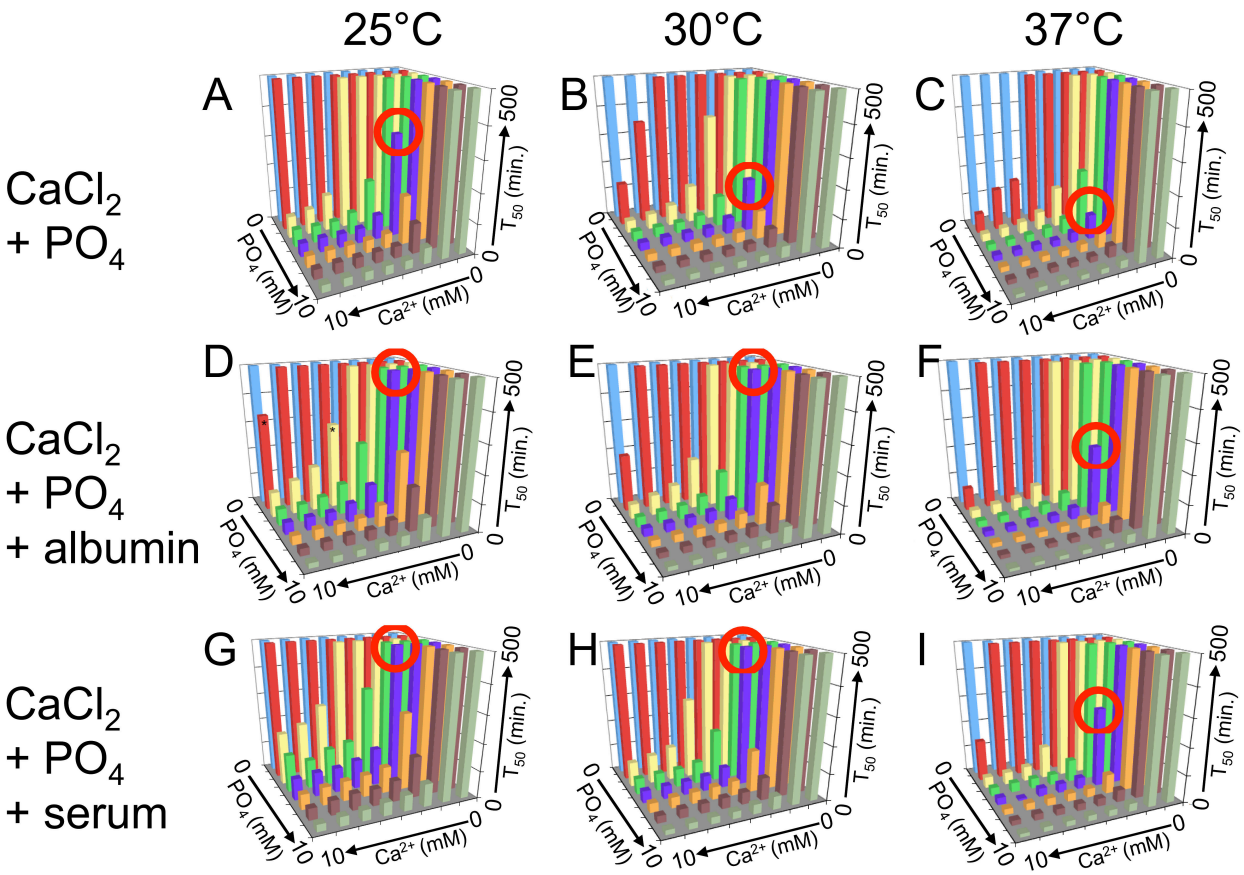
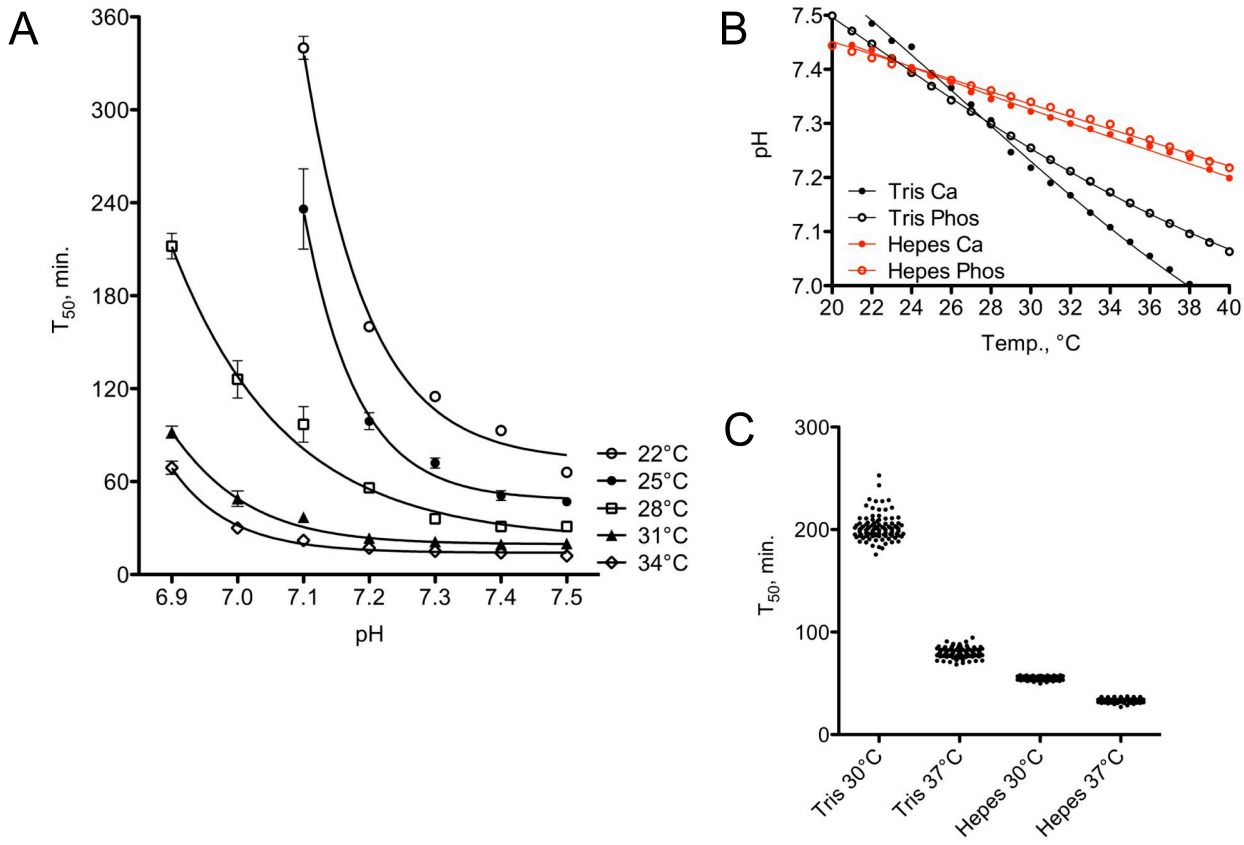
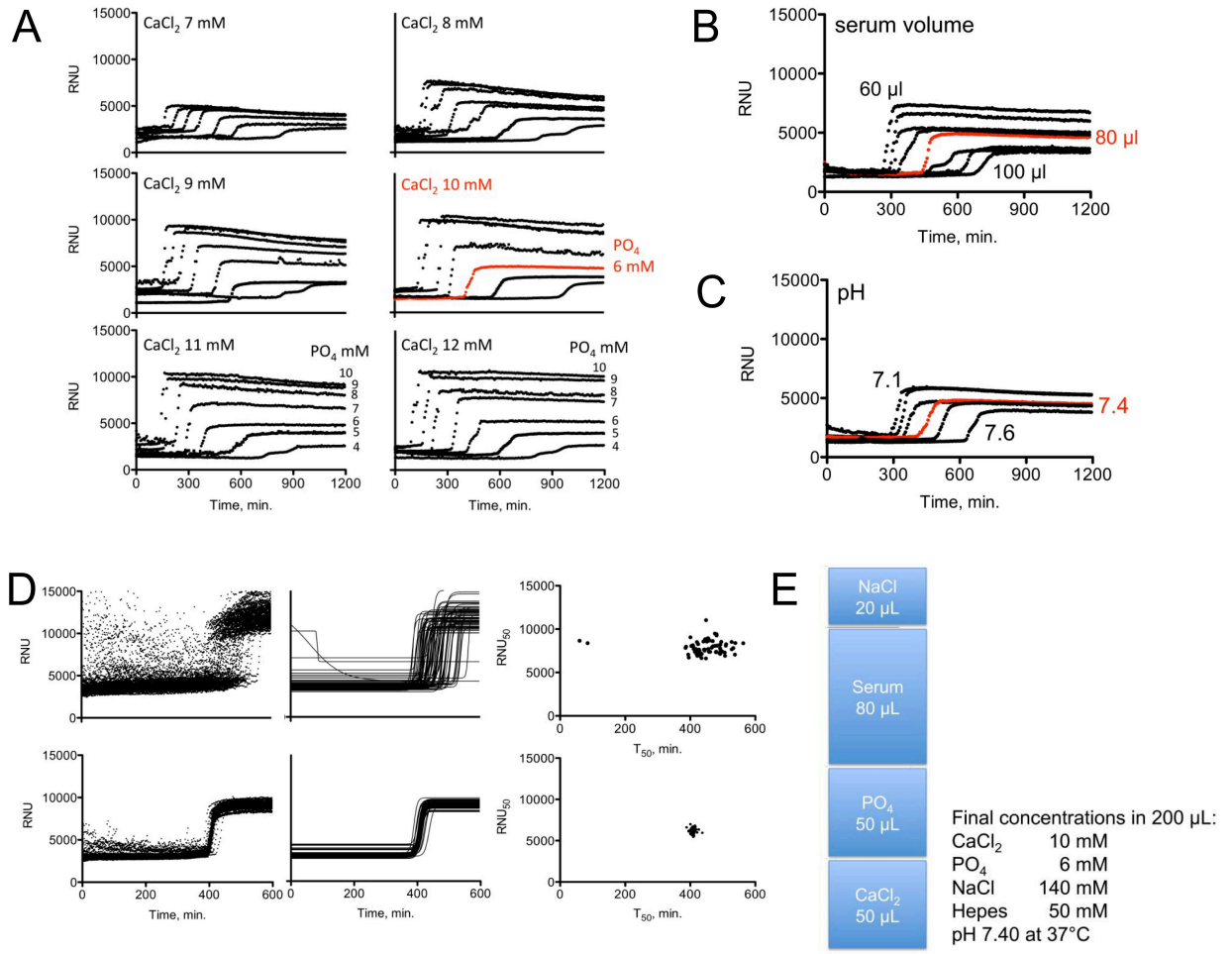


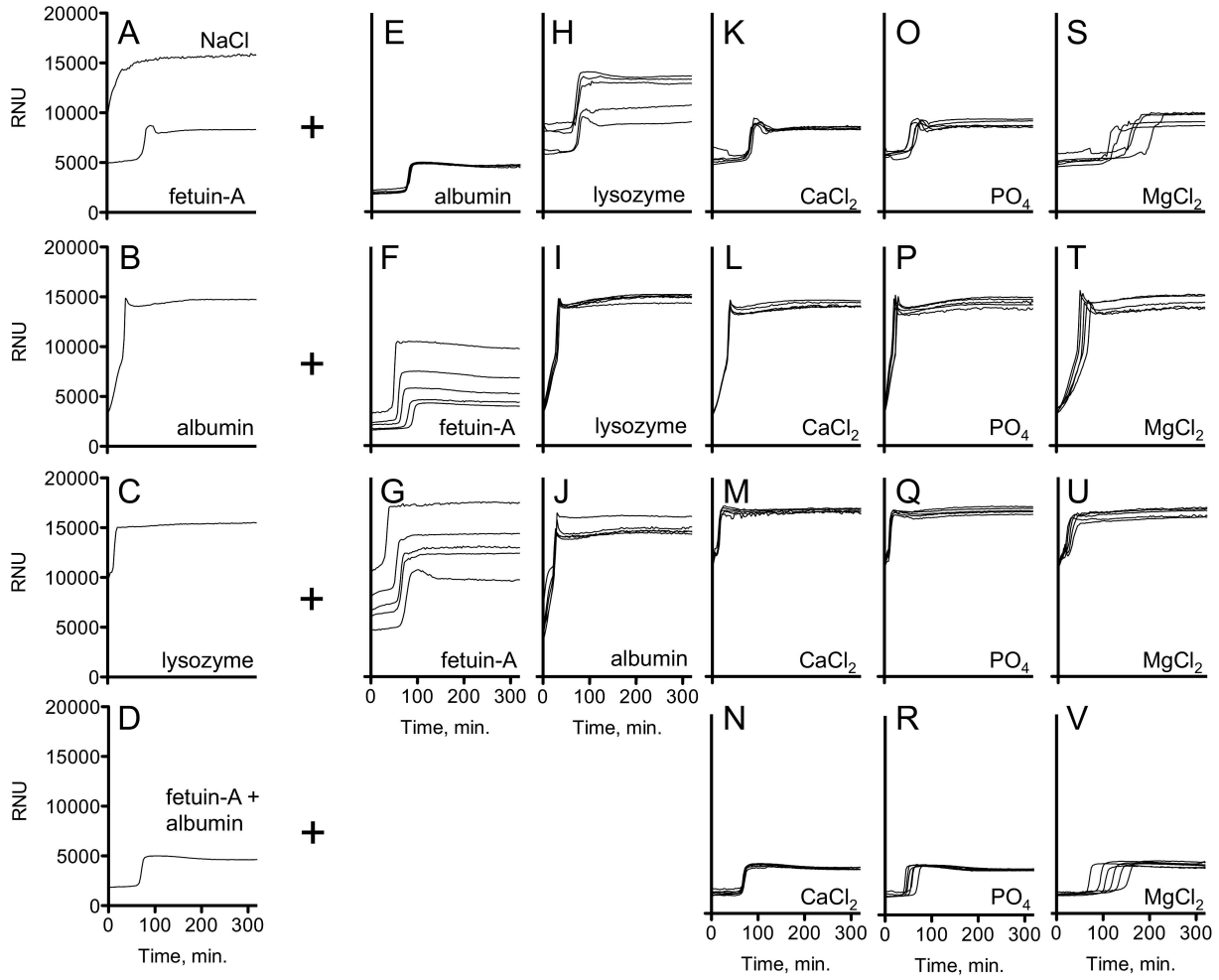
Suppl. Figure 1: Dependence of calcium phosphate precipitation on temperature, and calcium and phosphate concentrations.
When various concentrations of 100 µl calcium and 100 µl phosphate solutions were mixed at (A, D, G) 25°C, (B, E, H) 30°C and (C, F, I) 37°C, precipitation accelerated with increasing temperature. Addition of 10 µl 5% albumin (D through F) or 10 µl serum (G through I) uniformly delayed precipitation velocity at all temperatures. Based on these experiments, 100 µl of 2.5 mM calcium plus 100 µl of 5.0 mM phosphate (both solutions supplemented with 50 mM Tris, pH 7.4 at room temperature) plus 10 µl serum were initially chosen as standard conditions for our nephelometry precipitation assay. These conditions are marked by a red circle in A through I. Under these conditions with comparatively low serum and high calcium and phosphate concentrations, readouts were however extremely variable and hardly reproducible (data not shown). PO_4 and $CaCl_2$ concentrations used here: 0, 1.25, 2.5, 3.75, 5, 6.25, 7.5, 10 mmol/L



Suppl. Figure 2: Impact of temperature, pH and buffer on nephelometry assay.
(A) Isothermal lines for nephelometer assay runs performed from 22°C to 34°C with pH values adjusted at 6.9 to 7.5 (pH adjusted at measurement temperature) showed strong pH- and temperature-dependence of the assay (Hepes buffer); (B) pH-dependence on temperature in the presence of Tris and Hepes buffers (50 mM each). pH decline was more pronounced with Tris than with Hepes buffer both in calcium (2.5 mM) as well as phosphate (5.0 mM) solutions; (C) Precipitation times (T_{50}) of calcium plus phosphate solutions greatly differed with Tris or Hepes buffer when the pH was adjusted to 7.40 at room temperature, and the nephelometer assay subsequently performed at higher temperatures (30 and 37°C). Concentrations used in all experiments: calcium 2.5 mmol/L, phosphate 5.0 mmol/L, Tris or Hepes 50 mmol/L.
All experiments shown here were performed in the absence of serum.

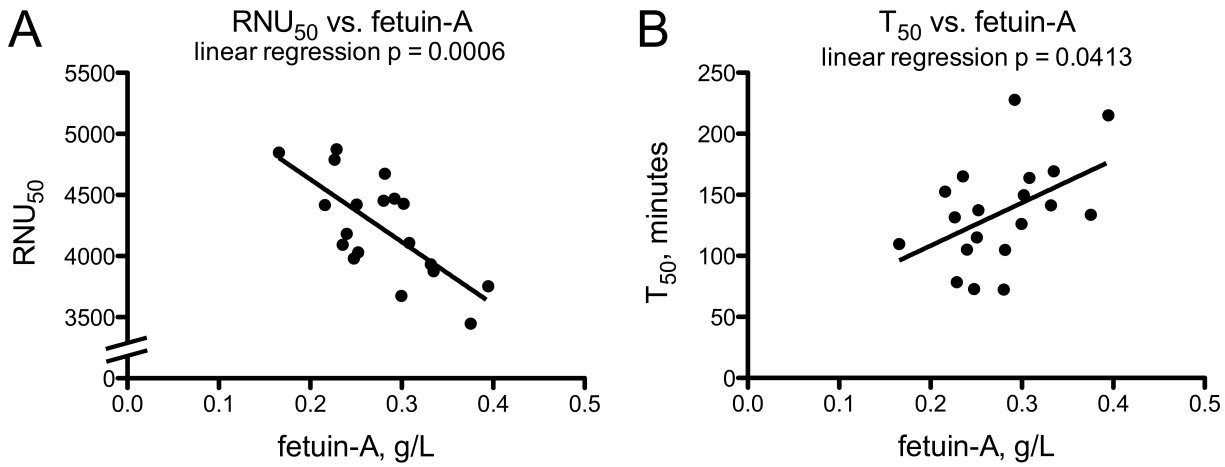


Suppl. Figure 3: Establishment of standard nephelometry assay conditions.
(A) Influence of calcium and phosphate concentrations on the assay readout. Calcium (50 µl, 7-12 mM) and phosphate (50 µl, 4-10 mM) solutions were mixed in the presence of 80 µl serum from healthy volunteers and 20 µl NaCl 140 mM; (B) impact of serum volume on assay readout; (C) impact of pH on assay readout; (D) scatter of curves from 96 well plates before (top) and after (bottom) standardization of the assay. From left to right: raw data, data transformed by nonlinear regression analysis, T_{50}/RNU_{50} values; (E) final conditions used for the nephelometry assay. Red curves in (A) through (C) indicate the final assay conditions (Ca^{2+} 10 mM, PO_4 6 mM, serum 80 µl, pH 7.40 adjusted at 37°C).



Suppl. Figure 4: Nephelometry in the presence of single and combined serum constituents.

(A) Fetuin A (0.5 g/L), (B) albumin (50 g/L), (C) lysozyme (50 g/L) and (D) a combination of fetuin-A and albumin (0.5 g/L plus 50 g/L) were spiked with (E, J) albumin (12.5, 25, 37.5, 50, 70 g/L), or (F, G) fetuin A (0.125, 0.25, 0.375, 0.5, 0.7 g/L), or (H, I) lysozyme (12.5, 25, 37.5, 50, 70 g/L), or (K-M) calcium (1, 1.5, 2, 2.5, 3 mmol/L), or (O-R) phosphate (1, 1.5, 2, 2.5, 3 mmol/L), or (S-V) magnesium (0.5, 0.75, 1, 1.25, 1.5 mmol/L). Albumin and lysozyme alone led to only minor changes of curve morphology when compared to NaCl 140 mmol/L as shown in A. Fetuin-A alone led to a concentration-dependent reduction of the RNU-signal and a delay of particle transformation (A, F, G). Albumin alone had no effect (B), but it acted synergistically with fetuin-A (E). The addition of calcium (K-M) had no marked effect, the addition of phosphate (O-R) led to a slight acceleration, and the addition of magnesium (S-V) to a dose-dependent delay of particle transformation.



Suppl. Figure 5: Correlation of RNU_{50} and T_{50} with fetuin-A serum concentrations.
Fetuin-A concentrations were measured in sera obtained from 20 hemodialysis patients and plotted against the RNU_{50} and T_{50} values obtained from our assay. Fetuin-A concentrations were highly correlated with (A) RNU_{50} ($p = 0.0006$) and (B) T_{50} ($p = 0.0413$). Patient sera were the same as used for the experiment shown in Figure 3B. Fetuin-A serum concentrations were measured by ELISA as described (Ketteler M, Bongartz P, Westenfeld R, Wildberger JE, Mahnken AH, Böhm R, Metzger T, Wanner C, Jahnen-Dechent W, Floege J: Association of low fetuin-A (AHSG) concentrations in serum with cardiovascular mortality in patients on dialysis: a cross-sectional study. Lancet 361: 827–833, 2003).

Fetuin-A serum concentrations and RNU_{50} and T_{50} correlate well. Note however, that the nephelometry should measure calcification propensity unbiased and beyond fetuin-A, including the contributions of calcium, phosphate, magnesium, albumin, and other yet unidentified components.