq} = \{x\}, \{-x\}$ . Frequency sign discrimination in the indirect dimension was performed with a TPPI scheme<sup>185</sup> applied on  $\phi_2$ .

**Spectrum analysis:** The two-field INAZEQUATE experiment provides a correlation between the single-quantum carbon-13 signals in the direct dimension and zero-quantum frequencies in the indirect dimension. The spectra were obtained with a sample of 1 M <sup>13</sup>C and <sup>15</sup>N labelled of glycine in

 $D_2O$  (Figure 3.18). The signals in the indirect dimension appear at 131.99 ppm, which is close to the difference of 130.99 ppm between the chemical shifts of  ${}^{13}C_{\alpha}$  ( $\delta$  = 41.46 ppm) and  ${}^{13}$ ' ( $\delta$  = 172.43 ppm). The broadening due to field inhomogeneities are largely suppressed since homonuclear zero-quantum coherences are not affected. The full line-width at half height of the  ${}^{13}C'_{signals}$  is 0.11 ppm in the indirect dimension. The comparison between two-field HSQC and two-field INADEQUATE illustrates the gain in resolution in the indirect dimension. The two-field INAZEQUATE experiment thus provides a two-field 2D spectrum with high resolution in both dimensions.



Figure 3.18 – (A) 2D-2F-INAZEQUATE spectrum of 1 M carbon-13 and nitrogen-15 labelled glycine in  $D_2O$ . The signals appear at 131.99 ppm in the vertical indirect dimension. (B) 1D spectrum extracted along the vertical dimension (at 41.30 ppm in the direct dimension) of another 2D 2F-INAZEQUATE spectrum obtained with a smaller spectral width to have a higher resolution, with the same pulse sequence and the same pulse lengths. The narrow peak obtained in B is folded many times so the absolute value of the chemical shift is irrelevant. (C) 1D spectrum extracted from the 2D-INAZEQUATE and 2D-INADEQUATE (with the same processing methods).

**Conclusion:** Two-field correlation spectra with single- or double-quantum coherences at low field display broad lines in the indirect dimension, because of the fairly crude 10 ppm homogeneity of the low magnetic field. Homonuclear correlations obtained in the 2F-INAZEQUATE experiment, by contrast, feature narrow lines in both dimensions. High-resolution in the indirect dimension is achieved by evolution of homonuclear zero-quantum coherences at low field, which is largely insensitive to line broadening due to moderate magnetic field inhomogeneities. The ability to carry out NMR experiments at several fields opens the way to the development of a new library of pulse sequences that are designed to perform different parts of a sequence (polarisation, chemical shift evolution, coherence transfer, etc.) at the most advantageous magnetic field.

#### 3.3.2 2F-HZQC

Introduction As described in the previous section, high-resolution zero-quantum spectra can be obtained in homononuclear systems, provided the effective chemical shift evolution is carefully tailored. Here, we introduce a two-field Heteronuclear Zero-Quantum Correlation experiment (2F-HZQC), where a zero-quantum <sup>13</sup>C-<sup>1</sup>H coherence evolves under the difference of the offsets of <sup>13</sup>C and <sup>1</sup>H with respect to the relevant carrier frequencies at low field, while single-quantum <sup>1</sup>H coherences are detected at high field. The experiment is based on a simple sequence for Heteronuclear Multiple Quantum Coherence (HMQC)<sup>186</sup> and related to methyl HZQC experiments<sup>66</sup> with the following modifications figure 3.19: a two-spin order  $2\hat{H}_z\hat{C}_z$  is generated before each transfer between the high- and low-field centres; a zero-quantum <sup>13</sup>C-<sup>1</sup>H coherence is selected by phase cycling at the lowest field<sup>187</sup>; and a  $\pi$  pulse is used to scale the <sup>1</sup>H offset down by a factor  $\gamma_C/\gamma_H = 0.2514$  in the indirect dimension.

**Pulse sequence:** The evolution of the coherence at low field is thus determined by a combination of the <sup>13</sup>C and <sup>1</sup>H chemical shifts:

$$\omega_{\rm LF}^{eff} = \omega_{\rm LF}(^{13}{\rm C}) - \frac{\gamma_C}{\gamma_H} \omega_{\rm LF}(^{1}{\rm H})$$
(3.4)

The effective propagator  $\hat{U}_{CS}^{^{13}C}$  that drives the carbon chemical shifts during delays  $\tau_1 - \tau_5$  at low field is:

$$\hat{U}_{CS}^{^{13}C} = \exp\left(-in_1\Delta t_1\Omega_C \hat{C}_z\right) \tag{3.5}$$

where  $\Omega_C$  is the carbon-13 offset,  $\Delta t_1 = 1/(2SW)$  is the increment in the indirect dimension, where SW is the spectral width in the indirect dimension. The effective propagator for proton chemical shifts is:

$$\hat{U}_{CS}^{1H} = \exp\left[-in_1\Delta t_1(2c-1)\Omega_H \hat{H}_z\right]$$
(3.6)

where  $\Omega_H$  is the proton offset and  $2c - 1 = \gamma_C / \gamma_H$ . The system does not evolve under any of the heteronuclear couplings  $J({}^{13}C, {}^{1}H)$ . The overall effective propagator is therefore

$$\hat{U}_{tot} = \exp\left[-in_1\Delta t_1 \left(\frac{\gamma_C}{\gamma_H}\Omega_H \hat{H}_z + \Omega_C \hat{C}_z\right)\right]$$
(3.7)

The evolution of a zero-quantum coherence under this propagator is insensitive to magnetic field inhomogeneities.

Two pulse sequences have been used for the 2F-HZQC sequence. The sequence used to record a high-resolution two-field spectrum of tert-butyl alcohol (Figure 3.20) is displayed in figure 3.19.A). A slight variant of the 2F-HZQC experiment was used to obtain the spectra shown in figure 3.19.B. No carbon-13 coherences were generated at high field to avoid losses due to chemical exchange.

A relatively long delay ( $2\delta = 300 \text{ ms}$ ) is used after the return of the sample to the high-field position. A proton inversion pulse in the centre of this delay reduces the recovery of longitudinal proton



Figure 3.19 – Pulse sequence for 2F-HZQC. Narrow black and wide open rectangles represent 90° and 180° pulses, respectively. All pulses are applied along the x-axis of the rotating frame unless otherwise indicated. The phases are cycled as follows:  $\phi_1 = x, -x$ ;  $\phi_2 = 4\{x\}, 4\{y\}; \phi_3 = 2\{x\}, 2\{-x\}, 2\{y\}, 2\{-y\};$  with the receiver phase  $\phi_{acq} = \{x\}, 2\{-x\}, \{x\}$ . The phase  $\phi_3$  was incremented following the TPPI scheme.<sup>188</sup> The delays were  $\tau_a = 1/(4J_{CH})$  with  $J_{CH} = 125$  Hz,  $\Delta$  comprises of the length of the gradient and the gradient recovery delay,  $\tau_1 = (\tau_0 + n_1 \Delta t_1)c$ ,  $\tau_2 = (\tau_0 + n_1 \Delta t_1)(1-c)$ ,  $\tau_3 = \tau_0(2c-1) + n_1 \Delta t_1(c-0.5)$ ,  $\tau_4 = \tau_0 c$ , and  $\tau_5 = \tau_0(1-c)$ ;  $\Delta t_1$  is the time increment in the indirect dimension; and  $n_1$  is the index of the time increment. The constant  $c = (\gamma_C/\gamma_H + 1)/2 = 0.6257$ . Gradients G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub> of 0.9 ms duration were applied along the z-axis with respective amplitudes 10.2 G.cm<sup>-1</sup>, 15.3 G.cm<sup>-1</sup>,  $G_3 = 2(\gamma_C/\gamma_H)G_1$ . GARP composite pulse decoupling<sup>189</sup> was applied on the <sup>13</sup>C channel during detection with  $\omega_1^{GARP}/2\pi = 2.08$  kHz. B)  $G_1 = 20.4$  G.cm<sup>-1</sup>,  $G_2 = 6.12$  G.cm<sup>-1</sup>;  $G_3 = 2.55$  G.cm<sup>-1</sup>;  $G_4 = 6.12$  G.cm<sup>-1</sup>. The additional delays are  $\epsilon = 3 \mu s$  and  $\delta = 150$  ms.

polarisation. Then, three subsequent INEPT-like steps are used to eliminate unwanted <sup>1</sup>H-<sup>12</sup>C signals. At the end of each of them the proton polarisation is aligned with the static magnetic field, while the

coherences of protons that are not directly coupled to <sup>13</sup>C nuclei is dephased by gradients. This filtration is supplemented by a 1 ms trim pulse at the end of the last INEPT step with a 1 ms duration and a 23.7 kHz rf amplitude.

**Spectrum and processing** The natural-abundance high-resolution  $[^{1}H, ^{13}C]$  2F-HZQC spectrum of 0.5 M tert-butanol and 0.5 M acetone in D<sub>2</sub>O is shown in figure 3.20. The methyl signals of both tert-butanol and acetone have full line-widths at half-height below 1 ppm. The single-quantum  $^{13}C$  chemical shifts at low field can be obtained after a shearing transformation of the spectrum, safely assuming that the proton chemical shifts do not depend on the magnetic field. The spectrum has a similar appearance as a conventional high-field heteronuclear single-quantum coherence (HSQC) correlation<sup>60</sup> spectrum.



Figure 3.20 – Natural abundance  $^{13}C_{\rm LF}$ ,  $^{1}H_{\rm HF}$  2F-HZQC spectrum of a sample of 0.5 M acetone and 0.5 M tert-butyl alcohol. Low-frequency one-dimensional  $^{13}C$  spectra (vertical cross-sections) extracted from the 2F-HZQC spectrum at  $\delta(^{1}H_{\rm HF})$  = 1.146 ppm (tert-butyl alcohol) and  $\delta(^{1}H_{\rm HF})$  = 2.128 ppm (acetone). A shearing transformation was used to obtain the  $^{13}C_{\rm LF}$  chemical shift in the indirect dimension. The comparison between 2F-HZQC (orange) and HSQC (blue) on the same sample is also represented.

The generality of two-field NMR was further demonstrated on a protein in solution (Figure 3.21). A high-resolution 2F-HZQC spectrum was obtained on a sample of human ubiquitin (concentration 1.2 mM, pH 4.5) with specific isotope labelling on the  $\delta_1$  <sup>13</sup>C<sup>1</sup>H<sub>3</sub> methyl groups of its seven isoleucine residues within a <sup>2</sup>H,<sup>12</sup>C background.<sup>52</sup> The good sharpness of lines in the indirect dimension is likely due to relaxation interference effects, in a transverse optimized relaxation spectroscopy (TROSY) manner.<sup>190</sup> The sensitivity is sufficient to obtain such a spectrum in 9 hours. Weak artefacts appear 2 ppm away from the most intense peaks in the indirect dimension. The details of this spectrum will be the subject of a forthcoming study.



Figure 3.21 – Two-dimension  ${}^{1}\text{H}{}^{-13}\text{C}$  correlation spectra obtained on human ubiquitin specifically labelled with  ${}^{13}\text{C}{}^{1}\text{H}_{3}$  groups on isoleucine  $\delta_{1}$  positions. An HSQC spectrum recorded at 14.1 T (red) is overlaid to a two-field HZQC spectrum recorded on the two-field spectrometer (blue).

#### 3.3.3 Exchange processes

Most chemical and biological systems are subject to exchange broadening. In many organic molecules and biomolecules, this effect can be subtle and merely gives rise to slight perturbations of NMR spectra. In other instances, e.g., when labile protons exchange with a solvent, or in so-called molten globules,<sup>191–194</sup> chemical exchange may lead to dramatic broadening of spectra that can rule out any NMR measurements. The line broadening  $R_{ex}$  strongly depends on the magnetic field B<sub>0</sub> and reducing the field may lead to dramatic line narrowing.



Figure 3.22 - Graphical representation of chemical exchange studied by NMR

For the sake of simplicity, we discuss the case of a two-site exchange with equal populations. The exchange regime is defined by the ratio between (i) the effective rate  $k_{ex} = k_{-1} + k_1 (k_{-1} \text{ and } k_1 \text{ are the first-order or pseudo-first order forward and backward rates of a chemical reaction or conformational exchange between two environments A and B) and (ii) the difference of precession frequencies of the$ 

exchanging sites:

$$\Delta \Omega = (\delta_A - \delta_B) \gamma B_0, \tag{3.8}$$

where  $\delta_{A,B}$  are the chemical shifts of the nucleus in two environments;  $\gamma_i$  is the gyromagnetic ratio of spin *i* and  $B_0$  is the static field. The maximum broadening, of the order of  $\Delta\Omega$ , occurs near coalescence<sup>195</sup> where  $k_{ex} = \Delta\Omega/(2)1/\sqrt{2}$ . In the fast exchange regime, the line-broadening rate  $R_{ex}$ is proportional to  $(\Delta\Omega)^2$ . Thus, reducing the field  $B_0$  may have dramatic effects on the line widths. To demonstrate the strength of the two-field system for the study of chemical exchange, we have studied a system in exchange, which is at the coalescence at high field and in fast exchange at low field.

**Triazene compound:** The triazene compound under study, also know as triazanylene, is an unsaturated inorganic compound with three nitrogen atoms connected with one double bond. They have been studied previously<sup>39</sup> in the case were the two substituent of the diazoamino group are methyl group, that face chemical exchange (Figure 3.23). The rate constant of this chemical exchange can be



Figure 3.23 – Schematic representation of the chemical exchange that takes place on methyl group substituents of the diazoamino group

tuned by adding an aromatic group to the diazoamino group and changing the substituent of the aromatic ring. Theses molecules can be synthesized without difficulties.<sup>196</sup> Two compounds have been synthesized with the procedure described in figure 3.24.

**Synthesis:** The synthesis was performed by Baptiste Haddou (ENS). The reaction was performed using 11.0 mmol of para-anisidine added to 12 mL of an ice-cooled 9 M HCl aqueous solution in a 100 mL round-bottom flask. After dissolution, a solution of 11.6 mmol (800 mg, 1.05 equiv) of sodium nitrite in 10 mL of ice-cold water was added drop-wise and the mixture was stirred for ca. 30 minutes. 11.6 mmol (2.4 mL, 1.05 equiv) of dimethylamine (40% solution in water) were then added drop-wise, followed by the addition of a 3 M aqueous solution of sodium hydroxide until neutralization. A precipitate appears during neutralization. The heterogeneous reaction mixture was then extracted three times with 25 mL diethyl ether in which the precipitate dissolves. The organic layers were combined, dried over anhydrous MgSO<sub>4</sub>. The volatile components were removed, leading to the product as a yellow-brownish solid. No further purification was necessary.



Figure 3.24 - Schematic representation of chemical reaction

**Chemical Exchange in the triazene compound:** The  $\{^{13}C, ^{1}H\}$  HSQC spectra at 600 MHz, and 26° have been measured and are displayed in figure 3.25 for the bromine or methoxy substitution at the para position of the aromatic group. We can observe that in both cases, the system is in slow exchange, and the signal of both methyl groups can be observed. Nevertheless, in the case of the methoxy substitution, the intensity of methyl groups is low and the peaks are severely broadened.



Figure 3.25 –  $\{^{13}C, ^{1}H\}$  HSQC recorded by a 5 mm TBI probe at 25° C. The sample has been prepared in benzene and has been confined in the shuttle tube and positioned inside a classical NMR tube. For the bromide substitution (A), the doublet is well defined in both dimension, the system is in slow exchange, far from coalescence(B). For the methoxy substitution (C), the signal of exchanging methyl groups are almost invisible(D). Comparison with the methoxy signal shows that the intensity is decreased by a factor ~550.

**2F-HZQC on Triazene:** Here, we use the sensitivity and resolution at high fields combined with a dramatic reduction of exchange-induced line broadening at low fields to study the two triazene compounds. The saturating vapour pressure of benzene forced us to work on a narrow temperature range (from 20 to 35°C) to avoid the confined sample to explode inside the spectrometer. For the bromide substitution, the coalescence cannot be reached at high field by increasing the temperature to 35°C. However, when the chemical shift is labelled at 0.33 T, the system is beyond coalescence, in the fast exchange regime (figure 3.25). In the case of the methoxy substitution, we have been able

to reach the coalescence, which lies on the range 31°C to 40° C. The 2F-HZQC provide any case a sharp and well defined signal (Figure 3.26). These were recorded with a suitable 2F-HZQC sequence (Figure 3.19.B).



Figure 3.26 – 2F-HZQC and HSQC spectra of the bromide substitued triazene (A), and on the methoxy substituted triazene (B).

Line-shape analysis on the methoxy substitued triazene: At 14.1 T, the carbon-13 signals of the two methyl groups of the dimethyl triazene compound with the methoxy group (Figure 3.25.B) are in slow exchange at 20°C. The kinetics of the pseudo-rotation of the two methyl groups in 4-methoxy-N,N-dimethylbenzotriazene was evaluated from a line-shape analysis of the high-field HSQC spectrum at 20°C. We found  $k_{ex}(21^{\circ}C) = (1.7 \pm 0.3)10^3 \text{ s}^{-1}$  (Figure 3.27). The system approaches coalescence at 26°C (Figure 3.27.C). At 31°C, the signals of the exchanging methyl groups are broadened beyond detection (Figure 3.27).

For comparison, the peak height of the signal of the non-exchanging methoxy group is at least 2000 times higher. The decay rate of the carbon-13 coherence is estimated to be about 1000 s<sup>-1</sup> at 14.1 T for an estimated  $k_{ex}k_{ex}(31^{\circ}\text{C}) \sim 3400 \text{ s}^{-1}$ . At 0.33 T, the exchange contribution is predicted to be dramatically reduced to 1.9 s<sup>-1</sup> for  $k_{ex}(31^{\circ}\text{C}) \sim 3400 \text{ s}^{-1}$ , corresponding to a line narrowing by a factor 500. Remarkably, the signal stemming from the exchanging methyl groups is readily observable and barely affected over the whole range of temperatures in the two-field correlation spectra (Figure 3.26).



Figure 3.27 – Line-shape analysis in the indirect carbon-13 dimension (at the <sup>1</sup>H frequency corresponding to the maximum peak intensity) of a high-field HSQC spectrum of 4-methoxy-N,N-dimethylbenzotriazene at 21°C. (A) The continuous line is the intensities predicted from rates determined by fitting using a Markov-chain Monte-Carlo (MCMC) analysis<sup>19733</sup>; the red dots with errors bars show a cross-section extracted from the HSQC, processed with no window function. (B) Distribution of the exchange rates resulting from the MCMC trajectory (the bins are separated by 50 Hz), (C) Histogram of the difference between the resonance frequencies of the two exchanging methyl groups resulting from the MCMC trajectory (the bins are separated by 10 Hz).

#### 3.3.4 Conclusion

Two-field NMR spectroscopy offers the possibility of manipulating nuclear spins such as <sup>15</sup>N, <sup>13</sup>C or <sup>31</sup>P at a field that is most appropriate for them, and detect signals of other nuclei such as <sup>1</sup>H at a higher field where the best resolution and sensitivity can be achieved. The effects of field inhomogeneities can be greatly reduced by exploiting zero-quantum coherences. The superb resolution and sensitivity that can be achieved at high fields can thus be combined with favourable properties offered by lower fields. Most high-field NMR systems may be turned into a two-field spectrometer with a plateau at 0.33 T. The design of the ferroshims requires a simple optimisation for each type of superconducting magnet. Two-field systems with a low-field centre above 0.5 T should rely on other technologies, which are currently available,<sup>198</sup> to obtain a second plateau of magnetic field. This study marks the beginning of a new generation of two-field NMR experiments. Our work opens the way to the characterisation of a wide variety of systems.

# 3.4 Relaxation experiments on small molecules

#### 3.4.1 Introduction

Relaxation times ( $T_1$  and  $T_2$ ) provide essential information on molecular dynamics of small molecules and macromolecules alike, as described in the introduction chapter. Relaxation of small molecules can be interesting for the study of solvent-solute interactions, contrast agents for magnetic resonance imaging<sup>199–201</sup> or ligand-protein interactions.<sup>202</sup> Relaxometry has been used extensively to probe contrast agents and ligand-protein interactions.<sup>76, 203, 204</sup> So far, low-field measurements of relaxation rates have been carried out with no control of relaxation pathways, which is not a problem in studies of proton relaxation in water. However, the presence of cross-relaxation pathways may lead to significant systematic errors in the measured relaxation rates. With the ability to apply rf pulses at low field, the two-field system can be used to measure relaxation rates of small molecules at low field, with the suppression of unwanted relaxation pathways.

In this part, we will discuss a few proof-of-principle  ${}^{13}C_{LF}$  relaxation measurements. Two-field relaxation experiments with  ${}^{13}C$  or  ${}^{1}H$  detection have been designed and compared to a simple field-cycling experiment without any rf pulse at low field. Results are display in this subsection.

#### 3.4.2 Relaxation experiments with <sup>1</sup>H detection

<sup>13</sup>C relaxation rate measurements:

**Longitudinal relaxation of**  ${}^{13}C_{\alpha}$  **in Glycine** We first recorded relaxation experiments similar to those typically employed in high-field studies: inverse experiments where the proton polarisation is transferred to a heteronucleus for relaxation and back to protons for detection. Here, to measure the longitudinal relaxation time T<sub>1</sub> of  ${}^{13}C_{\alpha,\text{LF}}$  in glycine, we generate a longitudinal  ${}^{13}C$  polarisation at high field with a refocused INEPT, followed by transfer to the low-field centre, relaxation at low field and transfer back to high field. This experiment is challenging with the  ${}^{13}C^{1}H_{2}$  spin system in Glycine, since pure carbon-13 longitudinal polarisation is necessarily accompanied with a term  $4C_z(H_x^1H_x^2+H_y^1H_y^1)$ , where the zero-quantum proton part of the operator cannot be suppressed, even by the strong gradient during shuttling.



Figure 3.28 – Longitudinal relaxation decay for the  ${}^{13}C_{\alpha}$  of glycine at 0.33 T.

A nice longitudinal relaxation decay was observed (Figure 3.28). The differential relaxation of the two components of the proton spectrum is due to dipolar cross-relaxation between proton and carbon-13 longitudinal polarisation at low field (the proton polarisation is later converted to antiphase polarisation by the back-INEPT sequence). This observation illustrates the need for the control of cross correlated relaxation pathways at low field.

<sup>15</sup>N relaxation in Urea In order to manipulate an AX spin system, we used a sample of 4M Urea (99% enriched in <sup>15</sup>N) in 90% D<sub>2</sub>O and 10% H<sub>2</sub>O sample. We performed high-field relaxation measurement of <sup>15</sup>N (with inverse detection) using the state-of-art relaxation experiment. Low-field relaxation measurements have been done using 180° pulses on both proton and nitrogen-15 channels at low field to prevent cross-correlated relaxation.



Figure 3.29 – Longitudinal relaxation decay of  $^{15}$ N in urea at 14.1 T (A) and at 0.33 T (B).

These results (Figure 3.29) show that the relaxation effect is apparently multi-exponential, and have to be interpreted carefully. In particular, proton/deuteron exchange is likely to take place during the relaxation delay. At the first order, this leads to a decay of the polarisation (since  ${}^{15}N^{2}H_{2}$  groups are not

observable) but at the second order, this leads to a very slow component in the decay, when two consecutive exchange events ( ${}^{15}N^{1}H_{2}$  to  ${}^{15}N^{2}H_{2}$  back to  ${}^{15}N^{1}H_{2}$ ) lead to the observation of polarisation that was conserved as the very slowly relaxing  ${}^{15}N^{2}H_{2}$  species.

**Relaxation measurements at low field with direct** <sup>13</sup>**C detection at high field:** We have measured longitudinal relaxation times  $T_1$  of <sup>13</sup>C nuclei in an experiment with direct <sup>13</sup>C detection at high field. To increase the sensitivity of the experiment, we have used the effective saturation of <sup>1</sup>H during the polarisation delay to benefit from the polarisation enhancement through the steady-state nuclear Overhauser effect (NOE) (Figure 3.31.A). In order to evaluate the need for the suppression of cross relaxation between <sup>1</sup>H and <sup>13</sup>C nuclei, this experiment has been performed with and without 180° pulses on the proton channel at low field during the relaxation delay. The same experiment have been performed using Waltz-16<sup>184</sup> decoupling at low field on <sup>1</sup>H<sub>LF</sub> (Figure 3.31.B).



Figure 3.30 – Longitudinal relaxation decays for  ${}^{13}C_{\alpha}$  nuclei in glycine in D<sub>2</sub>O. (A) The pulse sequence includes a series of 180° pulses on the proton channel at low field. (B) no rf pulse was applied at low field.

The longitudinal relaxation time obtained in the experiment with inversions on the proton channel during the relaxation delay is  $T_1 = 3.99 \pm 0.03$  s. A mono exponential fit of the decay obtained in the experiment with no pulse at low field gives  $T_1 = 4.71 \pm 0.03$  s. The longer  $T_1$  measured in the absence of pulses on the <sup>1</sup>H channel is likely due to the effect of dipolar cross-relaxation between the <sup>13</sup>C and the two protons. The results for the measurement of longitudinal relaxation time using composite pulse decoupling on the proton channel at low field is also a longer relaxation time, with  $T_1 = 4.47 \pm 0.03$  s.

Measurements of carbon-13 transverse relaxation rates are more challenging in carbon-13 labelled glycine. The evolution under the  $C_{\alpha}C'$  scalar coupling has to be refocused. This requires the use of band-selective pulses at low field, which was difficult because of the limited stability of the magnetic field.



Figure 3.31 – Pulse sequence for the measurement of the longitudinal relaxation time (T<sub>1</sub>) at low field with direct <sup>13</sup>C detection at high field. Narrow (filled) and wide (open) rectangles represent 90° and 180° pulses, respectively. The recovery delay with proton irradiation is 6 s and the stabilisation delay  $\tau_{st} = 100 \text{ ms}$  allows for convection currents and vibrations to settle. (A) The delay  $\tau_a$  is  $\tau_a = 20 \text{ ms}$ . The phase cycles were:  $\phi_1 = 2\{x\}, 2\{-x\}, 2\{y\}, 2\{-y\}; \phi_{acq} = \{x\}, 2\{-x\}, \{x\}, \{y\}, 2\{-y\}, \{y\}$  (B) The relaxation delay  $\tau_{rel}$  is varied from  $\tau_{rel} = 2 \text{ ms}$  s to  $\tau_{rel} = 9.2 \text{ s}$ . The phase cycles were:  $\phi_1 = \{x\}, \{y\}; \phi_2 = 2\{x\}, 2\{-x\}, 2\{-y\}; \phi_{acq} = \{x\}, 2\{-x\}, \{x\}, \{y\}, 2\{-y\}, \{y\}\}$ 

### 3.5 Conclusion:

As very high fields (1 GHz and above) become available, possible issue are complicated to imagine. In addition, carbon-13 relaxation due to chemical shift anisotropy (CSA) can become a major limitation. Many multi-dimensional NMR experiments include delays for evolution under chemical shifts that may not be optimal at one and the same field for all nuclei. In some cases, the best fields may be extremely high, (e.g., to reduce effects of second-order quadrupolar couplings<sup>205</sup>). For single-quantum evolution of carbonyl <sup>13</sup>C nuclei in large proteins, the optimal magnetic field lies below 14.1 T (Chapter *Introduction*). The transverse <sup>13</sup>C relaxation rates in proteins are predicted to be minimal between ca. 2 and 5 T. On the other hand, the optimal field may be close to 1 GHz for transverse optimised relaxation spectroscopy (TROSY) of amide <sup>15</sup>N-<sup>1</sup>H pairs. Moreover, chemical exchange can lead to severe line broadening.

In this chapter, I have presented a breakthrough innovation in NMR spectrometers with the introduction of two-field system. This collaboration with Bruker Biospin has been fruitful and has led not only to the work presented here but to patent applications on two-field NMR experiments. A satisfactory magnetic field homogeneity has been reached for the second working zone, and radiofrequency pulses can be applied at 0.33 T. A classical set of experiment has been set, including nutation experiments (direct or indirect), 2D/2F HSQC spectra or basic relaxation experiments.

This prototype gives access to unusual pulse properties, which led to the development of a 2F-TOCSY. This experiment provided unprecedented results with correlations between methyl and carbonyl carbon-13 nuclei in an amino acid. Two field chemical-shift correlations can be obtained, where the effects of field inhomogeneities are greatly reduced by exploiting zero-quantum coherences.

The superb resolution and sensitivity that can be achieved at high fields can thus be combined with favourable properties offered by lower fields.

This prototype can nevertheless be improved in order to fulfil needs of a protein NMR spectroscopist. The second working zone may be positioned at an higher magnetic field (1 or 2 T), which is possible using permanent magnets, or cryo-shims. This would increase the possibilities of the system, and decrease the relaxation rates at low field in proteins.

This system would also benefit from a new probe at high field. Indeed, the sensitivity of the system is reduced by the design of this probe, where the proton coil is the outer coil. Besides the low-field probe may include a broadband channel which would open the way to studies of nuclei with broad ranges of chemical shift and notoriously fast CSA relaxation, such as <sup>195</sup>Pt, which has a chemical shift range of more than 6000 ppm.

# **Conclusion and prospects**

This manuscript summarized most of my doctoral work from the past three years. This body of work includes relaxation theory, pulse sequence development, hardware innovations, wet lab, and biophysics. This work is structured along three main axes.

Redfield relaxation theory is briefly presented in chapter 1. It was used to adapt a program in which the relaxation matrices of various spin systems are computed analytically. This MATHEMATICA notebook called REDKITE can work on up to six nuclear spins (<sup>1</sup>H, <sup>2</sup>H, <sup>13</sup>C, <sup>15</sup>N) and includes the treatment of the geometry of the system in space. Several computations were performed: relaxation of methyl groups specifically labelled { $^{13}C^{1}H^{2}H_{2}$ }, comparison of relaxation properties with respect to the magnetic field, and work with long lived-states on a C<sup>2</sup>H<sub>2</sub> group. A published review and a submitted paper used this tool to calculate relaxation matrices.

Also, a series of isotopically labelled U-[<sup>2</sup>H, <sup>15</sup>N], Ile- $\delta_1$ [<sup>13</sup>C<sup>1</sup>H<sup>2</sup>H<sub>2</sub>]-ubiquitin sample was produced for the first time in our laboratory. An extensive dataset of relaxation experiments was acquired on a sample of ubiquitin specifically labelled on isoleucine- $\delta_1$  methyl groups, using a conventional high-field spectrometer and high-resolution relaxometry. Pulse sequences were adapted for carbon-13 measurements from nitrogen-15 experiments. This dataset was analyzed using REDKITE and a newly-written MATHEMATICA version of ICARUS. Thanks to this extensive amount of relaxation data, we were able to propose a precise form for the spectral density function assuming the non-correlation of the CC bond motion and methyl rotation. Motions on a timescale from tens of picoseconds to a few nanoseconds were identified in isoleucine sidechains. A manuscript is in the process of being written in collaboration with the group of Raphael Brüschweiler where MD simulations are combined with the analysis of relaxation rates for in-depth understanding of sub- and near- $\tau_c$  motion in ubiquitin.

Finally, we introduce two-field NMR spectroscopy. A two-field NMR spectrometer was developed by Bruker Biospin in the frame of a long-term collaboration with our group. We have been able to run the first experiments and develop tailored experiment on this unique device. The two-field spectrometer includes a commercial high-field spectrometer as well as a second magnetic centre at 0.33 T, which is combined with a shuttling system to move the sample between the two centres. A series of two-field NMR experiments were designed:

- nutation experiments at low magnetic field, with detection at high-field
- longitudinal relaxation experiments (with pulses at low magnetic field) performed on small molecules
- two-field correlation spectroscopy (2F-HSQC and low-field HSQC)

 two-field zero-quantum correlation experiments, homonuclear (2F-INAZEQUATE) and heteronuclear (2F-HZQC)

Properties of the small frequency range of the low magnetic field were exploited to perform a total correlation spectroscopy (2F-TOCSY) experiment, in which the signals of carbon-13 were correlated across the entire chemical-shift range in a mixture of carbon-13-enriched amino-acids. The 2F-HZQC sequence was applied successfully on ubiquitin specifically labelled on isoleucine- $\delta_1$  with {<sup>13</sup>C,<sup>1</sup>H}. Finally the same pulse sequence was applied on a system prone to severe line broadening due to chemical exchange at high field. The "invisible" signal was retrieved by sampling the indirect dimension at 0.33 T using zero-quantum coherences. A patent application was submitted for the first two-field experiments. A first article has just been published, another one submitted and one more is under preparation.

Numerous projects remain to be done in order to extend the usefulness of multiple-field NMR spectroscopy. The methodology for measuring relaxation rates of methyl groups in sidechains should be used in more challenging proteins with prevalent nanosecond timescale motions. With this goal in sight, I designed and optimized a plasmid for the production of Polymerase- $\eta$ .

The application of two-field NMR to relaxation is being initiated. Longitudinal and transverse relaxation rates will be measured at 0.33 T with full control of relaxation pathways. The results will be compared to the one obtained with ICARUS to test the correction on the longitudinal rates from high-resolution relaxometry at low field. Many more experiments are possible, including applications to biomolecules.

# Scientific contributions

- Patent: *Application EP15290102*, Ferrage F., Cousin S., Charlier C., Marquardsen T., Tyburn J.M., Method for two-field nuclear magnetic resonance measurement.
- Cyril Charlier and Samuel F. Cousin, Fabien Ferrage. "Protein Dynamics from Nuclear Magnetic Relaxation". *Chem. Soc. Rev.*, 2016. doi:10.1039/C5CS00832H.
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# Manuscripts submitted or under preparation:

• "Side-Effects of Cross-Polarization Enhanced Dissolution DNP of Deuterated Molecules", D Kurzbach, E Weber, A Jhajharia, S.F. Cousin, A. Sadet, S. Marhabaie, E. Canet, N. Birlirakis, J. Milani, S. Jannin, D. Eschenko, R. Melzi, S. Luetolf, M Sacher, J. Kempf, M. Weller, G. Bodenhausen, D. Abergel. *Submitted.* 

- "High-Resolution Two-Field Nuclear Magnetic Resonance Spectroscopy", S. F. Cousin, C. Charlier, P. Kadeřávek, T. Marquardsen, J.-M. Tyburn, P.-A. Bovier, S. Ulzega, T. Speck, 5 D. Wilhelm, F. Engelke, W. Maas, D. Sakellariou, G. Bodenhausen, P. Pelupessy, F. Ferrage. *Submitted*.
- "Study of Protein Motions by High-Resolution Relaxometry", S.F. Cousin, P. Kadeřávek, N. Bolik-Coulon, F. Ferrage. *Book chapter, invited contribution, work in progress*
- "Full broadband carbon-13 correlations with two-field NMR spectroscopy". Work in progress.
- "Protein sidechain dynamics from high-resolution relaxometry". Work in progress.

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