



生物复杂环境下的核磁共振技术和应用: 机遇与挑战并存

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摘要 核磁共振(nuclear magnetic resonance, NMR)技术是研究生物大分子结构、动力学和相互作用最理想的工具之一. 近年来, 高场NMR波谱仪的使用和NMR实验方法的不断创新, 在很大程度上提高了NMR技术检测的灵敏度和分辨率, 使NMR技术得到快速发展和广泛应用. 目前, 生命科学与物质科学的交叉融合使生命科学研究从观察、描述性科学转向定量、可预测性科学, 多种学科交叉渗透发展已成为科学研究领域十分普遍的现象. 在这种趋势下, 生物复杂环境下的磁共振谱学研究体系日趋成熟, 本文重点回顾和讨论了多学科交叉研究趋势下NMR技术在生物复杂体系中的应用和发展, 主要包括复杂膜环境下的膜蛋白研究、复杂细胞环境下的细胞内NMR(in-cell NMR)研究以及骨组织的固体NMR研究等.

关键词 核磁共振, 生物复杂环境, 膜蛋白, in-cell NMR, 骨生物材料

自1946年核磁共振现象被发现至今, 经过70余年的发展, NMR技术的应用范围已经涵盖了物理学、化学、生物学、医学和材料科学等多种科学研究领域. 早期由于技术条件的限制, NMR检测的灵敏度难以提升, 随后由于超导磁体的使用、傅里叶变换NMR的提出和多维NMR实验方法的建立使得NMR光谱的分辨率得以大幅提高. 20世纪90年代, Wüthrich教授领导的团队实现了利用NMR测定溶液中生物大分子的三维结构, 意味着NMR成为了结构生物学领域强有力的工具之一. 除了解析蛋白空间结构, NMR还可以用来研究蛋白的构象转变和不同时间尺度下的动力学^[1]. NMR技术的主要限制性因素是研究对象的分子量不宜过大, 为了突破限制, 研究人员开发出针对芳香基团^[2]、酰胺基团^[3,4]和甲基基团^[5]的横向弛豫优化谱(transverse relaxation optimized spectroscopy,

TROSY)脉冲序列以及甲基选择性标记的方法^[6], 达到了减缓横向弛豫提高灵敏度的目的.

近年来, 强磁场大科学装置的建立和发展可以协助人们开展生命科学与物质科学的交叉科学研究, 发展新的针对复杂环境的NMR研究方法, 完善生物复杂环境下磁共振谱学的研究体系. 本文着重介绍和讨论NMR技术在以下3种生物复杂环境中的应用和发展: 复杂膜环境下膜蛋白的结构和功能研究; 复杂细胞环境下的NMR研究; 复杂生物材料如骨组织和医用生物材料的固体NMR研究等, 揭示了NMR技术的独特优势及其在多学科领域的广泛适用性.

1 NMR应用于膜环境下膜蛋白的结构功能研究

在生物体已知基因组中, 约有30%开放阅读框

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编码的蛋白质是膜蛋白^[7],包括受体、离子通道和转运蛋白等.膜蛋白在生物体内承担着重要的生物学功能,比如跨膜信号识别和转导、离子和分子的跨膜转运、化学反应的催化、能量代谢等.研究证明,人类许多疾病的发生与膜蛋白的异常表达密切相关^[8,9],而且大多数靶向药物的设计研发是针对膜蛋白^[10],因此解析生理状态下的膜蛋白结构对于认识疾病发生的分子机制及靶向治疗意义重大.与X射线晶体和单颗粒冷冻电子显微镜(cryo-electron microscopy, cryo-EM)结构分析技术相比,NMR技术的主要优点是它与不含去污剂的膜蛋白样品具有相容性,这保证了膜蛋白结构功能研究结果的准确性.为了获得膜蛋白的高分辨光谱,首先要求样品具有高均一性,其次是提高NMR谱仪的信噪比,而最直接的方法就是发展超高场谱仪(1 GHz);固体NMR还涉及到超高旋转速率(>100 kHz)魔角旋转(magic angle spinning, MAS)探头的开发以及动态核极化(dynamic nuclear polarization, DNP)和基于¹H检测技术的快速发展.另外,膜蛋白生物功能的发挥会受到膜组分的影响和调控,与其所处的膜环境密切相关,溶液NMR能够提供溶液中蛋白-膜相互作用的原子层面信息. Shi等人^[11]利用溶液NMR研究了T细胞受体的活化机制,发现Ca²⁺通过与质膜间的电荷相互作用调控蛋白质与生物膜的结合(图1).

1.1 类生物膜环境中的膜蛋白溶液NMR研究

由于膜蛋白存在于体内细胞的生物膜上,具有很强的疏水性,体外不易溶于水,因此目前大部分膜蛋白的NMR研究需要在溶液中加入去污剂帮助膜蛋白溶解复性,这种研究体系通常被称为Detergent Micelle.近年来,研究人员利用这种体系解析了人源线粒体I型电压依赖性阴离子通道(voltage dependent anion channel-1, VDAC-1)的溶液结构,根据光谱中特定氨基酸的化学位移变化确定了该蛋白与小分子配体可能的结合位点^[12,13]. Ouyang等人^[14]发现C型肝炎病毒的p7通道在十二烷基磷酸胆碱(dodecylphosphocholine, DPC) Micelle中形成六聚体且能与该通道抑制剂金刚乙胺(rimantadine)发生特异性结合,表明该六聚体通道具有生物学功能.

然而去污剂体系并非通用的类生物膜模拟体系,例如,溶液NMR研究表明,去污剂会影响G蛋白偶联受体(G protein-coupled receptor, GPCR)发挥生物学

功能时的构象转化,干扰甚至破坏GPCR受体-配体相互作用^[15],类似的破坏作用也存在于其他膜蛋白的结构研究中^[16].最近,研究人员用两亲性高分子取代去污剂来模拟膜环境,成功获得了质量较高可用于结构解析的NMR光谱^[17].为了更接近生理膜环境, Vold等人^[18]将磷脂和去污剂以一定的比例混合得到类膜的磷脂双分子层体系Bicelle,证明该体系同样适用于膜蛋白的NMR研究. Sanders实验室^[19]利用Bicelle体系研究了整联蛋白β1和β3跨膜区和胞质末端结构域跨膜/胞质末端(trans-membrane/cytoplasmic tail, TM/CT)的结构和相互作用,通过顺磁标记,发现将β3上位于膜和水相界面的赖氨酸突变为谷氨酸后,会导致在该残基移出膜之后出现5个残基的疏水片;NMR滴定结果显示该赖氨酸突变会显著降低β3与talin的结合,而不是β1与talin的结合.

Nanodisc体系不包含去污剂,体系中的磷脂双分子层通过膜支架蛋白膜支架蛋白(membrane scaffold protein, MSP)缠绕固定,是目前体外研究膜蛋白最理想的类膜体系^[20].2009年, Raschle等人^[21]首次将人源线粒体VDAC-1组装到Nanodisc中,利用NMR证明了蛋白折叠的正确性以及与还原型辅酶I(nicotinamide adenine dinucleotide, NADH)结合的功能完整性.同年, Glück等人^[22]也成功将单跨膜蛋白CD4组装到Nanodisc中,获得了分散性较好的¹H-¹³C异核单量子相关谱(heteronuclear single quantum coherence, HSQC)二维谱,这些研究意味着利用Nanodisc体系研究膜蛋白结构和功能的可行性.随着NMR实验方法的不断优化^[23]和Nanodisc体系研究的日趋成熟,膜蛋白结构和功能研究也相应取得了一系列进展^[24-29].细菌外膜蛋白OmpX, OmpA和Ail的溶液结构相继被解析出来^[24,27],因为这些蛋白的跨膜区主要是由β片交联串联,NMR光谱中包含了丰富的距离约束信息,因此十分有利于结构计算. Zhang等人^[28]首次将细胞色素P-450B4和它的氧化还原伴侣细胞色素b5的复合物组装到Nanodisc中,利用NMR鉴定了复合物的结合界面. Wagner研究组^[24]开发出直径更小的Nanodisc,使细菌外膜蛋白OmpX和视紫红质蛋白的NMR共振指认和结构测定都得到很大程度地改进,这种工程化的小尺寸Nanodisc非常适用于研究膜蛋白与它的伴侣蛋白之间的相互作用.该研究组^[26]还设计了一种共价环化Nanodisc(covalently circularized nanodiscs, cNDs),这种Nanodisc具有较强的稳定性、限定

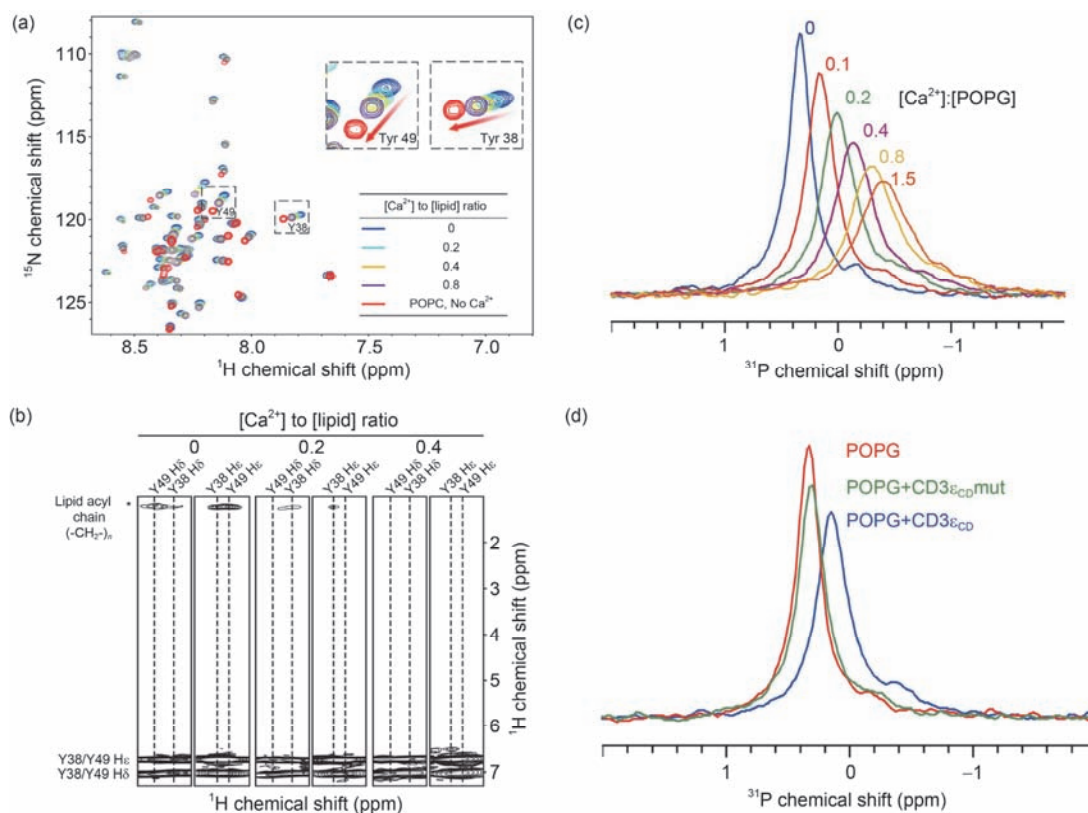


图 1 Ca^{2+} 通过改变磷脂的电荷属性调控T细胞受体活化^[11]。(a) 1-棕榈酰-2-油酰基甘油-3-磷酸胆碱 (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine, POPC) bicelles- $\text{CD3}\epsilon_{\text{CD}}$ (红色)和 1-棕榈酰-2-油酰基-Sn-甘油-3-磷-(1'-Rac 甘油)(1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol), POPG) bicelles- $\text{CD3}\epsilon_{\text{CD}}$ (蓝色的) ^{15}N - ^1H HSQC(heteronuclear single quantum coherence)光谱对比以及滴加不同浓度的 Ca^{2+} (青色、黄色和紫色)对 POPG bicelles- $\text{CD3}\epsilon_{\text{CD}}$ 光谱的影响。(b) 滴加不同浓度的 Ca^{2+} 对Tyr38 和Tyr49 芳香环质子 $\text{H}\epsilon/\text{H}\delta$ 与脂酰基链亚甲基质子的核欧佛豪瑟效应频谱(nuclear overhauser effect spectroscopy, NOESY)光谱的影响。(c) 随着 Ca^{2+} 滴定浓度的升高, Nanodisc中POPG的P信号化学位移扰动也随之增大。(d) 与 Ca^{2+} 滴定结果类似, $\text{CD3}\epsilon_{\text{CD}}$ 的滴定也会引起POPG的P信号发生化学位移的同向扰动且扰动的趋势一致, 表明 Ca^{2+} 和 $\text{CD3}\epsilon_{\text{CD}}$ 均是通过离子作用结合在磷脂头相同的部位

Figure 1 Ca^{2+} regulates T cell receptor activation by altering the charge properties of phospholipids^[11]. (a) Superimposed ^{15}N - ^1H HSQC (heteronuclear single quantum coherence) spectra of $\text{CD3}\epsilon_{\text{CD}}$ with POPG Bicelles (blue), $\text{CD3}\epsilon_{\text{CD}}$ with POPG(1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)) Bicelles and Ca^{2+} with different concentrations (cyan, yellow and purple), and $\text{CD3}\epsilon_{\text{CD}}$ with control POPC(1-palmitoyl-2-oleoyl-glycero-3-phosphocholine) Bicelles (red). (b) Strips from aromatic NOESY spectra showing NOEs between the aromatic protons $\text{H}\epsilon/\text{H}\delta$ of Y38 and Y49 and the methylene protons in the lipid acyl chains, Ca^{2+} was titrated in at a molar ratio relative to POPG of 0.2 to 0.4. (c) Ca^{2+} induced a substantial chemical shift change of the phosphorus signal of the anionic POPG. (d) The $\text{CD3}\epsilon_{\text{CD}}$ peptide induced a shift in the same direction for the POPG phosphorus signal as Ca^{2+} did, implying that both Ca^{2+} and basic residues in $\text{CD3}\epsilon_{\text{CD}}$ bound to the same region in the phospholipid headgroup via ionic interactions

的直径尺寸和形状的可调控性, 作者利用cNDs研究了简单无包膜病毒引发感染时基因组的跨膜转运机制。同样, 虽然许多膜蛋白在不同的膜体系中显示出相似的结构, 但去污剂的存在会对这些蛋白的生物活性造成破坏性的影响。细菌外膜蛋白Ail在Micelle体系^[30]和Nanodisc体系^[31]中的 ^1H - ^{15}N HSQC光谱十分类似, 溶液结构也基本相同, 但Micelle中的Ail与配体结合活力遭到去污剂破坏, 无法进行功能实验, 因此使用Nanodisc体系获得的膜蛋白结构更具有说服力。

1.2 膜蛋白的固体NMR研究

固体NMR(solid-state NMR, ssNMR)不受限于样品分子量的大小, 且样品中不含去污剂。近年来, 高场和高效射频探头的开发利用增加了更多实验可行性, 得到的特定位点共振指认为结构计算提供了更丰富的约束信息^[32]。在定向样品(oriented sample, OS)的ssNMR实验中, 定向约束提供的信息包含了蛋白三维结构、取向和动力学, 可以作为一种独立的方法验证结构的精确性^[33,34]。磷脂双分子层包埋的膜蛋白, 其单轴定向样品的NMR光谱可以直接反映出

蛋白的构象特性, 例如人的钠钾ATP酶调节蛋白FXD2的 ^1H - ^{15}N 魔角自旋交换极化翻转(polarization inversion with spin exchange and the magic angle, PISEMA)光谱就提供了非常丰富的蛋白与磷脂双分子层的取向信息^[35].

目前, 魔角旋转技术是ssNMR研究领域的主流研究方向, MAS的高分辨率光谱包含了关于各向同性化学位移、原子间距离和扭转角的信息. 由于MAS会移除 ^{13}C - ^{13}C 同核偶极偶联, 因此通过基于 ^{13}C 检测的一系列实验可以完成共振指认, 而利用标记原子之间偶极相互作用的重新耦合可以测量蛋白不同残基特定位点之间的精确距离($\leq 0.1\sim 5$ Å), 为蛋白结构计算提供极高分辨率的距离约束. 近年来, 膜蛋白的MAS固态NMR研究取得了一系列进展^[36-44]. 为了避免2D晶体或微晶样品制备体系的缺陷, 使蛋白的构象状态在NMR可探测的时间尺度上表现为整体的均一性, Yao等人^[45]将细菌外膜蛋白Ail组装到Liposome中, 通过采集二维 ^{13}C - ^{13}C 相关谱推断出Liposome样品的高均一性. 作者对样品采取 ^{15}N 和 ^{13}C 全标记以及部分 ^2H 标记的策略, 其中氘化可以稀释 ^1H 浓度, 抑制同核 ^1H - ^1H 之间的强偶极偶联, 从而延长 ^{13}C 和 ^{15}N 相干的寿命, 提高检测的灵敏度和分辨率.

最近, 基于 ^1H 检测的NMR实验已成为提高ssNMR灵敏度和分辨率的重要技术. 质子化蛋白的

MAS光谱缺乏位点特异性的分辨率, 因为同核 ^1H - ^1H 之间的强偶极偶联会导致较大的均一性谱线增宽. 氘化已被证明是去除 ^1H - ^1H 偶极相互作用, 减少质子线宽有效实用的方法, 由于较高的MAS速率会导致更长的质子相干寿命, 因此在60 kHz甚至更快的MAS下不需要这种额外的同位素稀释^[43]. 借助氘化-主链交换, 基于 ^1H 检测的ssNMR方法已被用于多种膜蛋白的结构研究^[36,43-48]. 如图2所示, Ail的 ^1H - ^{15}N CP-HSQC ssNMR光谱(900 MHz谱仪, 30°C)与其在Nanodiscs体系下的TROSY光谱具有很高的吻合度^[45], 显示出ssNMR在膜蛋白结构研究中的巨大潜力.

除了快速MAS下的 ^1H 检测技术, DNP技术可以使ssNMR的信号灵敏度提高2个数量级. DNP是将大量的电子自旋极化转移到核自旋, 从而产生超极化核自旋状态. 目前, DNP应用于生物样品的ssNMR检测方法已日趋成熟^[49], 使得膜蛋白的DNP ssNMR研究工作相继被报道出来^[50-57]. Griffin研究组^[50,51]首次用DNP增强的NMR研究了细菌视紫红质(bacteriorhodopsin, BR)的光循环, 鉴定了循环中BR的早期K中间态和4个之前未检测到的L中间态. DNP增强有利于采集二维 ^{15}N - ^{13}C 化学位移相关谱, 得到视网膜(retinal)中间态 ^{15}N 和 ^{13}C 的化学位移, 该结果与retinal的平面构象相吻合. 最近, Glaubitz研究组^[55]研究了绿色蛋白视紫红质(green proteorhodopsin, GPR)的寡

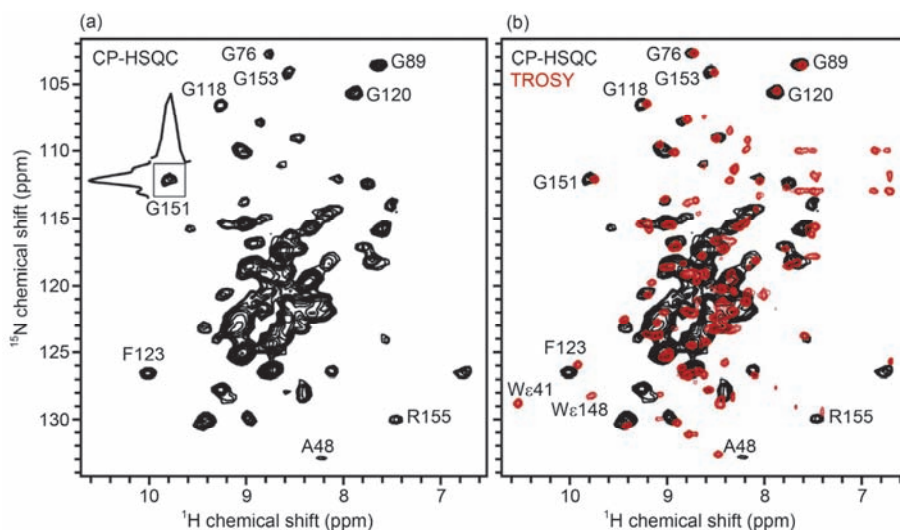


图2 Ail在磷脂双层膜中的2D ^1H - ^{15}N 相关谱^[45]. (a) Ail(^{15}N , ^{13}C 和 ^2H 标记)在脂质体中的CP-HSQC固体NMR光谱. (b) Ail在nanodiscs选用 ^2H 标记的磷脂)中的TROSY-HSQC溶液NMR光谱(红色)与(a)中的光谱(黑色)进行叠加比较

Figure 2 2D ^1H - ^{15}N correlation spectra of Ail in phospholipid bilayers^[45]. (a) Solid-state NMR ^1H -detected CP-HSQC spectrum of (^{15}N , ^{13}C , ^2H) labeled Ail in liposomes. (b) Solution NMR ^1H -detected TROSY-HSQC spectrum (red) of Ail in nanodiscs prepared with ^2H labeled lipids. The solid-state NMR CP-HSQC spectrum (black) is superimposed

聚状态, 并利用DNP NMR确定了蛋白脂质体中GPR寡聚界面的结构约束. DNP增强保证了由 ^{15}N 和 ^{13}C 标记的原体混合而成的寡聚物样品 ^{13}C - ^{15}N 距离测量实验的完成, 提供了寡聚界面的结构约束数据.

2 In-cell NMR的发展和應用

细胞是生命活动的最基本单元, 细胞内的一切生命活动在时间和空间上都受到精确的调控, 为了揭示生物体在生理和病理条件下的调控网络和精细机理, 就必须在活细胞水平上检测细胞内的信号转导、应激反应和能量代谢等生命活动. 研究显示, 细胞内环境虽然十分复杂和拥挤, 但细胞可以对胞内各种组分施加精确有序的物理化学约束, 从而在多重空间和时间尺度上控制其性质^[58]. 细胞内环境下的蛋白行为会受到诸多物理因素, 如大分子挤压、胞内黏度和大量生物反应的影响. 近年来, 科研人员逐渐把兴趣集中在活体细胞内蛋白结构和功能的研究, 其中in-cell NMR技术是研究活细胞内生物大分子最理想的工具, 因为它对细胞没有破坏性, 可以在原子分辨率水平上提供活细胞内生物大分子的结构和动力学信息^[59,60], 即实现原位结构分析. 实际应用中in-cell NMR也存在一定的局限性: 首先, 虽然蛋白的in-cell NMR研究是在活体细胞内完成的, 但NMR检测的并不是细胞原有的蛋白, 而是过表达的高浓度蛋白; 其次, 细菌体内大部分折叠完全的过表达蛋白会表现出NMR光谱的缺失, 其原因可能是细菌胞质的过度拥挤以及蛋白与胞质内其他蛋白相互作用造成的, 因此必须用稳定同位素(如 ^{13}C , ^{15}N 和 ^{19}F)充分标记靶蛋白, 同时保持细胞内其他分子的NMR惰性. 目前, in-cell NMR技术已被成功应用于原核细胞和真核细胞.

2.1 原核细胞和真核细胞的in-cell NMR研究

2001年, Serber等人^[61,62]首次在大肠杆菌(*Escherichia coli*, *E. coli*)细胞中进行了NMR研究. 研究人员成功在*E. coli*细胞内过量表达细菌的NmerA蛋白和人的钙调蛋白, 探索了实验过程中细胞活力的变化, 尝试了不同种类同位素标记并分析了NMR线宽变化. 此项工作之后, 原核细胞NMR技术得到快速发展, 包括多种蛋白在同一细胞内的有序表达、单个蛋白的选择性标记^[63,64]以及in-cell NMR方法的探索^[65], 使得在细胞内研究蛋白与蛋白之间相互作用、蛋白与小分子相互作用以及解析蛋白结构成为可能.

真核细胞NMR研究的难点在于如何将原核细胞表达系统富集的目的蛋白递送至真核细胞内, 目前已有4种方法被报道出来(图3). 2006年, Wagner研究组^[66]和Shirakawa研究组^[67]分别借助显微注射技术成功将靶蛋白递送至爪蟾卵母细胞中, 这种方法同样适用于核酸的in-cell NMR研究^[68]. 该方法具有优良的标记选择性和显微注射的小尺寸, 能有效去除背景NMR信号, 但为了保持细胞活性, 必须使用高度浓缩的蛋白和恒定的实验温度. 2009年, Inomata等人^[69]将一段细胞穿膜肽(cell-penetrating peptide, CPP)通过二硫键融合在靶蛋白表面, 借助CPP穿透细胞膜的特性将融合蛋白成功送入HeLa细胞内, CPP被切除后释放靶蛋白. 同年, Ogino等人^[70]利用链球菌溶血素O(streptolysin O)在细胞膜上的穿孔效应将靶蛋白转运至细胞内, 为防止细胞死亡, 需及时加入 Ca^{2+} 促进细胞膜愈合. 2016年, Selenko研究组^[71,72]发现利用电穿孔的方法可以有效的将靶蛋白转运至细胞内, 而且这种方法适用于更多种类的蛋白和真核细胞系. 近年来, 研究人员发现可以直接在真核细胞内富集靶蛋白并进行同位素标记, 相关工作已在酵母细胞^[73]、昆虫细胞^[74]和哺乳动物细胞^[75]中取得成功.

2.2 In-cell NMR应用于细胞内蛋白结构解析

蛋白的高分辨率结构是研究蛋白功能的基础, 目前结构解析的方法均需要将蛋白从细胞中分离出来,

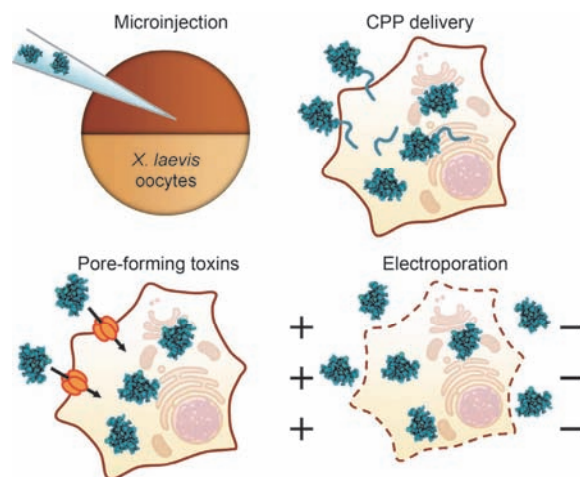


图3 外源表达的靶蛋白(蓝色)可通过显微注射递送到卵母细胞中, 或借助细胞穿膜肽(CPP)、成孔毒素和电穿孔等方法递送到人体细胞中^[59]
Figure 3 Exogenous proteins (blue) can be delivered to *X. laevis* oocytes by microinjection or to human cells exploiting either cell-penetrating peptides (CPP), cell permeabilization by pore-forming toxins or electroporation^[59]

因此这些结果是否真实反映了蛋白在活体细胞内的存在状态仍不确定. in-cell NMR由于其监测对象位于活体细胞内, 因此可以直接观测到细胞内的生物反应过程. 2009年, Sakakibara等人^[65]首次在*E. coli*细胞中利用NMR解析了重金属结合蛋白TTHA1718的溶液结构. 为了增加分辨率, 作者用同位素¹³C-¹⁵N标记样品, 用甲基¹³C选择性标记侧链, 并采用非线性采样方法缩短采样时间. 2016年, Pan等人^[76]和Müntener等人^[77]分别独立地提出一种适合非洲爪蟾卵母细胞的蛋白结构解析方法. 该方法主要是通过体外化学修饰, 将顺磁性镧系金属离子标签接到靶蛋白上, 然后将靶蛋白转运到爪蟾卵母细胞中进行NMR研究. 由于在蛋白内引入了含有未配对电子的顺磁性物质, 因此可以通过观测未配对电子与观测核间的顺磁效应, 如赝接触位移(pseudo-contact shifts, PCSs)和顺磁残余偶极耦合(para-magnetic residual dipolar couplings, pRDCs)得到非常有用的结构信息. Hikone等人^[78]利用类似的标记技术标记了泛素蛋白, 通过电穿孔方法将目的蛋白转运到HeLa细胞中, 并在蛋白的¹H-¹⁵N选择性优化翻转角短瞬态异核多量子相干谱(selective optimized flip-angle short- transient heteronuclear multiple quantum coherence, SOFAST-HMQC)光谱中观测到了赝接触位移. Ye等人^[79]通过在钙调蛋白-肌球蛋白轻链激酶(calmodulin-Myosin light chain kinase, CaM-MLCK)上引入顺磁性Tb³⁺, 首次在爪蟾卵母细胞中观测到CaM-MLCK的¹⁹F赝接触位移, 验证了钙调蛋白随Ca²⁺浓度变化而发生的构象转变(图4).

2012年, Reckel等人^[80]发现冷冻*E. coli*细胞内生物大分子复合物的蛋白信号可以被固体NMR检测到. 同年, Renault等人^[81]利用固体NMR解析了完整*E. coli*细胞内的细菌外膜蛋白Ompa结构. 最近研究发现, 通过添加顺磁性试剂, 结合微波照射促进未配对电子向核的高度极化转移, 可以使in-cell MAS NMR的信号灵敏度增加2个数量级^[82]. 借助于DNP MAS NMR技术, 膜整合蛋白PagL^[83]和细菌细胞色素b5蛋白^[84]在细胞内的结构和动力学信息得到了进一步验证. Frederick等人^[85]利用DNP NMR观测到酵母朊病毒蛋白Sup35在体外和细胞内存在结构差异, 证明了细胞内环境会影响Sup35的结构折叠. 固然, DNP技术可以显著提高in-cell NMR信号的灵敏度, 但极低温度(<100 K)的实验条件决定了靶蛋白所处的环境为冷冻状态下的细胞, 不利于细胞活性的维持. 因此

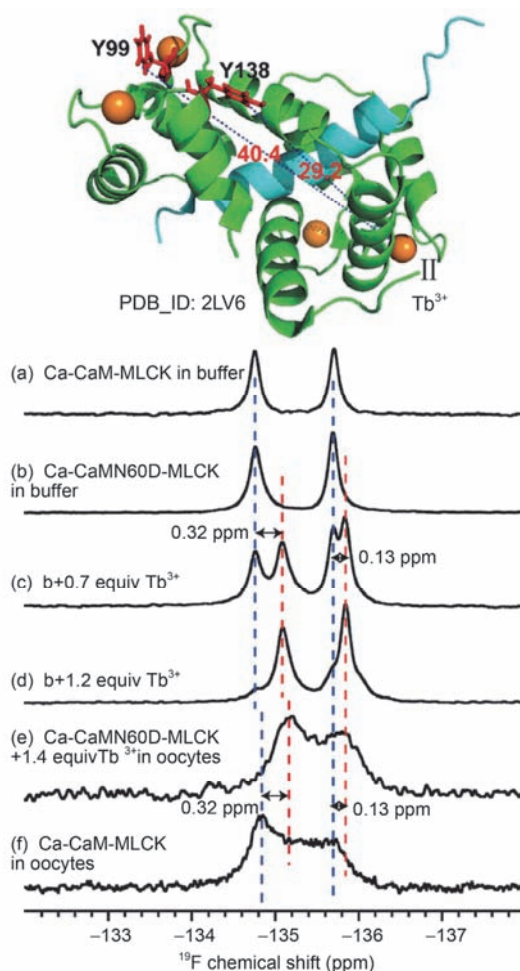


图4 一维¹⁹F NMR光谱. 在溶液中采集的Ca-CaM-MLCK(a)和Ca-CaMN60D-MLCK(b)以及包含0.7倍Tb³⁺(c)和1.2倍Tb³⁺(d)的Ca-CaMN60D-MLCK一维¹⁹F NMR光谱, 卵母细胞中采集的包含1.4倍Tb³⁺的Ca-CaMN60D-MLCK(Ca²⁺与CaMN60D-MLCK的摩尔比为5:1)(e)和抗磁性Ca-CaM-MLCK(f)一维¹⁹F NMR光谱. 顶部显示的是Ca-CaMN60D-MLCK模型(PDB_ID: 2LV6), 以及¹⁹F核与顺磁中心(II, Tb³⁺)之间的距离^[79]

Figure 4 One-dimensional ¹⁹F NMR spectra of Ca-CaM-MLCK (a), Ca-CaMN60D-MLCK (b), and Ca-CaMN60D-MLCK in the presence of 0.7 equiv of Tb³⁺ (c), 1.2 equiv of Tb³⁺ (d) in buffer, or in the presence of 1.4 equiv of Tb³⁺ in *Xenopus* oocytes (a molar ratio of Ca²⁺ to CaMN60D-MLCK of 5:1) (e) and diamagnetic Ca-CaM-MLCK in oocytes (f). The Ca-CaMN60D-MLCK model is shown at the top (PDB_ID: 2LV6). The distances between the ¹⁹F nuclei and the paramagnetic center (II, Tb³⁺) are indicated^[79]

从生理条件角度讲, 液体in-cell NMR更适合细胞内蛋白的原位结构分析和功能研究.

2.3 In-cell NMR应用于细胞内蛋白行为的研究

Shekhtman研究组^[63]发明了结构相互作用的NMR波谱分析(structural interactions using NMR spectroscopy,

STINT-NMR), 可以在细菌细胞内有序表达两种或者更多蛋白, 并且只标记需要观测的蛋白. 利用这种方法, 作者发现泛素蛋白(ubiquitin, Ub)与底物信号转导衔接分子(signal-transducing adaptor molecule, STAM2)和肝细胞生长因子调控的底物(hepatocyte growth factor regulated substrate, Hrs)的相互作用依赖于底物磷酸化^[86], 之后的另一项工作则揭示了原核细胞的泛素样蛋白Pup与结核分枝杆菌蛋白酶体不同亚基之间相互作用的关键氨基酸残基^[87]. Kubo等人^[88]发明了凝胶包封的生物反应器用于研究哺乳动物细胞内蛋白的相互作用, 这种反应器可以增加样品稳定性, 达到延长采样时间的目的.

In-cell NMR可以直接观测到活体细胞内蛋白-药物的相互作用, 以及细胞内环境对药物运输、定位和选择性结合的影响, 有助于揭示药物在细胞内的反应代谢过程, 显示了其在药物设计领域的重大应用潜力. Hubbard等人^[89]首次利用in-cell NMR探索了*E. coli*细胞内信号转导蛋白CheY与药物BRL-16492PA的相互作用机制, 通过对比CheY在体外与细胞内以及加药前后的¹H-¹⁵N HSQC光谱差异, 发现药物首先穿过细菌细胞膜进入胞内, 然后与靶蛋白CheY发生相互作用. Arnesano等人^[90]利用相同的方法观测到顺铂(cisplatin)可以穿过细胞膜并与铜伴侣分子Atox1(antioxidant-1)的CxxC金属结合基序发生相互作用.

由于蛋白在细胞内会与生物大分子和细胞骨架发生特异性和非特异性相互作用, 使得蛋白在细胞内的折叠稳定性与体外相比存在一定的差异, in-cell NMR检测蛋白稳定性的优势在于其可以在原子水平上对稳定性进行量化. Inomata等人^[69]利用氘气交换结合NMR检测了细胞内Ub的稳定性, 通过测量¹H-¹⁵N相关谱估算Ub蛋白主链酰胺上氢原子的交换速率, 结果显示细胞内检测到的交换速率高于体外, 表明细胞内的Ub与其他蛋白发生了相互作用, 导致折叠的不稳定性和较高的氘气交换速率. 另外, ¹⁹F标记也被广泛用于检测细胞内蛋白折叠的热力学和构象变化^[91,92]. Smith等人^[93]利用¹⁹F标记技术分析了蛋白表面在信号转导蛋白Drk N端的折叠动力学和热力学中的生理学作用, 发现体外溶液环境并不能使蛋白表面的生理学性质得到充分体现, 证明表面静电作用对细胞内蛋白折叠的稳定性具有至关重要的作用.

如上所言, NMR技术已在膜蛋白和蛋白的in-cell NMR研究这两种复杂体系中显示出巨大的应用潜力, 而对于更具挑战性的复杂体系如天然生物组织骨头, 固体NMR技术依然受到研究人员的青睐, 成为研究骨组织结构和动力学信息的重要工具.

3 固体NMR应用于骨组织材料的研究

骨头是一种组成复杂的复合物材料, 有着层次分明的结构. 骨头主要由两个部分组成: 有机成分和无机成分, 两者有序结合, 使骨头具有各种机械性能, 并且二者在骨头中还发挥着独特的生物作用. 骨头的有机成分主要由有机基质组成, 其中90%的有机基质是I型胶原蛋白, 剩下的10%大约是由两百种非胶原蛋白分子组成, 这些蛋白分子在骨头中发挥着重要的调控作用, 例如: 调控骨头矿化、促进骨头生长和骨头中钙和磷的吸收等. 骨头的无机成分主要由羟基磷灰石(hydroxyapatite, HA, Ca₁₀(PO₄)₆(OH)₂)纳米晶构成. 另外, 无机成分中通常还含有多种微量的阴离子和金属阳离子, 例如CO₃²⁻, F⁻, Na⁺和Mg²⁺等, 这些微量的离子对骨头的结构与功能也有重要的影响.

为了探测骨组织原子尺度的结构与动力学信息, 传统的生物物理分析技术已显示出其自身的局限性, ssNMR技术则脱颖而出, 成为研究骨头微观结构最理想的工具. ssNMR技术能在纳米尺度上对天然骨组织中的细胞外基质(extracellular matrix, ECM)进行深入观测并对生物矿化作用的结构和动力学信息进行定量分析^[94], 实现完整骨组织的无创检测, 这对于认识骨头中各种成分所起的作用, 以及预防和治疗骨疾病都具有重要的研究指导意义.

3.1 固体NMR应用于骨矿物相的研究

³¹P MAS和¹H-³¹P交叉极化(cross polarization, CP)MAS NMR实验是观测骨组织矿物相微观结构最常用的方法^[95,96], 通常在化学位移3.1 ppm左右的位置都会出现一个羟基磷灰石的³¹P特征峰. ¹H/³¹P CPMAS峰主要来自于靠近质子的³¹P物种, 而单脉冲激发的³¹P MAS峰则来自于骨样品中所有的³¹P物种, 并且前者峰的线宽大于后者, 据此推测在矿物纳米晶水合表面层的³¹P原子核附近存在水吸附位点和其他质子化类型. Kolmas等人^[97]还利用³¹P→¹H CP MAS NMR方法对人牙齿中的牙釉质、牙本质和牙骨

质中-OH含量进行了估算。

骨头的二维 ^1H - ^{31}P 异核相关谱(heteronuclear correlation, HETCOR)光谱可以证明骨磷灰石内存在大量的羟基^[98], 提供更多关于矿物纳米晶表面磷酸盐的分布信息. 牛皮质骨标本的 ^1H - ^{31}P HETCOR光谱显示, 骨矿物质中至少存在3种不同的局部磷酸盐环境, 与这些位点相关联的二维交叉峰可以揭示骨矿物晶体中靠近磷酸根离子的特定质子环境. 根据以往的研究^[98-101], 从 ^1H 间接维上观察(图5(b)), 0 ppm附近的交叉峰可以归属为矿物的 PO_4^{3-} 位点与磷灰石晶格中的结构 OH^- 离子; 5.4 ppm处较宽的交叉峰可归属为磷酸基团与矿物表面的氢键水分子; 而8~13 ppm区间一个十分微弱的峰则归属为 HPO_4^{2-} , 较宽的峰表明这些阴离子在矿物面不均匀分布或存在无定形的环境中. Maltsev等人^[99]利用二维 ^1H - ^{31}P HETCOR实验观测了一系列不同的马关节组织, 发现健康或患病样品之间的矿物成分差异很小. Pourpoint等人^[102]发现使用直径1.3 mm的小容量转子, 在17.6 T的超高磁场下对小鼠单个牙齿进行二维 ^1H - ^{31}P HETCOR检测, 仅需3 h左右即可完成.

Ca是骨矿物相中的重要组成元素, 但由于 ^{43}Ca 具有极低的天然丰度和拉莫频率, 导致 ^{43}Ca 的NMR灵敏度和信噪比相当低. 过去几年, 由于超高场NMR谱仪(18.8 T及以上)的推出和更大直径转子MAS探头的使用以及新的脉冲方法的发展, 使得 ^{43}Ca 的固体NMR检测变得更可行^[103,104]. Xu等人^[103]

在19.6 T的高场下通过 ^{43}Ca MAS NMR实验研究了天然牛皮质骨和去蛋白后的骨样品, 发现与纯的HA相比, 骨样品的峰呈现出较宽的线型, 而且 ^{43}Ca 的NMR各向同性化学位移向高场移动, 通过对实验谱图的模拟能给出骨头样品中钙物种的四极耦合常数(4 MHz). 作者还采用二维三量子MAS(3Q-MAS) ^{43}Ca 实验研究了骨钙蛋白与碳酸磷灰石的相互作用(图5(a)), 观察到两组特征峰, 其中信号较强的峰(I和II)归属于未结合骨钙蛋白的碳酸磷灰石的两个钙位点, 信号较弱的峰(I'和II')归属于与骨钙蛋白发生结合的碳酸磷灰石的两个钙位点, 这种方法也适用于研究其他骨蛋白与矿物质的相互作用. 为了研究 Na^+ 周围的局域结构, Laurencin等人^[104]利用 ^1H - ^{23}Na R³-HMQC实验直接证明了 Na^+ 位点与磷灰石晶格中的羟基基团距离十分接近.

3.2 固体NMR应用于骨有机基质和有机-矿物界面性质的研究

骨的有机基质主要由I型胶原蛋白组成, 还包括许多非胶原大分子如蛋白、磷脂、多糖和柠檬酸盐等. 利用高分辨固体NMR技术研究骨的有机基质, 不需要对样品进行任何化学预处理或者物理分离和纯化, 就可以在原子水平上提供天然骨组织中胶原和其他有机大分子的结构和动力学信息^[105]. 天然丰度 ^{13}C CPMAS NMR实验的信号主要来自于胶原基质中的氨基酸, 刚性组分糖胺聚糖和柠檬酸盐也有微弱

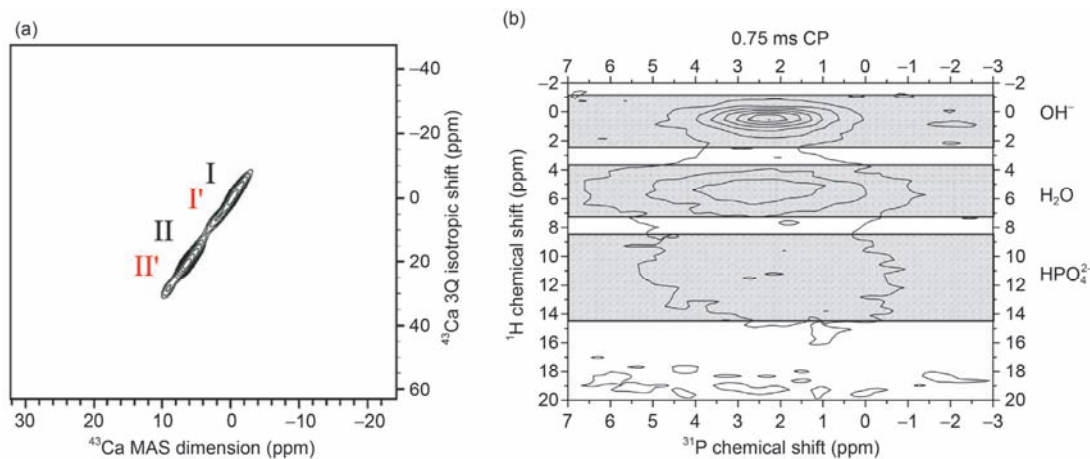


图5 (网络版彩色)骨组织的二维谱图. (a) 室温下 ^{43}Ca 富集的碳酸磷灰石与骨钙蛋白混合物的二维 ^{43}Ca MQMAS NMR谱图^[103]. (b) 马关节组织 ^1H - ^{31}P 相关谱的 ^1H 间接维指认^[99]

Figure 5 (Color online) Two-dimensional spectra of bone tissue. (a) Two-dimensional MQMAS spectrum of ^{43}Ca -enriched carbonated apatite mixed with osteocalcin protein at room temperature^[103]. (b) OH^- , water and HPO_4^{2-} ^1H spectral assignment on the ^1H - ^{31}P correlation spectra of the horse joints^[99]

的贡献。由于骨组织中存在一定量的水,因此 ^{13}C 固体MAS NMR实验对象可分为新鲜状态下的骨组织^[106-113]或者脱水处理后的样品^[114-118]。Ramamoorthy研究组^[115,116]研究了脱水对骨组织有机基质的二级结构和构象动力学的影响,发现 ^{13}C 峰的线宽与骨样品的水合状态密切相关,在脱水时会发生显著的线宽增宽;脱水后胶原蛋白氨基酸残基的流动性和糖胺聚糖的环状结构会相应降低,但再次吸水后分子结构和流动性又得到恢复。因此为了在测量过程中保持样品的完整性,控制实验过程中骨样品的水合状态就显得尤为重要^[113,114]。该研究组^[119]还把骨样品浸入含有顺磁Cu(II)中心的磷酸盐缓冲溶液中,发现可以将 ^1H 的纵向弛豫时间T1降低2~3倍,有利于在天然丰度下更快获取 ^{13}C CP MAS光谱,但是一些胶原蛋白的 ^{13}C 共振会受到附近 Cu^{2+} 的影响,不利于光谱的解析。骨中的水分为游离水、移动水、结合水或孔隙水,不同类型的水具有独特的NMR弛豫时间,可通过横向弛豫时间T2扩散和二维弛豫实验研究骨基质中的水动力学及其分布^[120]。Gul-E-Noor等人^[120]使用逆向拉普拉斯变换的二维 ^2H T1或T2实验分析了经不同方法处理后的完整骨样品,结果表明与骨有机成分相关的水更容易承受压力,骨的负载耐受能力来自于含胶原负载的部位,因此与胶原表面结合的水有益于骨骼的承载能力。Singh等人^[121]利用天然丰度 ^{15}N 光谱法研究了骨中天然胶原的水依赖性骨结构动力学,研究结果反映出骨干结构动力学的相互依赖性和影响,表明胶原由于其显著的动力学柔性可以作为负载组件吸收过多的机械能。

十多年来,骨中有机-矿物界面原子分子结构的NMR表征依然是这一领域的研究热点,旋转回波双共振(rotational echo double resonance, REDOR)脉冲序列被证明是在原子尺度上探测有机-矿物界面非常有用的工具^[99,106,107,110,111,114,121]。2005年,Duer研究组^[106]首次利用 ^{13}C $\{^{31}\text{P}\}$ REDOR实验研究了骨和牙齿的有机-矿物界面结构。Wise等人^[107]研究发现76 ppm处的 ^{13}C 信号在重新引入 ^{31}P 照射脉冲后发生了明显的失相,通过与一系列钙化组织的 ^{13}C NMR谱对比,推测该信号是来自于糖胺聚糖的碳原子,由此推断出骨的有机-矿物界面主要是多糖。然而,Schmidt-Rohr实验室^[110]根据多核固体NMR和距离测量的结果推测该信号来自于矿物相中的柠檬酸根离子,这些离子覆盖了磷灰石约六分之一的表面,会与骨中的磷灰

石纳米晶发生强烈结合,而且离子中的碳原子与磷灰石中磷酸盐层的距离约为0.4 nm。虽然关于这个信号的确切性质仍存在一些争议,但 ^{13}C $\{^{31}\text{P}\}$ REDOR实验中该峰的显著失相不仅存在于骨组织中^[107,110,111,114],而且在其他钙化组织中^[122]也能被观测到。Rai等人^[114]利用 ^{13}C $\{^{31}\text{P}\}$ REDOR实验探索了脱水对于完整山羊骨有机-矿物界面的影响,发现新鲜骨的胶原蛋白侧链与矿物表面的距离约为9 nm,并且二者之间的距离会随着样品的脱水而降低。

4 结论与展望

NMR技术的最新进展和各种膜体系的使用为膜蛋白的结构生物学研究开创了新篇章,溶液和固体NMR均可以提供膜蛋白的原子分辨率结构信息,有助于阐明生理膜环境下膜蛋白的动力学和分子机制。提高NMR检测的灵敏度一直是该领域研究人员努力攻关的难题,令人惊喜的是应用于膜蛋白研究的高场NMR谱仪已达到1 GHz。另外,与溶液NMR相比,固体NMR技术发展相对缓慢,但具有很大的提升空间。为了使ssNMR更好地应用于膜蛋白的生物学研究,除了文中讨论的DNP技术和快速MAS的 ^1H 检测技术,超过100 kHz的MAS NMR技术也显示出巨大的应用潜力^[123-125]。最近,天然膜环境中膜蛋白的ssNMR研究也取得了一系列进展^[126-128],主要得益于样品制备方案的不断改进、多维NMR实验的成功开展以及DNP的广泛应用。另据报道,用于ssNMR的CryoMAS探头已被Doty Scientific开发出来,何时能用于膜蛋白的研究也备受期待。

与其他原子分辨率的技术相比,in-cell NMR提供的是更接近生理条件下的数据,而且随着技术的发展,将会揭示更多关于细胞内蛋白的结构、动力学以及相互作用的信息。In-cell NMR在实际应用中存在一些缺陷,比如样品的泄漏、细胞寿命过短和蛋白翻滚速率过慢等,NMR谱仪硬件的改进如超高场磁体和低温探头的使用可以提高仪器的分辨率和灵敏度。为了防止样品泄漏,可以使用小切口机器人注射装置或采用荧光染料共注射方法监测蛋白的漏出;为了使细胞的活性和稳定性更持久,需要不断补充氧气和营养物质以及移除代谢副产物,生物反应器^[88]的出现巧妙地解决了这个问题;针对蛋白翻滚速率过慢的情况,可以借助顺磁NMR光谱的高灵敏度,来研究活细胞中蛋白复合物的结构、动力学和相互作用

用. 这些发展无疑会提高in-cell NMR数据的质量和结果的可靠性, 使我们坚信它可以成为研究细胞结构生物学强有力的工具之一.

固体NMR可以在原子层面上观测骨组织和相关生物材料的微观结构和动态构象, 同时NMR光谱学和其他谱学表征技术的发展为这些材料的详细光谱表征开辟了新的途径. 例如超快MAS探头的使用以及用于超快MAS研究的多脉冲序列的开发, 可以实现100 kHz甚至更高的样品旋转速率, 有助于获得骨的高分

辨率结构^[129]; 而且这些小转子的上样量极少, 因此可以有效地节约实验成本, 尤其是对需要同位素标记的生物固体样品. DNP技术可以使MAS NMR的信号增强因子达到50~100, 在骨组织的MAS NMR研究中发现, 极低温度下使用DNP可以使信号增强因子达到30, 并观测到胶原蛋白的CH- π 相互作用. 我们相信随着固体NMR技术的发展, 能完全实现对骨相关材料中低频四级核如³⁹K, ²⁵Mg, ¹⁷O和⁴³Ca的更精确观测, 揭示骨中金属离子的配位状态及其在各种骨疾病中的作用.

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Summary for “生物复杂环境下的核磁共振技术和应用: 机遇与挑战并存”

Nuclear magnetic resonance technology for biological complex systems: Opportunities and challenges

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Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool in learning the structure, dynamics and interaction of biological macromolecules. In virtue of its rapid development and wide applications, NMR has become an indispensable tool in numerous research fields, which in further benefits from the improvement of NMR spectrometers based on higher sensitivity and resolution, as well as the continuous innovation of NMR experiment methods. Nowadays life science researches are not limited to the traditional questions and technologies. Multidisciplinary studies of life science, physics, chemistry and materials science become new trends. Therefore, new tools are required to study the structure and function of macromolecules in its native or mimic environments, where NMR provides a powerful and versatile weapon.

Membrane protein mediates signal transduction, molecular transport and energy metabolism in cells. Hence, the therapeutic potential based on understanding the properties of membrane protein requires extensive investigation to characterize its structure and dynamics. Structural study of membrane protein presents challenging due to its low ability to form protein crystal in a mimetic membrane environment. However, NMR is a suitable tool and plays an important role in studying membrane protein, because it provides possibilities to determine the structure of membrane proteins in an environment similar to its native state. Specifically, optimal membrane mimetic including detergent micelles, bicelles and nanodiscs need to be considered in the structural study of membrane proteins, due to the size limits in solution-state NMR. On the other hand, solid-state NMR (ssNMR) has long been considered as another major method in the field of structural biology because it dominates in studying membrane proteins in native-like lipids. Moreover, oriented sample (OS) ssNMR can be applied to determine the secondary structure, orientation and topology of membrane protein in lipid bilayers, in which the sample should be uniformly aligned in the magnetic field. Recently, basing on the methodological and technological developments in the field of dynamic nuclear polarization (DNP) and proton detection, magic angle spinning (MAS) ssNMR has presented as a promising tool in completely characterization of the structure and dynamics of membrane proteins.

Exploring the structure, interaction and function of biomolecules in native environment requires understanding their mechanisms thoroughly in physiologically relevant. In-cell NMR is a branch of high-resolution biomolecular NMR spectroscopy, which brings atomic-resolution insights into the states of macromolecule in native cells. To date, in-cell NMR has been successfully applied to the investigation of bacterial and eukaryotic cells, accordingly, relevant structural and functional information has been obtained. Here we give a general discourse on the existing in-cell NMR approaches and its applications in protein structure determination, interaction and stability.

Bone is one of the most intricate natural materials, featuring by its highly hierarchical architecture. Nevertheless, its characteristic organic-inorganic organization presents challenging for most of conventionally analytical and biophysical techniques. Over the past several years, ssNMR has been extensively applied to materials science, enabling insights into the structure and dynamics of complicated biomaterials in atomic-scale. Particularly, ssNMR highlights its unique potential in the research of bone. Specifically, the organic matrix of bone can be explored using ¹³C-labeled NMR methods, while the inorganic bone mineral can be labeled by ⁴³Ca and ³¹P. Furthermore, the structural arrangement of the interface between organic matrix and inorganic mineral of bone can be probed using the method of ¹³C {³¹P} rotational echo double resonance (REDOR). The abundant structural information in atomic-scale derived from ssNMR has immensely contributed to the establishment of current structural model of bone, hence, aids understanding the molecular mechanisms of bone maturation and diseases.

nuclear magnetic resonance, biological complex environment, membrane protein, in-cell NMR, bone biomaterials

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