A RECOMBINANT FUSION PROTEIN THAT MIMICS THE INFLAMMATORY BIOMARKER CALPROTECTIN AS A TOOL TO HARMONIZE MRP-8/MRP-14 IMMUNOASSAYS

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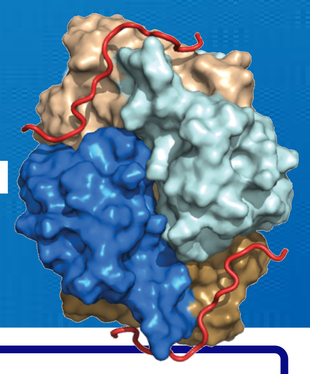


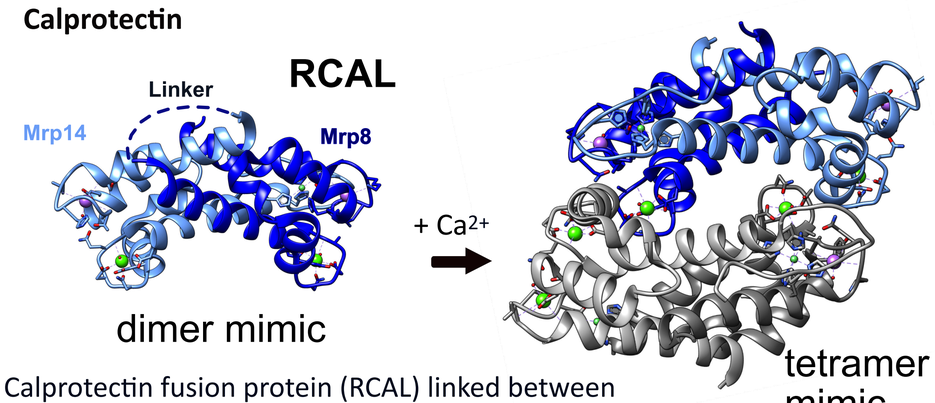
Background and aims

Calprotectin is a granulocyte-derived alarmin protein complex of Mrp-8 and Mrp-14 that indicates the inflammatory state of a patient. Fecal calprotectin is already established as the gold standard for diagnostics and monitoring of inflammatory bowel diseases and blood-circulating or serum calprotectin is emerging as an important biomarker for i.e. rheumatoid arthritis. However, standardization of calprotectin assays differs significantly among providers leading to varying clinical cut-offs. A suspected reason is that calprotectin's different oligomeric states can generate different quantitative results. The aim of this work was to produce a pure and stable calprotectin surrogate that fully mimics the manifold characteristics of this inflammatory biomarker.

Methods

The oligomeric state of a recombinant fusion protein between Mrp-8 and Mrp-14 that expressed solubly in *E. coli* was assessed by size-exclusion chromatography. Direct comparison to native calprotectin regarding antibody affinities were measured using BLI (bio-layer interferometry) and SPR (surface plasmon resonance) and its use as calibrator material in various monoclonal and polyclonal immunoassay formats was tested. Moreover, growth curves of *S. aureus* were recorded in the presence of native and recombinant calprotectin.

Calprotectin



Calprotectin fusion protein (RCAL) linked between Mrp-14 and Mrp-8 with a protease cleavable linker

Purification of RCAL

Soluble Bacterial Expression

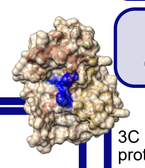
Ni-NTA

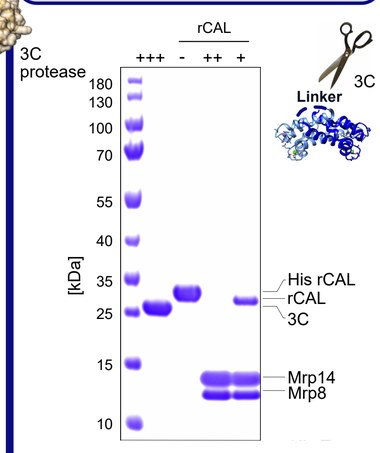
optional: Anion exchange

Size Exclusion Chromatography

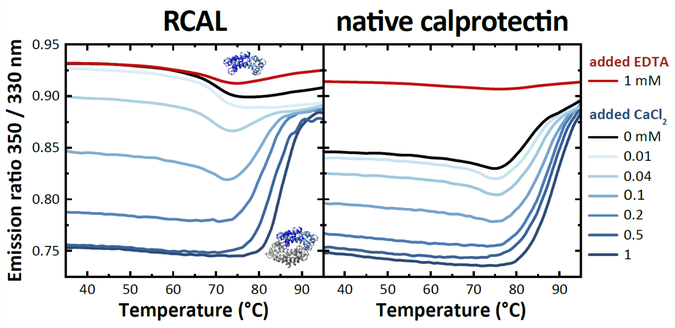
optional: 3C cleavage

optional: $CaCl_2$ induced oligomerization



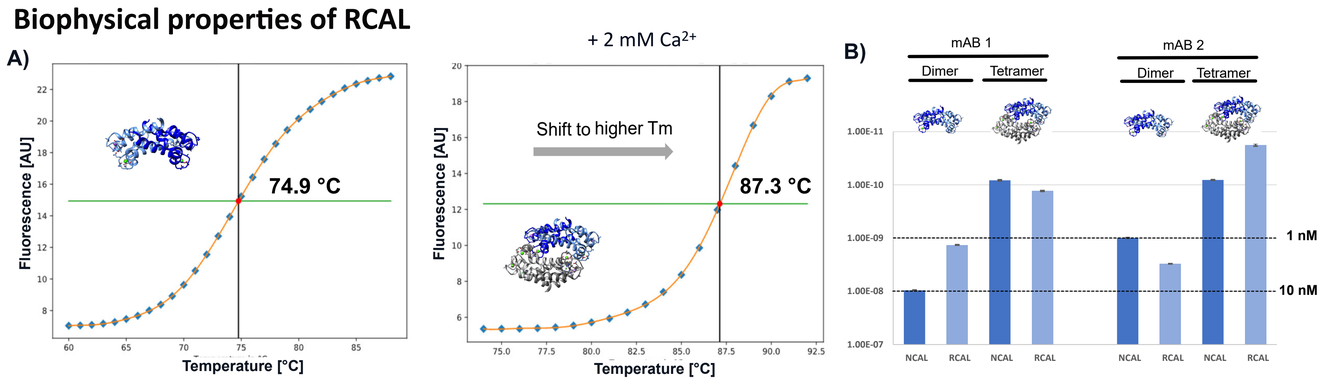


RCAL shows Ca(II) dependent oligomerization like native calprotectin



Differential scanning fluorimetry reveals a transition upon Ca^{2+} addition comparable to native calprotectin. RCAL shows little change upon EDTA addition due to higher purity of the dimeric state compared to native calprotectin.

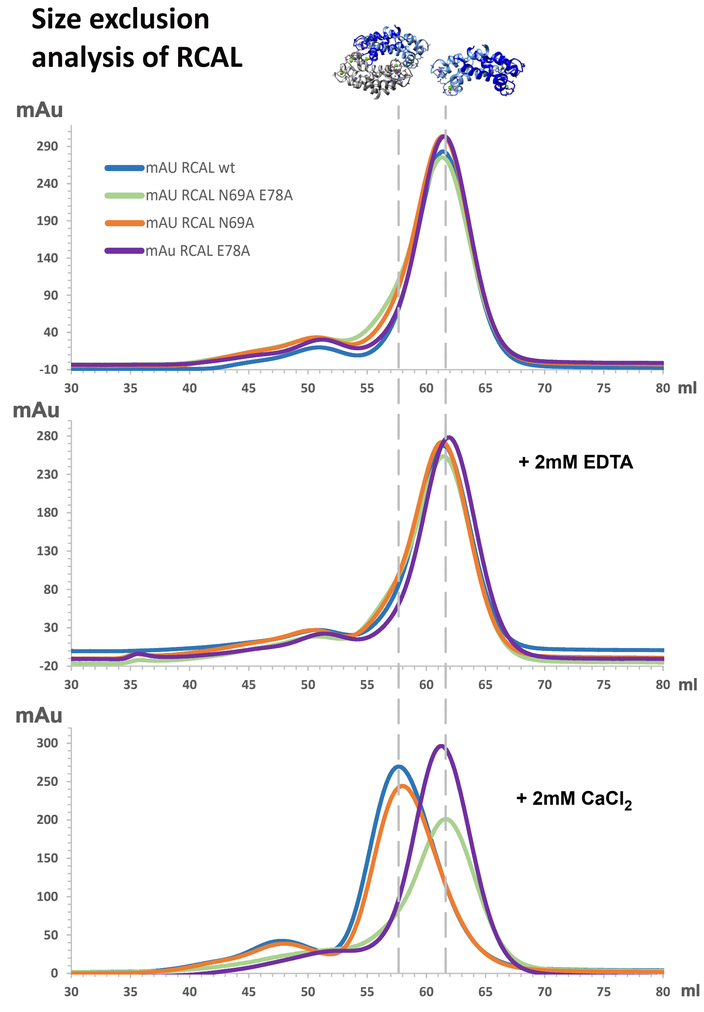
Biophysical properties of RCAL



A) Differential Scanning Fluorimetry reveals that RCAL thermostability increases in the tetrameric form.

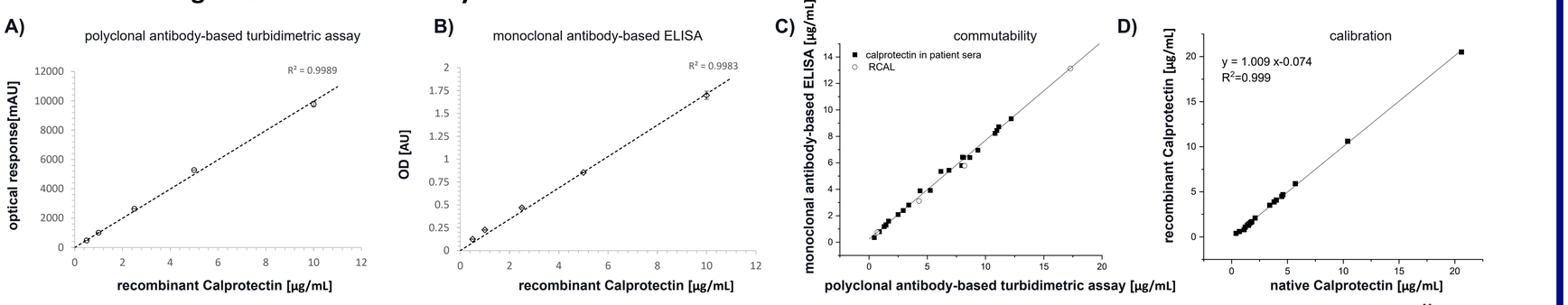
B) Affinity measurements by BLI and SPR reveal similar affinities for native (dark blue) and recombinant (light blue) calprotectin, which are dependent on the oligomeric state.

Size exclusion analysis of RCAL



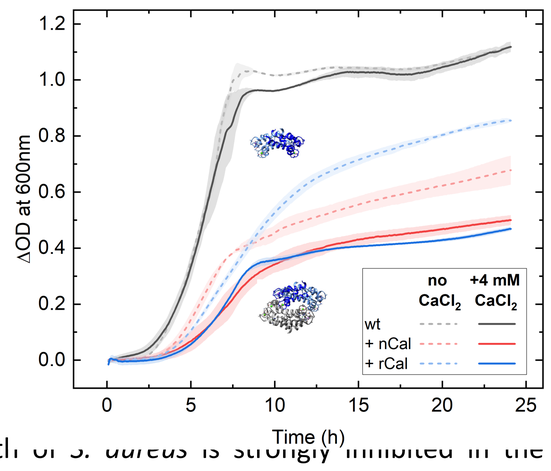
An intrinsic property of calprotectin is tetramerization in presence of Ca^{2+} ions. The recombinant protein elutes at higher SEC volumes in presence of 2 mM $CaCl_2$ indicating oligomerization. Mutations of the Ca^{2+} binding sites impair oligomerization in the case of S100A9 E78A, but not N69A mutations. The recombinant fusion protein therefore mimics native calprotectin with respect to oligomerization.

RCAL recognition in immunoassays



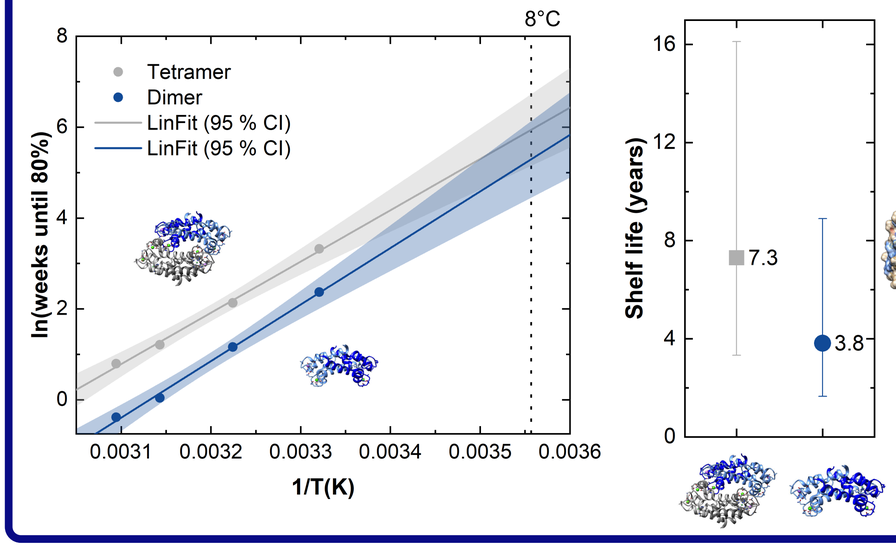
Concentration dependent measurements of recombinant calprotectin spiked incubation buffer shows linear behavior in the BÜHLMANN fCAL® turbo assay (A) as well as in the fCAL® ELISA (B). (C) Measurement of RCAL with both a polyclonal antibody-based turbidimetric assay as well as a monoclonal antibody-based ELISA shows comparable data to 23 patient samples. (D) Turbidimetric measurements of 23 patient samples based on calibration curves derived from recombinant or native calprotectin give rise to virtually identical values.

Bacteriocidal activity of recombinant and native calprotectin



Growth of *S. aureus* is strongly inhibited in the presence of native or recombinant Calprotectin tetramer.

Stability of RCAL as calibrator



Accelerated stability measurements at various temperatures (50°C, 45°C, 37°C and 28°C) give rise to stability estimates of more than three years for the dimer and more than seven years for the tetramer at 8°C.

Results

The fusion protein was purified as a calprotectin dimer mimic that oligomerizes in the presence of calcium ions. Affinities to monoclonal and polyclonal calprotectin antibodies were very comparable to native calprotectin. Spiking of different concentrations of recombinant calprotectin showed linear correlations in ELISA and turbidimetric assays. A perfect correlation (slope=1.009; R^2=0.999) was shown when measuring 23 human serum samples based on native and recombinant calprotectin calibrators with a turbidimetric assay. Both, recombinant and native calprotectin significantly hampered *S. aureus* growth most likely due to metal sequestration of the calprotectin tetramer.

Conclusions

The recombinant protein can be purified in large quantities in defined oligomeric states and shows immunological and biophysical properties identical to native calprotectin. Recombinant calprotectin thus presents a promising tool to overcome the prevalent fecal calprotectin standardization problem and may prevent future standardization discrepancies for serum calprotectin.