The effect of nanoscale surface curvature on the oligomerization of surface-bound proteins

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The influence of surface topography on protein conformation and association is used routinely in biological cells to orchestrate and coordinate biomolecular events. In the laboratory, controlling the surface curvature at the nanoscale offers new possibilities for manipulating protein–protein interactions and protein function at surfaces. We have studied the effect of surface curvature on the association of two proteins, α-lactalbumin (α-LA) and β-lactoglobulin (β-LG), which perform their function at the oil–water interface in milk emulsions. To control the surface curvature at the nanoscale, we have used a combination of polystyrene (PS) nanoparticles (NPs) and ultrathin PS films to fabricate chemically pure, hydrophobic surfaces that are highly curved and are stable in aqueous buffer. We have used single-molecule force spectroscopy to measure the contour lengths $L_c$ for α-LA and β-LG adsorbed on highly curved PS surfaces (NP diameters of 27 and 50 nm, capped with a 10 nm thick PS film), and we have compared these values in situ with those measured for the same proteins adsorbed onto flat PS surfaces in the same samples. The $L_c$ distributions for β-LG adsorbed onto a flat PS surface contain monomer and dimer peaks at 60 and 120 nm, respectively, while α-LA contains a large monomer peak near 50 nm and a dimer peak at 100 nm, with a tail extending out to 200 nm, corresponding to higher order oligomers, e.g. trimers and tetramers. When β-LG or α-LA is adsorbed onto the most highly curved surfaces, both monomer peaks are shifted to much smaller values of $L_c$. Furthermore, for β-LG, the dimer peak is strongly suppressed on the highly curved surface, whereas for α-LA the trimer and tetramer tail is suppressed with no significant change in the dimer peak. For both proteins, the number of higher order oligomers is significantly reduced as the curvature of the underlying surface is increased. These results suggest that the surface curvature provides a new method of manipulating protein–protein interactions and controlling the association of adsorbed proteins, with applications to the development of novel biosensors.

1. Introduction

The delicate balance between different interactions within and between biological molecules gives rise to intricate structures and dynamics. In many cases, subtle changes to their environment can give rise to dramatic rearrangements of the molecules. For example, changes in pH can alter the oligomerization state and binding of ions to proteins that changes their biological function. This sensitivity to environmental conditions is very common in biology, and cells have evolved elaborate, nanostructured machinery that allows them to respond to and even exploit environmental changes and stresses, such as changes in pH, temperature and pressure, to ensure their viability. In addition, the curvature of surfaces within cells is exploited to manipulate biomolecular events and this has the advantage of targeting specific locations within the cell.

Cells also respond to the topography of surfaces on which they grow. Micro-patterned of surfaces has been used to geometrically control the growth and apoptosis of epithelial cells [1] and to direct the migration of various mammalian cells [2] by controlling cell shape. Curvature has been specifically identified...
as a causal factor in the differentiation of mesenchymal stem cells, with cells that were trapped inside a convex shape differentiating preferentially into adipocytes while the concave curvature promoted osteoblast generation [3]. Nanoscale topography has been used to influence cell adhesion [4] and to induce morphological, genomic and proteomic changes in bacteria [5]. These studies have controlled the tissue micro-environment of cells to regulate their development, but an important biological question remains to be elucidated: do cells detect the geometry of the underlying surface by an internal mechanism (for example, signalling through cytoskeletal strain [3]), or do they detect biochemical or geometric changes in the membrane or protein film to which they are attached? Both effects are probably relevant and it is important to study each in detail. This means that it is important to understand the impact of substrate structure on cells as well as individual proteins that are many orders of magnitude smaller.

An excellent example of the effect of the underlying substrate structure is the localization of the peripheral membrane protein SpoVM at the outer forespore membrane as a result of recognition of convex curvature during sporulation of Bacillus subtilis [6]. Moreover, it has been shown in vitro that native SpoVM selectively targets membrane vesicles smaller than 5 μm, while a non-functional mutant is much less size selective and localizes in vesicles up to 20 μm in diameter [7]. Concave membrane curvature has also been shown to influence the localization of the peripheral membrane protein DivIVA [8]. In these cases, the mechanism by which the micrometre-scale curvature influences a nanometre-scale object has yet to be elucidated, though it may be that biological membranes act as curvature amplifiers by generating packing defects that can be detected by individual proteins [9]. Importantly, it remains to be shown whether surface-binding proteins respond to geometric curvature itself (perhaps by altering surface-induced folding landscapes) or whether phospholipid membranes must act as biochemical assistants or possibly transducers.

The availability of a wide variety of synthetic nanostructures [10] has made it possible to directly probe the effect of nanoscale surface geometry on the structure and function of adsorbed proteins. Recent studies have highlighted the importance of characterizing the complex and dynamic ‘corona’ of adsorbed proteins on nanoparticles (NPs) in vitro [11–13], emphasizing that cellular responses to nanomaterials in a biological medium are likely to stem from the adsorbed biomolecular layer rather than the material itself [14,15]. For example, it has been demonstrated that the adsorption of fibrinogen on talcum can be affected by nanoscale surface roughness, whereas the adsorption of bovine serum albumin (BSA) was not affected [16]. On titanium surfaces with similar nanoscale roughness, no changes in fibrinogen adsorption were observed with increasing roughness [17], whereas BSA adsorption was significantly increased on platinum surfaces with nanoscale roughness [18]. These results illustrate the subtle interaction of surface chemistry and geometry on the adsorption of proteins, with different substrate materials yielding different trends. Understanding the influence of nanoscale geometry on molecular events will not only further our understanding in vivo but also lead to technological developments, such as biomolecular NP conjugates for use in biosensing [19,20], drug delivery [21,22] and the creation of new classes of advanced biomaterials [23–25].

A number of studies have demonstrated changes in protein structure and function due to the adsorption of proteins onto NPs of diameter less than approximately 100 nm. Most of these investigations have used optical spectroscopy techniques to measure colloidal suspensions of protein-coated NPs, including tryptophan fluorescence [26,27], circular dichroism (CD) [28–31], ultraviolet–visible [29,32,33], infrared (IR) [27,29,34] and surface plasmon resonance [32,35,36] spectroscopies. Silica NPs have been used to induce structural changes in lysozyme [28,31,37–39], BSA [34,39–41], fibrinogen [34], human carbonic anhydrase I [30], ribonuclease A [42], haemoglobin [40] and β-lactoglobulin (β-LG) [27]. Changes in secondary structure from α-helix to β-sheet were detected in a model peptide covalently attached to thiolated Au NPs [43]. Other surfaces with nanoscale curvature, such as liposomes [44] and single-walled carbon nanotubes [45,46], have been investigated for their effect on the catalytic activity of enzymes. These measurements suggested that proteins could be stabilized on surfaces with nanoscale curvature more readily than on flat surfaces by suppressing unfavourable lateral protein–protein interactions. Recently, we have found that the surface curvature can also suppress favourable protein–protein interactions that hold together dimers and higher order oligomers [47].

In this study, we compare the effect of nanoscale surface curvature on two surface-binding proteins, α-lactalbumin (α-LA) and β-LG, using single-molecule force spectroscopy (SMFS). SMFS is an atomic force microscopy (AFM)-based technique that has been developed to study protein unfolding at the single-molecule level [48,49]. Protein molecules will attach spontaneously to an AFM tip that is brought into contact with a layer of adsorbed protein molecules, and the proteins can be unfolded by translating the tip away from the substrate while measuring the deflection of the AFM cantilever. This mechanical denaturation of proteins can be reversible for large proteins [49,50] and irreversible for small proteins [51]. As the retraction distance leading to a force peak is a measure of the length of an unfolded protein complex and the chain length of a monomer is known from the amino acid primary sequence, SMFS can be used to reliably measure the oligomerization state of a surface-bound protein.

β-LG and α-LA are ideal proteins for investigating interfacial phenomena. Although they are water soluble, they adsorb readily onto both hydrophilic and hydrophobic surfaces [52–55]. Their native environment is at the oil–water interface in bovine milk, where they act as emulsifiers. Bovine α-LA also serves as the regulatory component of lactose synthetase [56]. The structure of both proteins has been studied in detail (Protein Data Bank (PDB) codes 3BLG for β-LG and 1A4V for α-LA). Under physiological conditions, the ruminant variety of α-LA also forms a dimer in solution, but dissociates into monomers below pH 3 [57]. X-ray crystallography has revealed a hexameric quaternary structure for α-LA both with and without Ca²⁺ at physiological pH (PDB codes 1F6S and 1F6R, respectively [58]). Recently, α-LA has attracted renewed attention as a complex with oleic acid, in a technique called HAMLET/BAMLET (human/bovine α-LA made lethal to tumours), which selectively causes apoptosis in tumour cells as a very high-order oligomer [59–61].

Although the structures of β-LG and α-LA are known in solution, much less is known about the conformation of the proteins when they are adsorbed onto surfaces. Nuclear magnetic resonance (NMR) has been used to investigate α-LA when adsorbed onto the surface of polystyrene (PS) NPs [62] and the structure has been identified as a molten globule...
the population of dimers is not significantly affected. Mers and tetramers of as it did for equilibrium of surface-bound oligomers to smaller sizes for show that the nanoscale surface curvature shifts the equilibrium of surface-bound oligomers to smaller sizes for oligomers in solution suggested to us that the effect of nanoscale surface curvature on oligomerization could be even more dramatic for oligomers that were used to create the flat PS substrates (figure 1; also see Material and methods). The transfer of the ultrathin PS film onto the top of the PS NP surface ensured that the surfaces were chemically pure PS that was identical on the flat and curved regions of the samples, eliminating the effect of the ill-defined surface chemistry of the NPs. By choosing the thickness of the PS capping layer to be sufficiently small (10 nm thick) and by performing the water transfer procedure at an elevated temperature, we ensured that the capping film would conform to the shape of the underlying NPs for even the smallest NP diameters (27 nm).

The morphology of the PS-capped PS NP substrates was measured using both optical microscopy and AFM, and representative images are shown in figure 2. By carefully selecting the spin-coating conditions, it was possible to obtain NP-covered regions that contained well-ordered, close-packed layers of NPs perforated by large regions several tens of micrometres to hundreds of micrometres in size that exposed the flat PS substrate, caused by dewetting of the water-based suspension during the spin-coating process. We emphasize that, although it may be possible to create NP films with more uniform coverage, the presence of highly curved and flat regions in the same microscopic domains allowed us to perform in situ comparisons using SMFS of proteins adsorbed to both curved and flat surface topographies. The 10 nm thick PS capping film was clearly visible in the optical micrographs as a result of optical interference with the underlying film or NPs, which allowed us to target only the PS-capped surfaces for curved and flat regions in the AFM experiments. Figure 2 also shows an AFM image of the capping layer and its edge, demonstrating that the capping film conforms around the NPs and maintains the desired nanoscale curvature of the surface.

Recently, we used SMFS to determine that nanoscale curvature suppressed the oligomerization of β-LG on highly curved PS surfaces [47]. The propensity of α-LA to form hexameric oligomers in solution suggested to us that the effect of nanoscale surface curvature on oligomerization could be even more dramatic for α-LA than for β-LG. In this paper, we show that the nanoscale surface curvature shifts the equilibrium of surface-bound oligomers to smaller sizes for α-LA, as it did for β-LG, with an interesting difference: although trimers and tetramers of α-LA are suppressed on curved surfaces, the population of dimers is not significantly affected.

2. Results

The fabrication of highly curved, chemically pure hydrophobic surfaces that are stable in buffer allowed us to perform SMFS experiments on proteins immobilized on surfaces under biologically relevant conditions. A schematic of our sample preparation technique is shown in figure 1 (for details, see the Material and methods section). First, we spin-coated a thin (approx. 80 nm) film of pure, monodisperse PS onto a Si wafer to form a flat hydrophobic PS substrate. Next, we spin-coated a dilute aqueous suspension of surfactant-stabilized PS NPs onto the PS substrate, resulting in close-packed monolayers of the NPs separated by bare regions of the underlying substrate. As the NP suspensions used in this study are stabilized by surfactants, the surface chemistry of the NPs is necessarily different from that of pure PS. To produce chemically pure, highly curved surfaces, we used a water transfer procedure [69] to cap our PS NP surfaces with an ultrathin (10 nm thick) film of the same PS that was used to create the flat PS substrates (figure 1; also see Material and methods). The transfer of the ultrathin PS film onto the top of the PS NP surface ensured that the surfaces were chemically pure PS that was identical on the flat and curved regions of the samples, eliminating the effect of the ill-defined surface chemistry of the NPs. By choosing the thickness of the PS capping layer to be sufficiently small (10 nm thick) and by performing the water transfer procedure at an elevated temperature, we ensured that the capping film would conform to the shape of the underlying NPs for even the smallest NP diameters (27 nm).

In figure 3, we show distributions of the detachment length for the curved and flat regions of 27 and 50 nm diameter NP-coated samples for both α-LA and β-LG. To facilitate comparisons between the different histograms, we have divided the number of counts in a given histogram by the total number N (shown in the legends of each plot) of the force–distance curves included in that histogram. As
each $L_c$ distribution measured for the flat surfaces exhibits a well-defined peak that occurs at a value close to the contour length of a single protein (approx. 50 nm for $\alpha$-LA and approx. 60 nm for $\beta$-LG), we refer to this peak as the monomer peak, as in Kurylowicz et al. [47]. Smaller peaks observed at larger values of $L_c$ are identified as higher order oligomers, e.g. dimers, trimers, etc., indicating that the AFM tip has pulled on two or more proteins that are associated with the surface [47]. For the 50 nm diameter NP surfaces (figure 3a,b), the numbers of measured $L_c$ values that would correspond to the dimer peak for $\beta$-LG and the trimer peak for $\alpha$-LA are reduced with respect to those for the flat surface, with the distributions shifted to smaller $L_c$ values while preserving their overall shape. For the 27 nm diameter NP surfaces (figure 3c,d), the distributions are shifted to even smaller $L_c$ values, with the numbers of measured $L_c$ values that would correspond to the dimer peak for $\beta$-LG and the higher order peaks, e.g. trimers and tetramers, for $\alpha$-LA strongly suppressed or not present at all. Therefore, for both proteins, the number of higher order oligomers is significantly reduced as the curvature of the underlying surface is increased. It is interesting to note that the peaks in the $L_c$ histograms for the 27 nm diameter NP surfaces occur at $L_c$ values (approx. 30 nm for $\beta$-LG and approx. 12 nm for $\alpha$-LA) that are considerably less than the contour lengths of the $\alpha$-LA and $\beta$-LG molecules. It is possible that

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**Figure 2.** Optical microscopy image (left) and AFM topography image (right) of a 50 nm diameter NP surface, with part of the surface covered with a 10 nm PS capping film, indicating both the capped/uncapped and curved/flat regions. The AFM line scan in the lower right corner corresponds to the white line indicated in the AFM image directly above the line scan.

**Figure 3.** Comparison of histograms of detachment lengths $L_c$ measured for $\alpha$-LA and $\beta$-LG on flat PS surfaces with those measured on (a,b) capped 50 nm diameter NP-coated PS surfaces and (c,d) capped 27 nm diameter NP-coated curved PS surfaces. The number of force–distance curves included in each histogram is shown in the inset to each plot.
3. Discussion

One of the key aspects of this study is the development of a unique sample geometry that provides several advantages for studies of proteins on highly curved surfaces. Through the use of arrays of polymer NPs capped by ultrathin polymer films, we can directly compare the results for proteins adsorbed onto flat surfaces with those obtained for proteins adsorbed onto highly curved surfaces that are chemically pure. This is a major advance in the study of the properties of proteins adsorbed onto NP surfaces, e.g. protein–NP conjugates. Although NMR [62,63] and optical spectroscopy [26–36] have been used to characterize the ensemble average behavior of suspensions of protein–NP conjugates, our use of SMFS provides the first investigation of the properties of proteins adsorbed to NP surfaces at the single-molecule level. Moreover, previous studies of bulk suspensions of protein–NP conjugates have suffered from the presence of impurities because suspensions of NPs are typically stabilized by charge or surfactants before the introduction of proteins. In the case of polymeric NPs, there are additional impurities arising from the emulsion polymerization process. Our ability to ensure the chemical purity of the nanoscale curved surfaces by capping the surface-bound NPs with a pure, ultrathin PS film solves a major problem in controlled experimental investigations of protein–NP conjugates. The capping of highly curved surfaces with ultrathin polymer films also offers the possibility for surface curvature investigations using different polymeric materials.

Using this sample geometry, we have compared the association of two different surface-bound proteins, α-LA and β-LG, on flat and highly curved surfaces. It can be seen from the histograms shown in figures 3 and 4 that the effect of increasing the curvature of the underlying surface disrupts the oligomerization of the proteins because the relative size of the monomer peak increased for both proteins at the expense of the higher order peaks for the most highly curved surfaces (27 nm diameter NP surfaces). This effect can be understood in simple terms: each adsorbed protein molecule has more available space and therefore fewer tendencies to interact with neighboring molecules relative to its local environment on a flat surface. This results in fewer higher order peaks observed in the detachment length histograms for the surfaces with the highest curvature. As the size of the protein molecules is small compared with the diameter of even the smallest NPs, it is likely that the surface coverage of β-LG molecules is comparable on NPs of different diameters and that the dominant effect with decreasing NP diameter is weakened lateral interactions between adsorbed proteins [47].

Our SMFS data also show the expected increase in the detachment force F_d with increasing retraction rate (electronic supplementary material, figure S3). The corresponding
detachment length $L_c$ results (electronic supplementary material, figure S3) show that the longest detachment lengths are measured for the fastest retraction rates, suggesting that the dissociation time of an oligomer is comparable to the tip retraction time. It is for this reason that we performed the SMFS experiments using the fastest retraction rate.

Proteins can bind to different vertical locations on the AFM tip because of the vertical compression of the protein layer due to the pressing of the AFM tip onto the sample surface. This compression can allow neighbouring proteins to bind to the AFM tip at locations that are displaced vertically from the AFM tip apex. As the maximum compression of the protein layer is equal to the protein diameter (only several nanometres), this limits the attachment of neighbouring proteins to points on the AFM tip that are displaced vertically from the tip apex by the maximum value of several nanometres. In our histograms of the data presented in this paper, the bin size is 10 nm (considerably larger than the maximum difference in vertical binding distance on the AFM tip) and the changes in $L_c$ values due to changes in the surface curvature are approximately 25 nm (much larger than the maximum difference in the vertical binding distance on the AFM tip). Therefore, the possible variation in the vertical binding location of the proteins is considerably smaller than measured shifts in the $L_c$ values as a result of the surface curvature, with the uncertainty in the $L_c$ values limited to several nanometres.

As the oligomerization state of a protein often modulates its function [72], the manipulation of protein–protein interactions is of great interest for medical and biotechnological applications. Many possibilities are suggested by the ways in which dimerization is used in biological cells. For example, dimerization can regulate signal transduction in cell surface receptors, where ligands induce dimerization in the extra-cellular domain of the protein that triggers kinase activity within the cytoplasm [73,74]. Dimerization enables such remarkable effects by catalysing reactions by bringing substrates and active sites together in favourable orientations and enhancing specificity by increasing the effective surface area of interaction [75]. Transient changes from the monomeric to the dimeric state of the protein can also provide dynamic triggers for conformational changes in the local environment of the protein, can facilitate chemical modifications or exchanges and can provide temporary storage or stability for the monomeric state [72].

Protein–protein interactions can also be altered by external changes. In biomedical products, for example pharmaceutical formulations, chemicals or co-solvents can be added to either stabilize or compete with contacts between proteins [76]. Oligomerization of proteins can also be modified by changing the primary sequence of the protein at specific protein-coupling interfaces [76,77]. These changes will, of course, affect entire protein populations. The advantage of using substrate curvature to control protein oligomerization is that native protein structures can be targeted at specific locations within cells and on biosensing surfaces.

### 4. Conclusion

In this study, we have used SMFS to study changes in the oligomerization of surface-bound $\alpha$-LA and $\beta$-LG adsorbed onto hydrophobic PS substrates, as the curvature of the surface was varied at the nanoscale. A key aspect of this study was the preparation of monolayers of monodisperse PS NPs that allowed us to achieve a well-defined, controllable surface curvature. Capping of the PS NP monolayers with an ultrathin PS film allowed us to obtain chemically pure, highly curved, hydrophobic PS surfaces that were stable in buffer. The samples contained regions of close-packed NP monolayers as well as bare flat regions, allowing for in situ comparisons of the effect of surface curvature on the oligomerization of $\alpha$-LA and $\beta$-LG. By pulling on individual proteins using SMFS, we obtained clear evidence that the association of neighbouring protein molecules was significantly reduced for both $\alpha$-LA and $\beta$-LG molecules on the highly curved surface compared with the flat surface. These results are consistent with the curvature of the surface providing more space for adsorbed proteins, reducing their interactions with neighbouring proteins. In addition, the highest curved surfaces reveal the dominance of $L_c$ values that are considerably smaller than the contour lengths of the protein molecules, which may correspond to the distances between well-defined anchoring points of the proteins with the underlying surface. The ability to alter protein oligomerization with surface curvature provides promising applications to biosensing applications, modifying biological function by tailoring surface topography.

### 5. Material and methods

A 1.5% (by mass) solution of PS ($M_w/M_m = 1.12$, $M_w = 675 000$) in toluene was spin-coated at 1500 r.p.m. onto a 1 $\times$ 1 cm oxidized Si(100) wafer and annealed for 18 h at 105°C under an oil-free vacuum. The thickness of the resulting films was between 70 and 80 nm, as measured by ellipsometry and AFM. The resulting hydrophobic surface was then used as a substrate to spin-coat PS NPs of two different diameters: 27 and 50 nm.

PS NPs (Bangs Labs) were purchased with a concentration 10% by mass. The 27 nm diameter NPs were spin-coated directly from this stock suspension. The 50 nm diameter NP suspensions were diluted to 1% by mass with Milli-Q water, then mixed in a 1:1 ratio with a solution of 400 parts methanol to 1 part dioleoyl-phosphatidylcholine (DOPC) by mass (Avanti; lyophilized powder). The DOPC acted as a surfactant to allow 50 nm diameter NP suspensions to wet the hydrophobic PS film substrate with a much lower contact angle than the pure aqueous suspensions, allowing for convective self-assembly of NPs at the evaporative front during spin-coating. The stock 27 nm diameter NP suspension had a much lower contact angle than the others, and we hypothesized that the 27 nm diameter NPs are small enough to act as their own surfactant, as in Pickering emulsions. After spin-coating, all samples were flushed thoroughly with methanol to dissolve the DOPC and then dried in clean nitrogen gas, including the 27 nm diameter NP samples to which DOPC was not added.

For the capping film, a 0.25% (by mass) solution of PS ($M_w/M_m = 1.12$, $M_w = 675 000$) in toluene was spin-coated at 2000 r.p.m. onto a freshly cleaved 2 $\times$ 10 cm piece of muscovite mica and annealed for 18 h at 105°C under an oil-free vacuum. The resulting film was 10 nm thick as measured using AFM (see figure 2). A razor blade was used to score this film into a grid of twenty 1 cm² squares. The film-coated mica was then lowered at a 45° angle into a pool of Milli-Q water at 70°C. As mica is hydrophilic and PS is hydrophobic, the PS film floats onto the water surface. After 1 min of thermal equilibration under water, the 1 cm² Si-PS-NP-PS substrates were lifted at an angle of approximately 30° under a piece of floating PS film to transfer the PS film onto the solid substrate. Samples were put under vacuum for 1 h to remove water and then stored in air. Although the
conformation of the PS film around the highly curved NPs was not possible for all samples or regions of one sample, it was always possible to identify (with AFM imaging) regions with sufficient conformation of the PS film for SMFS measurements.

The morphology of PS-NP-PS substrates was measured using both reflected light microscopy and AFM. The reflected light microscopy measurements were performed using an Olympus BX-60 microscope using bright-field illumination. The AFM measurements were performed in contact mode in liquid using a Veeco Multimode AFM with a Nanoscope IV controller. Nanorod film NP-TR triangular cantilevers with a nominal spring constant of 0.08 nN nm$^{-1}$ and a nominal radius of curvature of 10 nm were used. The spring constant was measured for each tip $\textit{in situ}$ using the thermal tuning method and results were always within 20% of the nominal value. Deflection sensitivity was also measured for each tip (approx. 45 V nm$^{-1}$) and the resulting force exerted on the surface during imaging was always less than 2 nN. Images were typically collected using a lateral range of 1–2 μm using a scan rate of 1–1.5 Hz, resulting in a tip speed less than 5 μm s$^{-1}$.

A 1% solution of α-LA or β-LG (by mass) in 20 mM imidazole buffer (pH = 6.8) was prepared immediately prior to use. After warming to room temperature, 20 μl of the protein solution was deposited onto the PS-NP-PS substrates and allowed to incubate for 1 h. Excess liquid was then removed from the substrate by wicking with a Kimwipe tissue and the substrate was rinsed repeatedly (3×) with imidazole buffer, leaving only adsorbed protein on the substrate. The sample was then inserted into an AFM fluid cell and allowed to equilibrate for 30 min before measurement using AFM.

The protein layers on the PS-NP-PS substrates were measured with AFM in contact mode in liquid for both imaging and SMFS measurements. A new AFM cantilever was used for each curved and flat surface within a single experiment. For AFM imaging of the protein layers, we used the same operating parameters as for imaging the PS-NP-PS substrates. For the SMFS experiments, a vertical deflection sensitivity of 0.04 V nN$^{-1}$ was used (corresponding to a 3 Hz retraction rate), with a 2 s wait time at the surface to allow protein molecules to attach to the AFM tip. Because of the compact nature of the proteins adsorbed onto the underlying surface, with spatial extents of only several nanometres, there will be very little variation (maximum of several nanometres) in the vertical location of the adsorption site of the protein to the AFM tip. Additional SMFS measurements were performed using retraction rates of 0.5 Hz to measure the effect of the retraction rate on the resulting force–distance curves.

We note that the hydrophobic interaction between the NPs and the surrounding liquid was strong enough to ensure their adhesion to the PS substrate such that the mechanical perturbation associated with AFM contact imaging did not dislodge the NPs from the surface. The tapping film further increased the mechanical stability of the NP-coated surfaces, such that AFM imaging and SMFS measurements of the proteins were possible. The Digital Instruments Nanoscope ‘Point-and-Shoot’ software module (Bruker AXS) was used to image a region of the NP monolayer and then target the top of NPs that had six close-packed neighbours. Typically approximately 100 force–distance curves were collected per NP, with approximately 10% success rate for protein attachment. Owing to a small amount of lateral drift of the AFM system, no NP was probed for more than 2–3 min. Approximately 10 NPs were probed for each AFM image and three to five images were collected at different parts of the sample, for both curved and flat surfaces. This resulted in 100–200 curves for both curved and flat surfaces per sample, of which one-quarter to one-third contained a well-defined WLC curve (see equation (5.1)) at detachment and were used for further data analysis. Between five and 10 samples were measured for each size of NP, with typically 50–100 curves that satisfied our selection criteria for each sample. For each NP diameter and flat PS surfaces, we combined the measurements obtained on different samples to produce the histograms shown in figures 3 and 4, and the electronic supplementary material, figure S3. This resulted in good statistics for both the NP-coated and flat PS surfaces. In half of the experiments, SMFS measurements were performed on the curved surface first and then on the flat surface, and then this order was reversed in the other half of the experiments, to ensure that ageing of the protein layer did not affect our observations. As it was necessary to image NPs before performing the SMFS measurement on the protein layer, flat areas were also scanned with a similar force before performing the SMFS measurements so that all protein molecules were treated in a similar fashion on both flat and curved surfaces.

An objective, automated method was developed to identify and analyse force–distance curves that contained a well-behaved detachment peak [47]. The criteria for deciding whether or not to include a given curve in the detachment length $L_c$ histogram were as follows: the data points in the detachment peak had a maximum force that was larger than 50 pN; the maximum force occurred at an AFM tip–sample separation greater than 5 nm; the data points beyond the detachment peak in the curve corresponded to a flat baseline with near-zero force, and the data points within the detachment peak tracked continuously to the baseline and were well described by the WLC function [78]

$$\frac{F_{L_{c}P}}{k_B T} = \frac{1}{4} \left[ 1 - \frac{x}{L_c} \right]^{-2} - \frac{1}{4} + \frac{x}{L_c},$$

where $F$ is the measured force and $x$ is the tip–sample separation, $T$ is temperature, $k_B$ is Boltzmann’s constant and $L_c$ is the persistence length of the chain (fixed at a value of 0.4 nm for amino acids). The best-fit value of the detachment length $L_c$ was obtained by fitting the detachment peak data to equation (5.1).

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