

SI Text

Results

The viscosity of the solutions with and without the four kinds of copolymer nanoparticles was measured at particle concentrations of 0.01 mg/ml as described below. The buffer itself had a viscosity of 0.001043 Pa's, whereas the buffer with the particles at 0.01 mg/ml had viscosities ranging from 0.001016 to 0.001082 Pa's. These values are within the errors of the measurement, demonstrating that the enhanced fibrillation rate observed in the presence of nanoparticles cannot be ascribed to differences in the solution viscosity.

Materials and Methods

Protein. For this work human β_2m was initially expressed in *E. coli* BL21 pLysS and purified as described (1). The protein samples were stored as lyophilized powder and dissolved directly into the buffer immediately before the experiments. Fibrillation experiments were also conducted by using protein produced from a synthetic gene with a modified purification scheme as follows. The synthetic gene for human β_2m (with codons optimized for expression in *E. coli*) was constructed from four overlapping oligonucleotides a, b, c, d (SI Fig. 7) and two end primers (start and stop) using PCR, and cloned into the PetSac vector (modified Pet3a vector with NdeI and SacI cloning sites) as outlined in SI Fig. 7. The protein was expressed from this synthetic gene in *E. coli* BL21 DE3 PLysS Star and obtained as inclusion bodies. The harvested cell pellet was resuspended in Millipore water (400 ml of H₂O for pellet from 13-liter culture) and sonicated. Inclusion bodies were retrieved by centrifugation at 15,000 rpm in a Sorvall SS34 rotor for 5 min, resonicated three times on ice in 10 mM Tris/HCl, 1 mM EDTA, pH 7.3 (300 ml buffer for each sonication step), and collected by centrifugation as before. The resulting pellet was rocked in 100 ml of 8 M urea, 10 mM Tris/HCl, 0.5 mM EDTA, pH 7.3 for 2 h until all pellet was dissolved, pumped onto a 2.7 × 10 cm DEAE cellulose column packed in the same buffer with 8 M urea. The column was washed with 300 ml of

the same buffer with 8 M urea, then eluted with a linear salt gradient from 0 to 100 mM NaCl in 8 M urea, 10 mM Tris/HCl, 0.3 mM EDTA, pH 7.3, total volume 500 ml. The fractions were analyzed by absorbance spectroscopy, nonreducing SDS/PAGE, and agarose gel electrophoresis. Fractions containing predominantly β_2m were pooled and diluted with 8 M urea, 10 mM Tris/HCl pH 7.3, to a total volume of 3.4 liters, to yield an absorbance of 0.8 at 277 nm corresponding to a protein concentration of ≈ 0.5 mg/ml (1,700 mg total). The pH was adjusted to 8.0 with Tris base and 2 mM DTT was added. The protein solution was dialyzed against standing deionized water (pH 7.3) for 10 h in a dialysis bath only twice as large as the volume inside the dialysis tubing. The dialysis was then continued by six water exchanges, lasting for about 1 h each with larger excess of deionized water. This procedure leads to high yields of correctly folded protein. The solution was then rocked with 60 ml of DEAE cellulose for 2 h, collected on a funnel, packed in a column, washed with 5 mM Tris/HCl pH 7.3, and eluted with a linear NaCl gradient from 0 to 100 mM in 5 mM Tris/HCl pH 7.3, total gradient volume 600 ml. Fractions containing correctly folded β_2m were pooled (total 1,200 mg), concentrated to 12 mg/ml, aliquoted, and frozen. The total time from starting the production cell cultures in 13 liters of LB medium to the aliquotation and freezing of the correctly folded protein was 60 h. While developing the protocol we observed that overall speed and especially limited time spent between harvest of the cells and elution from the first exchange column in urea are the major factors preventing deamidation. For each set of fibrillation experiments, one aliquot (72 mg) was thawed and purified by gel filtration on a Superdex 75 column to remove any traces of oligomers and aggregates. The monomer fraction was collected and used immediately in the fibrillation assays.

Nanoparticles. NIPAM/BAM) copolymer particles of 70 and 200 nm in diameter and with two different ratios of the comonomers (85:15 and 50:50 NIPAM/BAM) were synthesized in SDS micelles as described (2), although higher SDS concentrations were used in the present work, resulting in similarly sized particles. The procedure for the synthesis was as follows: 2.8 g of monomers (in the appropriate wt/wt ratio) and 0.28 g of cross-linker (*N,N*-methylenebisacrylamide) were dissolved in 190 ml of MilliQ water with either 0.8 g of SDS (for the 70-nm particles) or 0.32 g of SDS (for the 200-nm

particles) and degassed by bubbling with N₂ for 30 min. Polymerisation was induced by adding 0.095 g of ammonium persulfate initiator in 10 ml of MilliQ water and heating at 70°C for 4 h (3). After polymerization, particles were extensively dialyzed against MilliQ water for several weeks, with the water changed daily, until no traces of any of the monomers, cross-linker, initiator, or SDS could be detected in the dialysis water or nanoparticle solution by proton NMR in comparison with spectra of each starting component. All spectra were acquired in D₂O with a 500-MHz Varian Inova spectrometer. Particles were freeze-dried and stored in the refrigerator until use.

Sixteen-nanometer hydrophilic polymer-coated quantum dots were provided by W. Parak, Ludwig Maximilians University, and 16-nm cerium oxide particles and multiwalled carbon nanotubes of 6 nm diameter were provided by J. Hanrahan of Glantreo Ltd.

Thiol-Linked Nanoparticles for SPR Studies. NIPAM/BAM/acrylic acid copolymer nanoparticles were synthesized in water by using SDS micelles for controlling particle size as described, with the addition of appropriate amounts of acrylic acid to obtain maximum one carboxyl group per particle surface (on average; ref. 4). Acrylic acid was distilled under reduced pressure before use to remove stabilizers. A stock solution of 1 mg/ml acrylic acid was then prepared, and 10 µl (70-nm particles) or 1.4 µl (200-nm particles) of this solution was added to the monomer solution. Reaction proceeded at 70°C for 4 h as above. After polymerization, particles were extensively dialyzed against MilliQ water for a couple of weeks, and then the covalent attachment of homocysteine to the acrylic acid groups was achieved by the formation of amide bonds between the primary amino group of the amino acid and a carboxylic acid group on a particle according to the procedure described by Bernkop-Schnürch *et al.* (5) with minor modifications. Briefly, the pH of a 50-ml sample of the particle solution (after dialysis) was adjusted to 5 by the addition of 5 M NaOH. Then 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was added to a final concentration of 150 mM to activate the carboxylic acid moieties. After 1 h of incubation with stirring at 4°C, 0.4 g of homocysteine was added and the pH was readjusted to 5. The reaction mixture was

incubated for 5 h at room temperature under stirring. Finally, the particles were dialyzed extensively against MilliQ water to ensure that no residual chemicals remained and freeze-dried.

Fibrillation Experiments in Plates. In these fibrillation experiments, 80 μM (1 mg/ml) $\beta_2\text{m}$ was incubated at 37°C in 10 mM sodium phosphate buffer, pH 2.5, with 0.02% NaN_3 , and 10 μM thioflavin T without or with 0.01 mg/ml nanoparticles in a multiwell plate. The plates were agitated continuously in an incubator at 200 rpm. The thioflavin T fluorescence was measured at 480 nm with excitation at 440 nm.

Fibrillation Experiments in Tubes. In the vast majority of fibrillation experiments, $\beta_2\text{m}$ fibrillation was studied at 37°C in the absence and presence of nanoparticles under a set of solution conditions in Eppendorf tubes. The protein concentration was 40 or 125 μM (as determined by absorbance at 277 nm), in 6, 10, or 20 mM sodium phosphate buffer, pH 2.5, with 0.02% NaN_3 , and no added or 50 mM NaCl (see SI Table 1). The protein solution was subjected to gel filtration just before setting up the experiments to isolate the protein monomer and remove any traces of oligomers or aggregates. Buffer stock was added to the monomer solution, pH was checked on a thoroughly rinsed pH electrode, and the solution was then filtered (0.2- μM filter Satorius Minisart, which was washed with buffer before the sample) before it was incubated at 37°C with or without 0.01 mg/ml copolymer nanoparticles. The experiments were set up in groups from the same solution, with 48-120 samples in each group out of which one fraction without nanoparticles and other fractions supplemented with nanoparticles. In total, 732 samples were followed for experiments with and without copolymer nanoparticles, and 116 samples were followed in the experiments with and without carbon nanotubes, cerium oxide particles, and quantum dots. Each sample of 0.5 ml was shaken in a 1.5-ml Eppendorf tube at 250 rpm with close to horizontal orientation. To monitor the appearance and growth of fibrils, aliquots from the tubes were taken at different time points, added to a black 96-well plate, and the thioflavin T fluorescence (20 μM thioflavin T added) was measured at 475 nm with excitation at 435 nm in a plate reader.

The protein concentrations used in the fibrillation experiments are similar to those found in patients with renal failure (up to 20 μM). Particle concentrations of < 0.001 mg/ml are typically of interest in nanotoxicology. We present data at slightly higher concentrations to move nucleation into a convenient time range; however, test experiments show that the lag phase is significantly shortened also at a particle concentration of 0.001 mg/ml.

Viscosity Measurements. The viscosity was measured for 10 mM sodium phosphate buffer, pH 2.5, with 0.02% NaN_3 , and the same buffer with the four kinds of particles (70 and 200 nm 85:15; 70 and 200 nm 50:50) at 0.01 mg/ml. Viscosity measurements were performed on a Carrie-Med CSL controlled stress rheometer with a cone and plate geometry (4 mm, 1° acrylic cone), and a Peltier element for temperature control. Measurements were carried out in the shear stress sweep mode at 37°C. For each sample the stress at an angular velocity of 30 Rad/s was determined (below the breaking point of the sample), and this value was used as the maximum stress in the flow experiments.

Electron Microscopy. Negative stain electron microscopy images were taken in a Philips CM-10 microscope. Sample grids were prepared as described (6).

Fluorescence Titrations. $\beta_2\text{m}$ at different concentrations ranging from 0 to 4 μM was mixed with nanoparticles (70 nm 50:50 or 70 nm 85:15 NIPAM/BAM at 0.9 nM concentration) in 10 mM sodium phosphate buffer, pH 2.5, with 50 mM NaCl and 0.02% NaN_3 . The samples were incubated at 37°C for 30 min before fluorescence spectra were recorded at 37°C with a PerkinElmer LS50B spectrofluorimeter. Tryptophan fluorescence was excited at 280 nm (slit 2.5 nm), and spectra were recorded between 300 and 420 nm (slit 5 nm).

Conjugation of Nanoparticles to Gold Surfaces for SPR Studies. The SIA Au kit (BIAcore AB, Uppsala, Sweden) was used for sensor chip preparation. Thiol-linked nanoparticles were dissolved at 1 mg/ml in 20 mM sodium phosphate buffer, 100 mM NaCl, pH 7.5 on ice, and 200 μl of this solution was placed on a 10 \times 10-mm gold surface. The thiol groups on the particles were allowed to react with the gold for 1 h,

before the surface was rinsed with H₂O, dried and assembled in a sensorchip cassette using the tools provided in the SIA Au kit.

SPR Experiments. SPR studies of β_2m associating to and dissociating from nanoparticles were performed by using a BIAcore 3000 instrument at 25°C. The flow buffer contained 10 mM sodium phosphate buffer, pH 2.5, with 50 mM NaCl, and was filtered (0.2 μ m filter) and degassed for at least 30 min before use. Each sensorchip surface with attached particles was washed for at least 5 h at a flow rate of 50-100 μ l/min and then equilibrated at 10 μ l/min for at least 30 min or until the baseline was stable. β_2m was injected for 30 min to study the association kinetics. After 30 min, buffer was flown over the sensorchip surface for 10-24 h. All experiments were performed at a constant flow rate of 10 μ l/min and all solutions were passed over the four flow channels in sequence.

The baseline response before injection of protein was subtracted from the data and Eqs. **1** or **2** were fitted to association and dissociation phase response [$R(t)$], respectively.

$$R(t) = C1 (k^{1on}/(k^{1on} + k^{1off})) (1 - \exp(-(k^{1on} + k^{1off})t)) + C2 (k^{2on}/(k^{2on} + k^{2off})) (1 - \exp(-(k^{2on} + k^{2off})t)) \quad [1]$$

$$R(t) = A1 \exp(-k^{1off} t) + A2 \exp(-k^{2off} t), \quad [2]$$

where a small k indicates a rate constant, and C1, C2, A1 and A2 are amplitudes.

In the calculation of the amount of bound proteins per particle, both protein and nanoparticles were assumed to yield a signal of 1 response unit for a bound amount of 1 pg/mm².

Isothermal Titration Calorimetry. Adsorption of β_2m to the 70-nm copolymer particles was studied by titrating β_2m (from a 160 μ M stock) into a nanoparticle solution in 10 mM sodium phosphate buffer, pH 2.5, with 50 mM NaCl at 5°C by using a Microcal VP

ITC instrument. The reaction cell (1.4 ml) contained 9 nM (1 mg/ml) of the 70-nm nanoparticles with 50:50 or 85:15 NIPAM/BAM ratio, and the protein was injected in steps of 8 or 15 μ l. Data were fitted by using Eq. 3, which assumes a simple 1:1 binding isotherm with affinity, stoichiometry and ΔH as variable parameters, yielding the change in heat content from injection $i - 1$ to i as

$$\Delta Q(i) = Q(i) - Q(i-1) + \frac{dV_i}{V_0} \left[\frac{Q(i) + Q(i-1)}{2} \right] \quad [3]$$

Each heat of injection is

$$Q = \frac{V_{cell} [np]_{tot} N \Delta H^o}{2} \left(1 + \frac{[b2m]_{tot}}{N [np]_{tot}} + \frac{1}{K_A N [np]_{tot}} - \sqrt{\left(1 + \frac{[b2m]_{tot}}{N [np]_{tot}} + \frac{1}{K_A N [np]_{tot}} \right)^2 - \frac{4 [b2m]_{tot}}{N [np]_{tot}}} \right)$$

[4]

where N is the number of moles of adsorbed β_2m per particle, ΔH^o is the enthalpy change upon binding (J/mol protein), V_{cell} is the cell volume, and K_A is the equilibrium association constant.

$$K_A = \frac{[np \bullet b2m]}{[np][b2m]}$$

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