Electronic Supplementary Material (ESI) for Analyst.

Supplementary information for manuscript "Multi-step conformational transitions in heat-treated protein therapeutics can be monitored in real time with temperature-controlled electrospray ionization mass spectrometry"

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Estimation of the center of charge state distribution of the compact mAb ions resulted from the first thermal transition

Based on the empirical correlation between the surface area and detected charge state of both native and denatured protein ions,[1, 2] the relationship between the increase in mAb surface area and increase in charge state resulted by the two thermal transitions are expressed as:

$$(\lg z_1 - \lg z_0) = k(\lg A_1 - \lg A_0) \quad (1)$$

$$(\lg z_2 - \lg z_0) = k(\lg A_2 - \lg A_0) \quad (2)$$

where *A* is surface area, *z* is the average charge state, *k* is the slope of the linear correlation, and the subscripts 0, 1 and 2 denotes the states prior to the first transition, between the two transitions, and after the second transitions respectively.

Arranging simultaneous Equations (1) and (2) yields:

$$\frac{A_2 - A_0}{A_1 - A_0} = \frac{z_2^{1/k} - z_0^{1/k}}{z_1^{1/k} - z_0^{1/k}} \quad (3)$$

According to the experimental data, z_0=25, z_2=38 (Figure 3B). According to the literature data, *k*=0.69±0.02. Based on the ratio of calorimetric enthalpies contributed by the two transitions (Figure 3A), we also made an assumption that the surface area increase contributed by the second transition is 4 times that by the first transition. Then $\frac{A_2 - A_0}{A_1 - A_0} = 5$. Plugging these values in Equation (3) results in z_1=28.

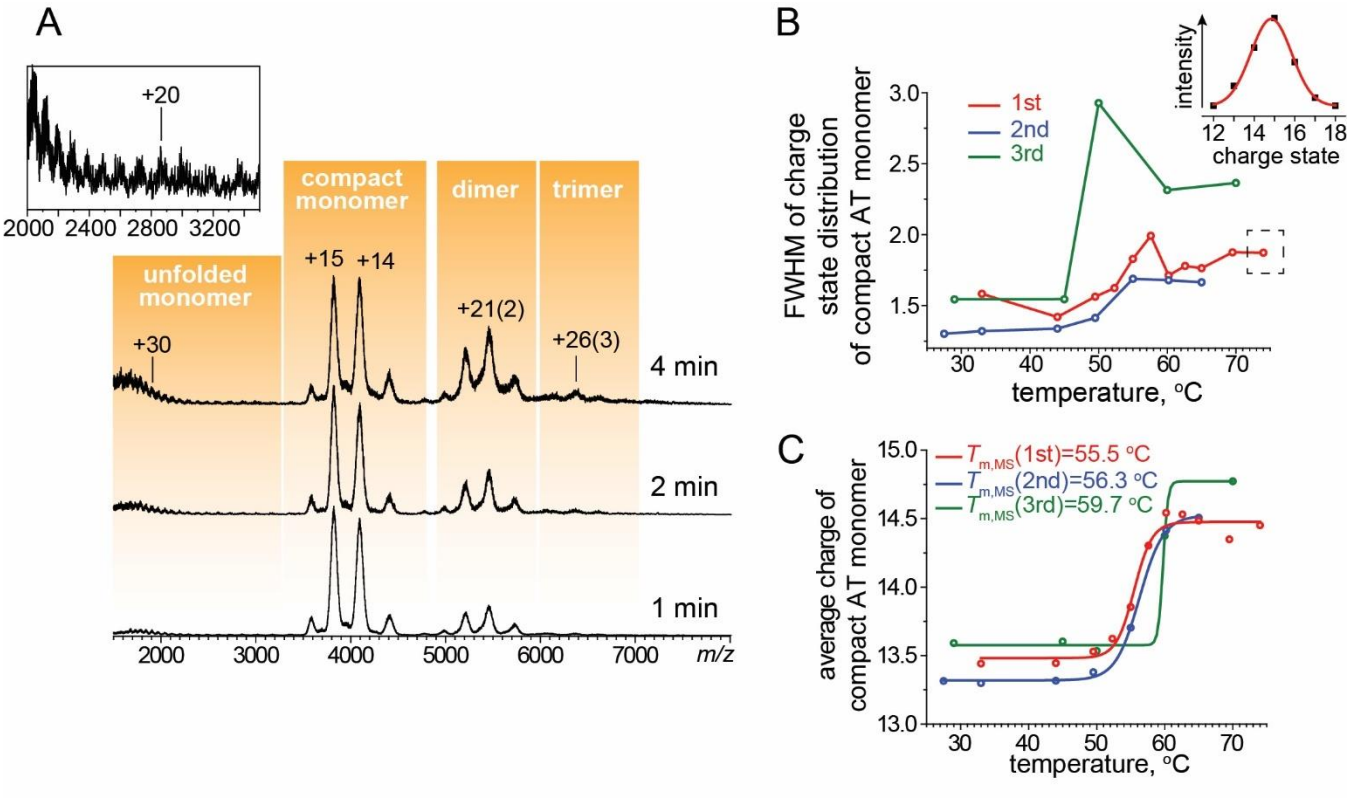

Fig. S1 A: ESI mass spectra of AT (in 150 mM NH_4Ac, pH 8.0) acquired at 65 °C with different incubation times. The inset shows a close-up view of the charge distribution of AT ions centered around 20+. **B:** Overlaid plots of average charge of compact AT monomer *vs.* temperature derived from 3 independent measurements conducted on 3 different days over 3 months. Although these measurements did not result in the same absolute value of average charge at the same temperature due to the factors including inconstant positions of ESI emitters and spray voltages among different sets of measurements, they yielded the same sigmoidal trend of data points throughout the investigated temperature range and similar $T_{m,MS}$ values. These $T_{m,MS}$ values were used to calculate the average and standard deviation of $T_{m,MS}$ reported in Figure 1A.

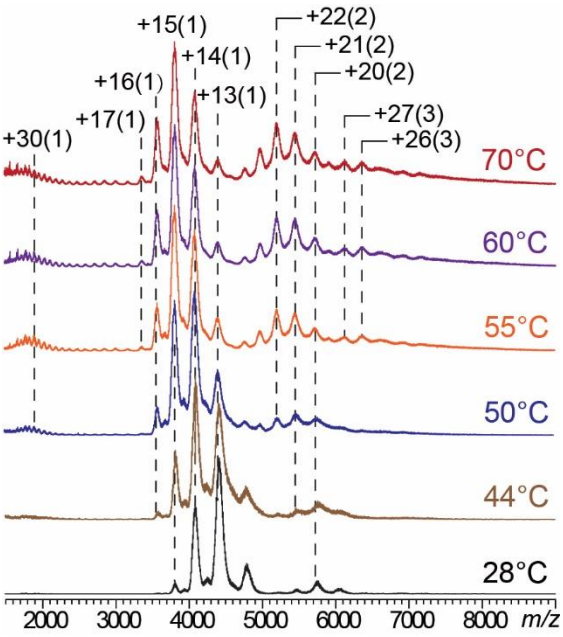

Fig. S2 ESI mass spectra of AT (in 20 mM NH_4Ac, pH 8.0) acquired at different temperatures (as indicated for each spectrum). The peak labels indicate ionic charge states; the oligomeric state of ions are shown in parentheses.

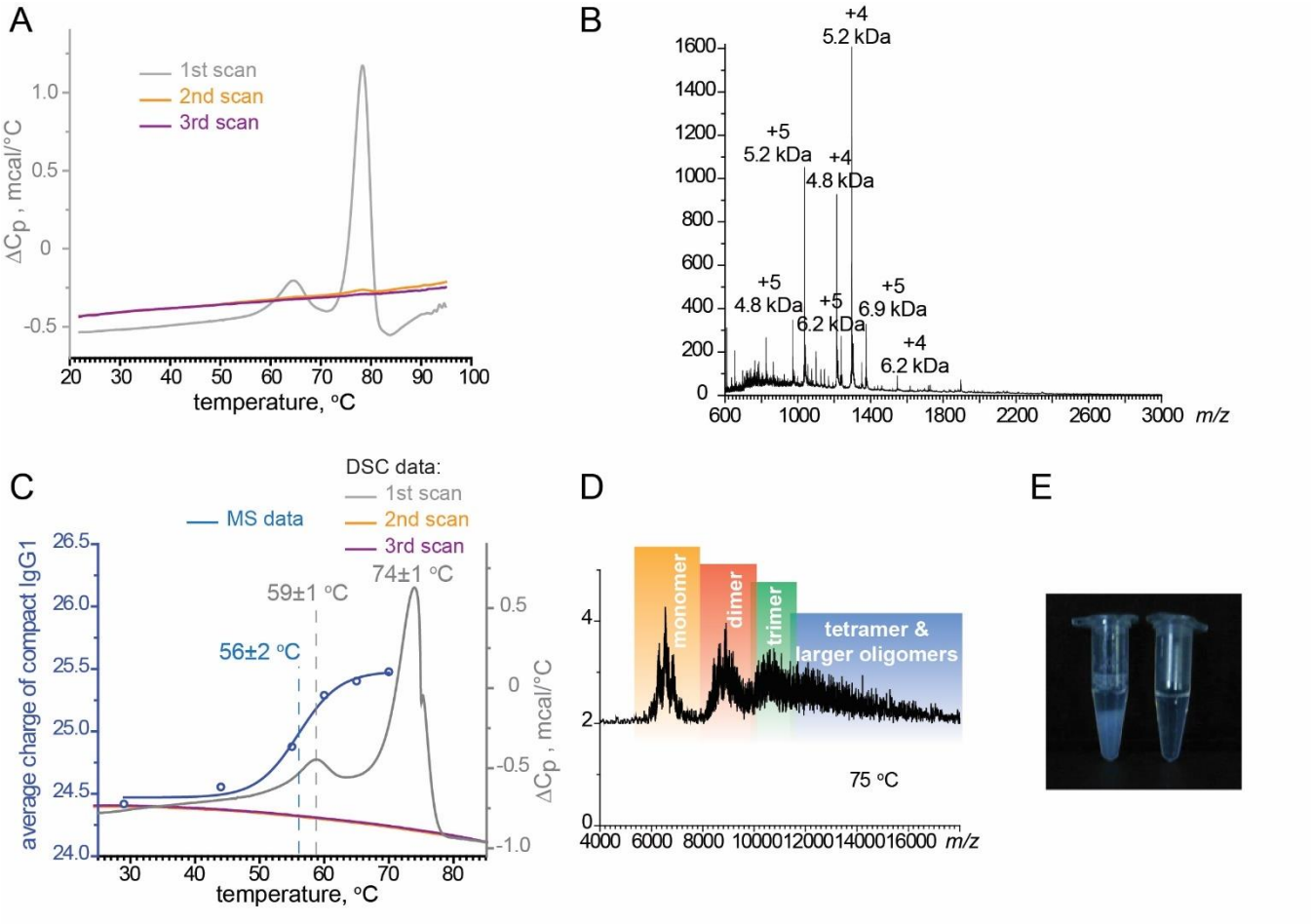

Fig. S3 A: DSC thermograms of three consecutive heating scans (gray, orange and purple; a programmed downscan (cooling process) was carried after each heating scan; see **Experimental** section for detail) of mAb (in 20 mM NH_4Ac, pH 4.7). **B:** ESI mass spectrum of mAb (in 20 mM NH_4Ac, pH 4.7) solution that was exposed to three DSC cycles, where almost all signal peaks represent peptide fragments. Representative peaks are labeled with the charge states and MW values. **C:** a plot of average charge of compact protein vs. temperature (blue trace) and DSC thermograms of three consecutive heating scans (gray, orange and purple traces) of IgG (in 200 mM ammonium acetate, pH 4.7). **D:** ESI mass spectrum of mAb (in 200 mM NH_4Ac, pH 4.7) acquired at 75 °C with signals accumulated for the same period of time as the other spectra presented in this article. The y-scale shows the absolute levels of baseline and signal intensity. **E:** appearance of mAb solutions prepared in 200 mM ammonium acetate (left) and 20 mM ammonium acetate (right) that were exposed to three DSC cycles.

References

1. I. A. Kaltashov and A. Mohimen, *Anal. Chem.*, 2005, **77**, 5370-5379.
2. L. Testa, S. Brocca and R. Grandori, *Anal. Chem.*, 2011, **83**, 6459-6463.