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Comment on mr-2022-11

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Referee comment on "Site-selective generation of lanthanoid binding sites on proteins using 4-fluoro-2,6-dicyanopyridine" by Sreelakshmi Mekkattu Tharayil et al., Magn. Reson. Discuss., https://doi.org/10.5194/mr-2022-11-RC2, 2022

This paper represents a significant attempt at the generation of lanthanide binding sites on proteins with selectivity and limited tag mobility under biocompatible conditions. The ability to generate several variants of the lanthanide tag after the initial labeling reaction is of high interest in the efficient localization of NMR PCS tensors for structural studies, and the potential ability to label selenocysteines may allow for fewer required host protein mutations. The produced tags appear to be more permissive towards solution studies of labeled proteins than prior attempts, although room for improvement is still apparent, as noted by the need to stay at metal: protein ratios of < 0.6 to avoid sample precipitation (lines 217-219).

The cysteine labeling reaction for a surface exposed cysteine is noted to complete overnight at RT, and the selenocysteine reaction is noted to have completed in 10 minutes (lines 175-180). This is stated to indicate that the reaction is selective for selenocysteine, but what is the actual timescale of the cysteine reaction? Is there a sufficient difference to avoid replacement of native cysteine residues? Additionally, buried cysteines are shown to label poorly or not at all with FDCP; is this the same for selenocys mutants?

The high salt requirement for the cyano group reaction with the free cysteine was noted. Was this to reduce aggregation of the protein in the reaction condition?

For the EPR experiments, several concerns were apparent.

First, in the NMR section, it is noted that metal: protein ratios > 0.6:1 resulted in

significant protein precipitation, yet the EPR studies used stoichiometric or excess metal? Were there issues with precipitation in these cases, and could any of the peaks presented in the DEER distributions reflect protein aggregates? The authors indicate that multiple tags may be able to jointly coordinate additional lanthanides, but these results could also be explained by protein dimerization that leads to tags in close proximity. Would this problem be alleviated if the concentration of protein in the metal titration be lowered significantly?

As the authors acknowledge, the modulation depths of the EPR experiments used to check for tag mobility and lanthanide stoichiometry are very small. The authors are correct in their assertion that while these tags do not appear to be highly efficient for DEER experiments, they do appear very promising for paramagnetic NMR. Still, the pulsed EPR measurements are presented in this work as evidence for: the limited mobility of the lanthanide tags, the stoichiometry of the tags, and for the presence of expected distance distributions in each protein of interest. The very small modulation depths shown limit the ability to determine accurate widths for the distance distribution, and also the ability to draw conclusions regarding stoichiometry, as the vast majority of the ensemble is of unknown state. These experiments do indicate that at least some of the protein samples are tagged and in the expected structural state, but this section would benefit from an additional method for validation of coordination stoichiometry and tag mobility.