Structural modeling of EDTA aggregates that lead to artifacts in a fluorescence-based biophysical assay

Tobias Kroeger[1], Benedikt Frieg[1], Finn Hansen[1], Andreas Marmann[2], Georg Groth[3], Sander H. J. Smits[4], Holger Gohlke[1]

[1]Institute for Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, Düsseldorf, Germany
[2]Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University Düsseldorf, Düsseldorf, Germany
[3]Institute for Biochemical Plant Physiology, Heinrich Heine University Düsseldorf, Düsseldorf, Germany
[4]Institute of Biochemistry, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

Differential scanning fluorimetry (or thermofluor assay (TA)) is a fast and cost efficient approach to investigate the melting point of purified proteins or protein complexes [1]. The assay is based on a fluorescent dye, Sypro Orange, that interacts with hydrophobic residues becoming available when a protein unfolds with increasing temperature. However, we observed a fluorescence signal also in the presence of EDTA at high pH even if no protein is present, leading to an artifact in TA. Here, we used a combined experimental and computational approach to investigate the origin of this artifact.

Our experimental approach revealed an EDTA concentration-dependent effect, where the EC_{50} = 36.3 mM is within the range of concentrations practically applied. Furthermore, the artifact emerges at pH > 9, indicating that the $EDTA^{4-}$ sup-population of EDTA causes the fluorescence signal. This signal is also observed in the presence of EGTA. For both EDTA and EGTA, the fluorescence signal can be quenched by adding Ca^{2+} ions (EC_{50}= 100 mM).

In molecular dynamics simulations of free diffusion of EDTA-Na^{+} and Sypro Orange of in total 27 μs length, we observe an aggregation of the $EDTA^{4-}$ molecules that leads to the formation of an inverted bilayer. While the observed aggregation of EDTA is in agreement with previous experimental studies [2], our results for the first time provide a structural model at the atomic level. The results are independent of the applied force field parameters for ions.

In all, we provide evidence that suggests that $EDTA^{4-}$, but not $EDTA^{3-}$, at basic, yet physiologically relevant pH, forms aggregates that interact with Sypro Orange, which can lead to a fluorescence signal in TA. As EDTA is widely used in the fields of biology and pharmacy, e.g. for investigating proteins with a calcium-dependent activity and structure [2], these results are highly relevant for future applications of TA.

1) Cimmperman, P., e*t al., Biophys J*, **2008**. *95(7),* 3222-31.
2) Muller, M. and A. Haeberli, *FEBS Lett*, **1994**. *340(1-2),*17-21.