# Design and synthesis of paramagnetic probes for structural biololgy

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# Chapter VI

# General discussion, conclusions and prospects

The majority of the work presented in this thesis involves the design and synthesis of paramagnetic NMR probes, including lanthanoids caged probes and spin labels. An overview of the development of different types of lanthanoids caged probes is given in Chapter 1. In order to design a proper paramagnetic probe, several issues have to be considered: *i*) The ligand must rigidly bind to the lanthanoids with high-affinity. *ii*) The symmetry of the probe is important. *iii*) Site-specific labeling is necessary for generating unambiguous paramagnetic restraints. DOTA, an octadentate ligand, is an outstanding lanthanoids cage compared with others, such as DTPA. It rigidly coordinates to the lanthanoids due to the macrocyclic ring and presents high thermal stability. Among all of the reported lanthanoid probes, the caged lanthanoid NMR probe version 5 (CLaNP-5) shows the largest paramagnetic effects due to the fact that it is tagged to proteins *via* two-point attachment and its free complex presents a single conformation. The attachment site also plays an important role in getting optimal paramagnetic effects. Generalized observations for a CLaNP binding site have been reported by Keizers *et al*.[11] It was reported that the distance between the Cα atoms of the two cysteines should be roughly 8 Å. Moreover, the incorporated cysteines should be solvent-exposed, with sufficient room for the CLaNP, so not in a cleft. For rigid attachment, an α-helix (at positions i & i+3 or i & i+4) or a β-sheet (at positions i & i+2) are ideal locations. Cross-strand attachments have not been reported for CLaNPs. Such cross-links could interfere with the natural dynamics of the protein. In the case of CLaNP-5, two pyridine-*N*-oxides were introduced to the opposite sites of DOTA ring for reasons of symmetry and disulfide bridges were employed in site-specific labeling. Although CLaNP-5 is successfully applied to study proteins and protein complexes, the net charge of Ln CLaNP-5 complex and the weak disulfide linker are the drawbacks.

*Development of next generation lanthanoid-chelating probes*

In Chapter 2, the replacement of pyridine-*N*-oxide with *p*-nitrophenol produced a next generation probe, CLaNP-7. The phenolic groups are deprotonated upon coordinating to lanthanoids, giving a lanthanoid complex with one positive charge. In the case of CLaNP-9 (Chapter 3), the linkers were rearranged to the *meta*-position of pyridine-*N*-oxides, keeping the carboxylic acids as the ligands to reduce the net charge of lanthanoid complexes. The introduction of carboxylic acid or negative functional groups such as phosphonic and sulfonic groups on the cyclen ring or the

ethylene of chelating arms may be an alternative approach to reduce the net charge. Another advantage for adding hydrophilic groups is that the undesired hydrophobicity, which arises from the exposed cyclen ring after attachment, can be balanced.

Cysteine is the most commonly modified residue for paramagnetic probe attachment. The thiol group can undergo disulfide exchange to form disulfide bridges as well as Michael additional with α,β-unsaturated carbonyl compounds or electron-poor ene to form a thio-ether. In order to enhance the stability of probe tagged protein, thio-ether linkage using α-ketobromide was utilized in CLaNP-9 (Chapter 3). Two vinyl functionalized dipicolinic acid based probes were also reported to form stable products.[47,54] Besides electron-poor vinyl group, acrylates/acrylamides and methacrylates/methacrylamides can also be considered.[46]

Bioorthogonal reactions are a new approach to rigidly tag a probe on protein surface at specific site. So far, only one example was reported based on this approach by using copper catalyst click chemistry.[50] It is clear that this method remains unexploited and different types of bioorthogonal reactions such as photoclick chemistry, norbornene cycloaddition, and [4+1] cycloaddition have to be considered in the future.[48] The advantage of the use of bioorthogonal reaction is that the probe could be attached to specific site without any side-products. Also dual probe attachment, using both a disulphide linkage and a bioorthogonal reaction are then possible. Disadvantages are that most of the unnatural amino acids are not commercially available and that the linkers are generally longer than disulfide bridges and thioether bonds. Rigid probe attachment requires two-armed attachment, but we found that incorporation of two unnatural amino acids close together in an amino acid sequence affects the yield of the engineered protein quite negatively.

*The rigidity and chirality of next generation probes*

CLaNP-7 and CLaNP-5-OMe (Chapter 2 and 4) tagged to a protein showed pH-dependent and temperature-dependent magnetic susceptibility tensors, respectively, due to the influence of neighboring amino acids. As a consequence two sets of paramagnetic restraints were observed. These results point out that not only the attachment site but also the conformation of the probe is important to obtain unambiguous paramagnetic NMR restraints. Generally, DOTA-based probe undergoes enantiomerization and the exchange rate is on the NMR time scale. In

solution the lanthanoid caged probes present a single enantiomeric pair but the symmetry of probe is broken upon tagging on a protein surface and a pair of diastereoisomers is obtained. As long as these diastereoisomers are similar to each other, they may still present a single set of paramagnetic effects. If the change in the ligand orientation between the two diastereoisomers is somehow correlated to the orientations of the arms and the protein, the two forms can exhibit different $\Delta\chi$-tensors and a second set of paramagnetic effects will be present. In order to avoid the enantiomerization, the decoration of amide arms with chiral centers in CLaNP-5-like paramagnetic probes may be one solution (Figure 1). The chirality will force the probe in a single conformation and the complementary conformation can be easily provided by using opposite chirality. In this way, two different orientated magnetic susceptibility tensors can be obtained.

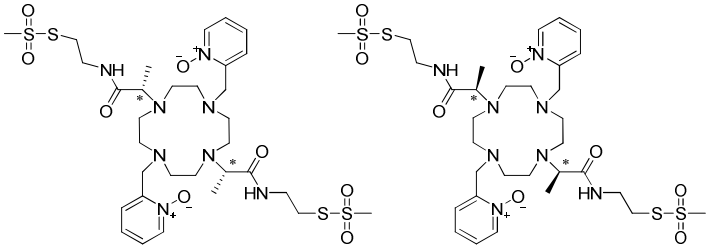

**Figure 1**. Structures of methyl functionalized CLaNP-5 like chiral probes.

*Development of substrate- and inhibitor- based paramagnetic probes*

In Chapter 5, the co-crystal structures of protein with inhibitor-based spin label probes were presented. These results show promise for an approach in which protein-ligand interactions are studied by using substrate- and inhibitor- based paramagnetic probes. Also, the dynamics of small compounds have recently been studied by paramagnetic restraints.[209-211] Instead of functionalized protein, the lanthanoids were linked to the oligosaccharides. Dedicated paramagnetic ligand derivatives represent a new alternative way for paramagnetic NMR to study protein-ligand interactions. In order to obtain large and unambiguous effects the paramagnetic center should be linked rigidly. CLaNP-11 and CLaNP-12 can be employed in this approach *via* copper-click chemistry due to the rigid and stereoselective cyclic products. However, the aqueous solubility and other properties of substrates and inhibitors may be influenced by the paramagnetic center, especially

for lanthanoid ligands which are relatively large. Nevertheless, it can be foreseen that the future will bring the development of paramagnetic ligands for specific proteins.