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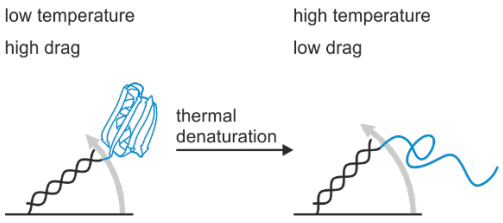
Application Note

Inhibitor induced shifts in the thermal stability of carbonic anhydrase

The thermal stability of proteins can be investigated by measuring the switching dynamics of protein-nanolever conjugates as a function of temperature. Here, we determine the melting temperature T_M of human carbonic anhydrase 1 (hCA1) with **switch**SENSE® and examine the influence of different inhibitors with pharmaceutical relevance on the thermal denaturation of hCA1.

Hydrodynamic friction is a valuable indicator revealing significant information about the size and shape of a protein. **switch**SENSE® probes the hydrodynamic drag of proteins that are attached to the top end of electrically actuated DNA nanolevers (NL) by measuring the upward and downward switching motions by time-resolved means [1,2]. Changes in the 'Dynamic Response' (DR) value, which designates the switching velocity of the protein-nanolever complex, indicate changes in the protein size or shape. Hence, it is possible to monitor conformational changes of proteins as a function of temperature and the binding of small molecule ligands.



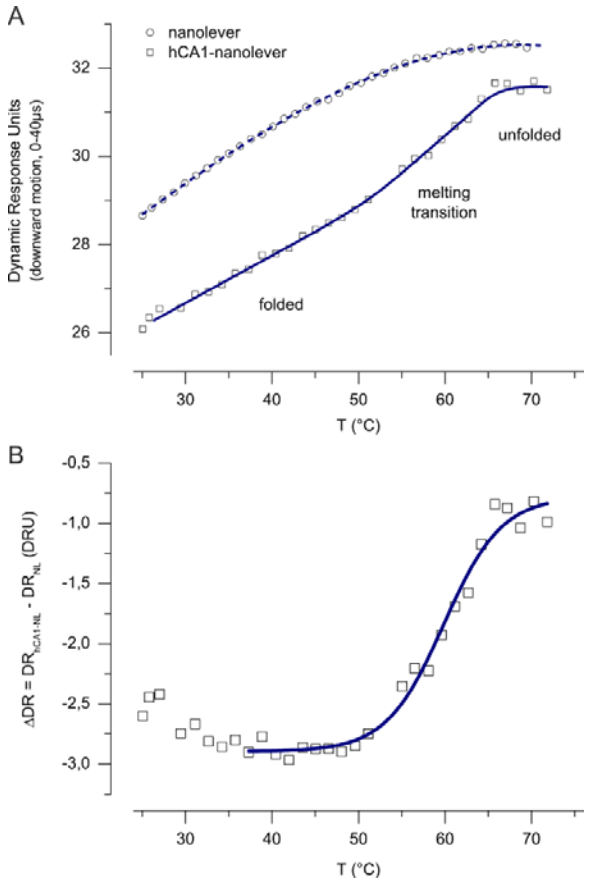
Scheme 1 | A globular protein exerts more hydrodynamic drag on an electrically actuated DNA nanolever than an unfolded peptide chain. Consequently, the switching dynamics accelerate upon disintegration (melting) of the protein conformation.

For the investigation of thermally induced unfolding processes, the protein is subjected to a gradually increasing thermal stress by ramping up the sample temperature.
When the thermal energy overwhelms cohesive forces which stabilize its three-dimensional structure, the protein disintegrates. This process may involve the spatial separation of protein domains from each other, the unfolding of individual domains, or even a complete denaturation to a linear peptide chain. The conformational transition from a rigid globular structure to a more flexible elongated shape alters the switching dynamics of the nanolever-protein conjugate. Because a flexible chain-like structure exerts less drag on the nanolever than a cohesive bead-like protein, an increase in switching velocity can be observed in a time-resolved **switch**SENSE® experiment.

This application note describes how to analyze a melting transition from a temperature dependent **switch**SENSE® experiment: In general the nanolever switching accelerates upon heating the sample, because the viscosity of water de-

creases with increasing temperature, which lessens hydrodynamic friction. On one hand, this directly affects the nanolever motion and on the other it also promotes a faster charging of the microelectrode. The raw data depicted in **Figure 1A** show the gradual increase in DR-values of a bare DNA nanolever (dashed line) upon slowly heating an S2 chip in a DRX 2400 instrument. Standard nanolevers maintain their double stranded helical conformation even at high temperatures and can be used up 75°C in T20/5 buffer (70°C in T40 buffer).

The temperature dependent Dynamic Response of the unmodified DNA nanolever serves as an internal reference for the temperature curve of the nanolever-hCA1 conjugate, which is shown as a solid line.



As expected, the DR-values of the nanolever-hCA1 conjugate lie below the Dynamic Response of the bare nanolever, because the hydrodynamic drag of the protein slows the nanolever motion. The interesting feature occurs from 55° to 65°C, where the hCA1 curve 'accelerates' and approaches the curve of the bare nanolever – it corresponds to the thermal melting transition of hCA1. At higher temperatures around 70°C, the curve of the now unfolded hCA1 saturates at a constant lag to the bare nanolever, which is still faster.

Figure 1 | Dynamic Response as a function of temperature of bare DNA nanolevers and nanolever-hCA1 conjugates. **A** Dynamic Response values of the nanolever downward motion as determined from time-resolved switching measurements. **B** DR-lag between the bare nanolever and the nanolever-hCA1 conjugate (=DR_{hCA1} – DR_{DNA}). The solid line is a Boltzmann fit.

It is useful to reference the switching velocity of the hCA1-nanolever conjugate with that of the bare nanolever. **Figure 1B**. shows the difference in DR-values, ΔDR = $DR_{hCA1\text{-}NL}$ – DR_{NL}. A clear melting transition is apparent with a midpoint melting temperature of T_M = 60°C.

In order to investigate the influence of small molecule drugs on the thermal stability of hCA1, four different inhibitors with pharmaceutical relevance were tested: sulfanilamide (172 Da), punicalagin (1085 Da), brinzolamide (384 Da), and acetazolamide (222 Da). To this end, hCA1-nanolever layers were incubated with solutions containing the respective inhibitors in concentrations well above the known K_D values. **Figure 2** shows the obtained melting curves including Boltzmann fits for the analysis of melting temperatures, which are listed in **Table 1**.

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Table 1 |
Comparison of melting temperatures (T_M)

	Thermo-fluor™ T_M (°C)	switchSENSE® T_M (°C)
hCA1 (without inhibitor)	60.3	59.9
Sulfanilamide (500 µM)	62.9	63.8
Punicalagin (2.3 µM)	58.8	58.7
Acetazolamide (100 µM)	64.6	66.7
Brinzolamide (4 µM)	62.7	n.d.

For comparison fluorescence thermal shift (Thermofluor™) assays were performed in solution using the dye Sypro Orange™. Good agreement between switchSENSE® and Thermofluor results is found for bare hCA1 and for hCA1 complexed with the inhibitors sulfanilamide, punicalagin, and acetazolamide, cf. **Figure 3**. In case of brinzolamide the broad width of the melting transition prevents the analysis of a reasonable T_M value. Interestingly, for punicalagin switchSENSE® data indicate a second conformation transition around 65°C, which causes the nanolever switching to slow down again.

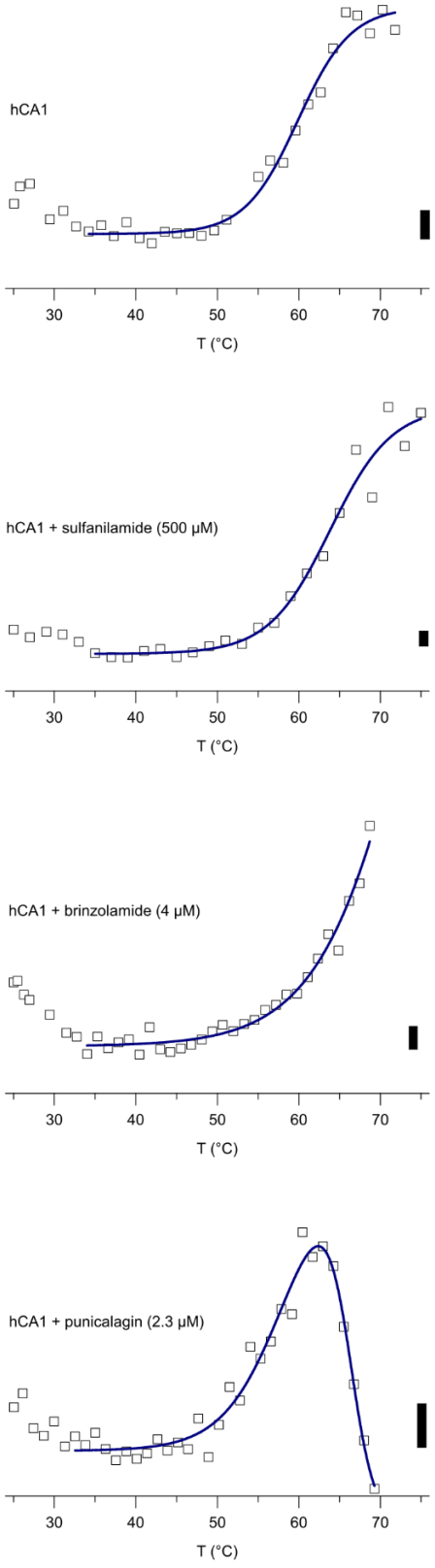

Figure 2 | Melting curves of hCA1 in complex with different inhibitors. Dynamic Response values were referenced by the DR of bare nanolevers (cf. Fig. 1), scale bars indicate relative DR magnitudes.

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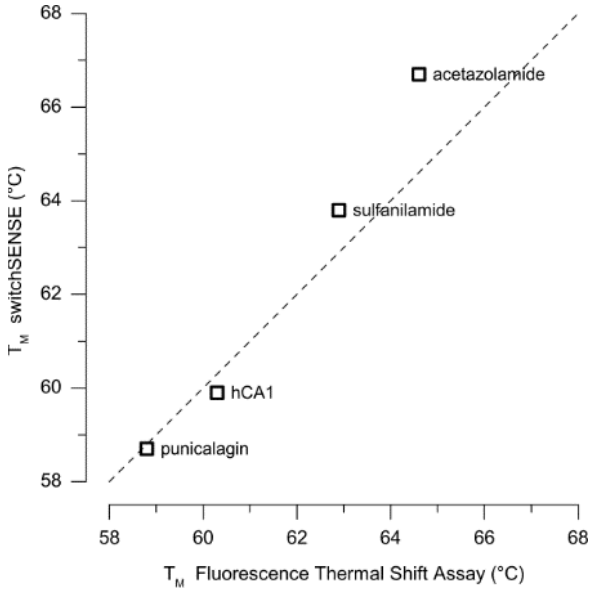

Figure 3 | Comparison of melting temperatures determined by **switch**SENSE® and Thermofluor.

METHODS

Measurements were performed with the DRX 2400 **switch**SENSE® analyzer and the S2 chip. Temperature ramps were 2°C/min. Running buffer was DBS buffer T20/5 (20 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris, pH 7.4) Human carbonic anhydrase 1 and all inhibitors were obtained from Sigma Aldrich. hCA1 was coupled to nanolever seq. B with the DBS 'amine-reactive in-vitro kit'.

REFERENCES

1. Langer, A. et al. Molecular Dynamics of DNA-Protein Conjugates on Electrified Surfaces – Solutions to the Drift-Diffusion Equation *Journal of Physical Chemistry B*, DOI: 10.1021/jp410640z (2013)
2. Langer, A. et al. Protein analysis by time-resolved measurements with an electro-switchable DNA chip *Nature Communications*, **4**:2099 (2013)

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