Magn. Reson. Discuss.,
https://doi.org/10.5194/mr-2020-26-RC2, 2020

Open Access MAGNETIC RESONANCE Discussions

Interactive comment on "Phosphoserine for the generation of lanthanide binding sites on proteins for paramagnetic NMR" *by* Sreelakshmi Mekkattu Tharayil et al.

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Received and published: 25 November 2020

A new lanthanoid binding tag is proposed, which does not require cysteine residues for attachment to the protein, based on the incorporation of phosphoserine in close proximity of negatively charged residues. The vicinity of the binding site to the protein backbone is expected to ensure a good immobilization of the tag. On the other hand, the authors show that a double phosphoserine mutation can increase the magnitude of the magnetic susceptibility anisotropy, and thus of the pcs, but also show that it is difficult to produce most of these mutants.

The tag here presented is of potential importance for the improvement of tagging strate-

gies of proteins; the manuscript is interesting and well written, and thus recommended for publication.

The authors should comment that the axial anisotropies of the proposed tag attached to ubiquitin with a single phosphoserine mutation are significantly smaller than those of other previously proposed rigid tags (more than a factor 2 for Tb probes, more than a factor 5 for Tm), and should discuss the origin of this difference.

The tensor for the GB1 K10D/T11Sep(Tb3+) should be reported with an axial component of -33.7 and a rhombic component of 14.7 to fulfill the axis labeling convention providing a rhombic component up to 2/3 of the axial component in absolute value. If the authors prefer to report the tensor as in Table 1, they should at least explain why. In any case, the tensor anisotropy is surprisingly large considering that the measured pcs span a range smaller than that measured for ubiquitin, and surprisingly rather rhombic. In the double phosphoserine K10Sep/T11Sep(Tb3+) mutant, the measured values of the pcs span a range which is roughly double, but the tensor is less than half with respect to that of K10D/T11Sep(Tb3+). Please, double check that no mix-up of data has occurred.

Can you comment on the reason of the different sign of the tensor axial components between K10Sep/T11Sep(Tb3+) and A24Sep/K28Sep(Tb3+)? On the other hand, the sign of the axial components of Tb and Tm are usually opposite. Why are they the same in A24Sep/K28Sep?

Minor points:

Pag.2, line 1: "As lanthanide ions display particularly large. . ." not all lanthanoids, only some of them!

Pag. 2, line 2: "While paramagnetic lanthanide ions generate paramagnetic relaxation enhancements (PRE) in the protein irrespective of metal mobility" This sentence may be read that PREs do not depend on mobility, which is slightly inaccurate, because

internal mobility changes the correlation time of dipole-dipole relaxation (see Fragai et al. Coord. Chem. Rev. 2013, 257, 2652 for a thorough discussion). Please, clarify this point.

Caption to Fig. 3: please indicate all panel letters.

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