Conformational flexibility of fatty acid-free bovine serum albumin proteins enables superior antifouling coatings

Gamaliel Junren Ma1, Abdul Rahim Ferhan1, Joshua A. Jackman2✉ & Nam-Joon Cho1✉

Bovine serum albumin (BSA) protein is widely used to fabricate antifouling coatings for nanobiotechnology applications. Numerous BSA protein options are commercially available and obtained through different purification methods, however, there is no guidance on which ones are preferable for antifouling coatings. Herein, we investigate the real-time fabrication of antifouling coatings composed of BSA proteins obtained through different purification methods, and report significant differences in ultrathin film coating properties and ability to mitigate serum biofouling and to prevent nanoparticle-induced immune reactions. Mechanistic studies unravel the source of these performance variations, which are related to some BSA proteins containing fatty acid stabilizers while other BSA proteins are fatty acid-free depending on the purification method. Fatty acid-free BSA proteins exhibit greater conformational flexibility and less charge repulsion, which allow them to form more rigidly attached and tightly packed coatings on flat surfaces and nanoparticles that result in superior application performance.
Ulitrathin film coatings are widely used to coat flat, nanostructured, and nanoparticle surfaces in order to confer advantageous properties, such as antifouling or stealth immune profile features. Many types of coating materials, ranging from natural proteins to synthetic nanomaterials such as polymers with finely tuned architectures, have been explored and one of the most popular options for antifouling applications is bovine serum albumin (BSA) protein. BSA has advantageous properties such as natural abundance, simple coating procedure, low cost, well-established purification methods, and broad availability. Indeed, BSA coatings—often in the form of a single protein monolayer—have long been used in numerous biological assays, including enzyme-linked immunosorbent assays (ELISA), blots, immunohistochemistry, and polymerase chain reaction, in order to minimize nonspecific binding events and thus enhance assay performance. In recent years, BSA has also found extensive usage as an antifouling coating material in biosensing and nanomedicine applications. For example, the simplicity of forming BSA-based coatings has inspired the development of advanced antifouling coatings that enable high-performance electrochemical biosensing in complex biological fluids. In addition, BSA coatings are used to improve nanoparticle stability and biocompatibility and to passivate surfaces to inhibit nonspecific adsorption of bioanalytes.

An often-overlooked aspect of BSA is that there are many commercially available versions which differ in how they were isolated from bovine plasma, even for those from the same company. Guidelines to predict which BSA version would be most suitable for antifouling applications are lacking despite broad interest in developing a nanodescriptor index to predict protein adsorption at nano-bio interfaces. To date, most related studies have only evaluated a single BSA version and compared BSA adsorption and surface-induced conformational changes on different surfaces or investigated the effects of isolated parameters such as ionic strength or heating. However, the comparison of BSA proteins obtained through different purification methods remains unexplored despite being the greatest source of variability. As such, the selection of BSA protein type is largely a matter of trial-and-error and laboratory precedent, and there is oftentimes limited reporting on which particular BSA was used in scientific publications.

Herein, we scrutinize the fabrication of BSA-based antifouling coatings from BSA proteins obtained through different purification methods, and comprehensively investigate BSA conformational and adsorption properties on flat and nanoparticle surfaces in order to identify key mechanistic factors that drive the formation of high-performance ultrathin film coatings with superior antifouling properties.

Results

Evaluation strategy. BSA is widely used to form surface passivation coatings on bulk (e.g., glass coverslip) and nanostructured (e.g., inorganic nanoparticles) surfaces. In practice, the surface is incubated with an aqueous BSA solution, whereby protein molecules noncovalently adsorb and undergo a conformational change (“denature”) on the surface to form an ultrathin protein monolayer coating. Coating performance is sensitive to various material parameters such as atomic composition, surface roughness, and nano-curvature effects, along with environmental parameters such as solution pH and ionic strength.

Our evaluation strategy focused on characterizing the conformational, adsorption, and antifouling properties of six BSA proteins purified through different methods. The six BSA types were purified through three different fractionation routes, without or with a fatty acid removal step (Fig. 1c and Supplementary Table 1). The fractionation step is necessary to separate BSA from other serum components and there are two main options: cold ethanol fractionation and heat-shock fractionation. Briefly, cold ethanol fractionation involves reducing the sample temperature to approximately ~5 °C followed by the addition of ethanol and adjusting the solution pH and ionic strength to isolate BSA protein. Conversely, heat-shock fractionation involves heating the sample (typically to ~60 °C) to isolate BSA protein and is conducted in the presence of a fatty acid stabilizer (caprylic acid). These two fractionation processes can be completed alone or sequentially (cold ethanol, then heat-shock).

Notably, after the fractionation step(s) is completed, the purified BSA proteins still contain fatty acids that were either naturally bound or were added as a stabilizer. We tested fatty acid-containing (“fatted”) BSA proteins prepared by (1) cold ethanol fractionation, (2) heat-shock fractionation, and (3) cold ethanol fractionation followed by heat-shock fractionation and these proteins were designated as BSA 1, 2, 3, respectively. To remove fatty acids, an extra processing step is required, which involves the addition of activated charcoal followed by adjusting solution pH and temperature. We also tested the fatty acid-free (“defatted”) versions of BSA 1, 2, 3, designated as BSA 4, 5, 6, respectively. Infrared spectroscopic characterization—through the analysis of relevant spectral features—confirmed the removal of fatty acids from defatted BSA proteins, which is in line with gas chromatography results (Supplementary Fig. 1 and Supplementary Note 1).

Conformational stability of fatted and defatted BSA proteins. Like other globular proteins, BSA folds in aqueous solution to minimize its conformational free energy. The folded structure imparts conformational stability and we first investigated the relative degree of conformational stability of the different BSA proteins by dynamic light scattering (DLS) and circular dichroism (CD) spectroscopy. The experiments were initially conducted at 25 °C and then repeated at higher temperatures in 5 °C increments. DLS detected temperature-induced loss of conformational stability when protein molecules irreversibly unfold and begin to aggregate and a higher onset temperature indicates greater conformational and/or colloidal stability. CD spectroscopy further evaluated corresponding changes in the secondary structure of protein molecules.

Across the temperature range of 25 to 55 °C, the DLS data showed that all six BSA proteins have ~8 nm diameter, which agrees well with the expected size of BSA monomers (Fig. 2b and Supplementary Fig. 2). At 60 °C, a marked increase in the sizes of defatted BSA proteins (4-6) was detected, with average diameters around 20 nm that indicated the onset of protein aggregation. By contrast, the sizes of fatted BSA proteins (1–3) remained stable at 60 °C. At 65 °C, BSA 4–6 increased in size to around 45 nm diameter and the onset of protein aggregation occurred for BSA 1, with an average diameter of around 35 nm. By contrast, the onset temperatures for BSA 2 and 3 were 70 °C and 75 °C, respectively. Kinetic experiments further verified that defatted BSA proteins underwent more rapid and extensive aggregation, which points to fatty acid molecules conferring BSA proteins with greater conformational stability and/or colloidal stability (Supplementary Fig. 3).
onset temperature of aggregation) and irreversible (at and above the onset temperature) conformational changes contribute to protein unfolding so we compared the helicity data at 65 °C. All three defatted BSA proteins had helical percentages below 50% on average, while all three fatted BSA proteins had helical percentages above 51% on average (Supplementary Table 2). More pronounced differences in helicity were observed at 70 °C, and the findings support that, among the fatted BSA proteins, BSA 1 had the lowest α-helicity and hence, lowest conformational stability. The relatively low conformational stability of BSA 1 is consistent with cold ethanol fractionation, whereby only natural fatty acids remain bound (i.e., no additional fatty acid stabilizer). Collectively, the DLS and CD spectroscopy data support that fatted BSA proteins had greater conformational stability than defatted BSA proteins.

Adsorption behavior of fatted and defatted BSA proteins. We then investigated the adsorption behavior of BSA proteins on silica surfaces (Fig. 3a). The experiments were conducted using a BSA protein concentration of 100 µM (~6.6 mg mL\(^{-1}\)) in an aqueous buffer (10 mM Tris [pH 7.5] with 150 mM NaCl) which is representative of the typical solution conditions used in BSA coating protocols. When a protein with lower conformational stability adsorbs onto a solid surface, it undergoes greater unfolding and spreading on the surface due to entropic gains and other factors such as protein-surface and protein-protein interactions can also affect the outcome. We conducted quartz crystal microbalance-dissipation (QCM-D) measurements to track the adsorption kinetics and corresponding mass and viscoelastic properties of adsorbed BSA protein molecules. The QCM-D technique measures real-time changes in the resonance frequency (ΔF) and energy dissipation (ΔD) signals of an oscillating, silica-coated sensor surface, providing insight into the viscoelastic character within the protein adlayers. Greater viscoelasticity, indicating less surface-induced denaturation, was observed for defatted BSA proteins (4-6), indicating greater adsorption, compared to 15 to 35 Hz for fatted BSA proteins (1-3) (Fig. 3c). The corresponding QCM-D energy dissipation signals indicated some degree of viscoelastic character within the protein adlayers (Fig. 3d). Further analysis of [ΔF\(_{\text{max}}\)/ΔD\(_{\text{max}}\)] ratios and time-independent frequency-energy dissipation (F–D) curves revealed that adsorbed, defatted BSA proteins underwent more surface-induced denaturation, as indicated by lower energy dissipation shifts relative to the frequency shift, which is consistent with lower solution-phase conformational stability (Fig. 3e and Supplementary Fig. 6). Conversely, fatted BSA proteins exhibited greater viscoelasticity, indicating less surface-induced denaturation in the adsorbed state.

To corroborate the QCM-D results and extend the results to nanostructured surfaces, we performed localized surface plasmon resonance (LSPR) experiments to measure BSA protein...
adsorption onto silica-coated gold nanodisk array surfaces. The LSPR technique detects the wavelength shift ($\Delta \lambda$) that is associated with changes in local refractive index near the sensor surface and the measurement signal is sensitive to the adsorbed protein mass and degree of adsorption-related protein spreading\(^{38}\). For all six BSA proteins, a positive monotonic $\Delta \lambda$ shift occurred upon adsorption and most protein molecules remained bound irreversibly after buffer washing (Fig. 3f). Importantly, the $\Delta \lambda_{\text{max}}$ shifts showed that defatted BSA proteins exhibited greater adsorption uptake than fatted BSA proteins (~1.2 ± 0.2 nm versus ~0.6 ± 0.2 nm), which supports the QCM-D data (Fig. 3g). Thus, defatted BSA proteins exhibited higher adsorption uptake and tighter adlayer packing compared to their fatted counterparts.

**Adsorption-related protein conformational changes.** To compare the extent of surface-induced protein denaturation for each BSA protein, we further analyzed the LSPR-tracked initial rate of adsorption uptake, denoted as $(d\lambda/dt)$. A larger initial rate is indicative of greater denaturation\(^{38-40}\). The LSPR measurements...
Fig. 3 Real-time monitoring of BSA protein adsorption onto flat and nanostructured silica surfaces. a Overview of experimental protocol. b-e QCM-D experiments tracking frequency (ΔF) and energy dissipation (ΔD) shifts related to BSA adsorption. b Time-resolved QCM-D ΔF shifts and c, corresponding [ΔF_max] shifts at saturation. d Time-resolved QCM-D ΔD shifts. e [ΔF_max]/ΔD_max] ratios obtained from saturation data in panels b and d. f-g LSPR experiments were conducted to measure wavelength shifts (Δλ) related to BSA adsorption. f Time-resolved LSPR Δλ shifts and g, corresponding Δλ_max shifts at saturation. Data in (c, e, g) are reported as mean ± s.d. (n = 3 biological replicates, one-way ANOVA with Tukey’s multiple comparisons test). P values are reported for defatted BSA proteins (versus fatted BSA proteins from the same fractionation method). Dots represent individual data points.

Fig. 4 Evaluation of adsorption-related conformational changes in BSA protein structure. a Maximum rate of change in the LSPR wavelength shift (dΔλ/dt)max during the initial adsorption stage. Values are computed from data in Fig. 3f. Data are reported as mean ± s.d. (n = 3 biological replicates, one-way ANOVA with Tukey’s multiple comparisons test). b Fractional percentage of α-helicity in BSA protein molecules in solution and in the adsorbed state, as determined in ATR-FTIR spectroscopy experiments. Data are reported as mean ± s.d. (n = 3 biological replicates, two-way ANOVA with Tukey’s multiple comparisons test). P values are reported for defatted BSA proteins (versus fatted BSA proteins from the same fractionation method) in (a), and are separately reported for defatted BSA proteins in solution (versus fatted BSA proteins in solution from the same fractionation method) and for defatted BSA proteins in the adsorbed state (versus fatted BSA proteins in the adsorbed state from the same fractionation method) in (b). Dots represent individual data points.

yielded (dΔλ/dt)max values of around 0.6–0.7 nm min⁻¹ for defatted BSA proteins, compared to around 0.2–0.3 nm min⁻¹ for fatted BSA proteins which indicates more extensive surface-induced denaturation for defatted BSA proteins (Fig. 4a and Supplementary Fig. 7).

In addition, changes in BSA protein structure due to adsorption-related conformational changes were measured by attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy. Generally, in the solution phase, the α-helocities of fatted and defatted BSA proteins were around 63%...
and 60%, respectively, agreeing with literature values22 (Fig. 4b, Supplementary Fig. 8, and Supplementary Table 3). Upon adsorption, fatted BSA proteins underwent surface-induced denaturation and the fractional helicities decreased to around 53% (i.e., a net helical loss of ~10%). By contrast, defatted BSA proteins underwent greater surface-induced denaturation, resulting in fractional helicities around 45%, (i.e., a net helical loss of ~15%). The adsorption-related decrease in α-helicity was related to protein unfolding, as indicated by a corresponding increase in the random coil fraction22,41,42. Specifically, fatted and defatted BSA proteins experienced an increase in random coil fraction by ~9% and ~12%, respectively, supporting that fatty acids partially stabilize BSA proteins against surface-induced denaturation by reducing the extent of the helix-to-random coil secondary structure transition (Supplementary Note 2). Thus, multiple lines of experimental data support that defatted BSA proteins exhibit greater surface-induced denaturation than fatted BSA proteins. This finding is consistent with the lower solution-phase conformational stability of defatted BSA proteins, and supports that decreased solution-phase conformational stability translates into more pronounced adsorption-related denaturation on both flat and nanostructured surfaces.

Antifouling coating performance. We next investigated the application performance of the six BSA proteins in surface-based and nanoparticle-based assays related to antifouling properties. We first measured the surface passivation efficiency of adsorbed BSA coatings to inhibit serum biofouling on silica surfaces (Fig. 5a and Supplementary Fig. 9). A bare silica surface was first coated with BSA protein molecules before incubation in 100% fetal bovine serum (FBS) for 80 min, followed by a washing step. The resulting amount of serum biofouling on the BSA-coated silica surface was evaluated by the QCM-D technique. The passivation efficiency of the BSA coating was determined from their ability to inhibit serum biofouling. We found that defatted BSA proteins outperformed fatted BSA proteins, with defatted BSA 5 and 6 exhibiting ~90% passivation efficiency (Fig. 5b). By contrast, fatted BSA 1 and 3 demonstrated less than 40% passivation efficiency. These findings are consistent with greater adsorption uptake and higher packing density of adsorbed, defatted BSA protein molecules.

In addition, we tested the surface passivation ("blocking") performance of fatted and defatted BSA proteins in Western blot experiments involving a hydrophobic nitrocellulose membrane surface (Supplementary Figs. 10–12 and Supplementary Note 3). We first exposed the membrane surface to human serum by electrophoretic transfer, "blocked" the remaining unexposed surface with BSA proteins, and then incubated the membranes with a primary C3 monoclonal antibody followed by a relevant secondary antibody before the intensities of specific and nonspecific bands—produced by enhanced chemiluminescence—were quantified. We found that the membranes "blocked" by
defatted BSA proteins produced lower intensity nonspecific bands, supporting that the higher packing density of adsorbed defatted BSA proteins more effectively prevented nonspecific interactions between the primary and/or secondary antibodies and the membrane surface. Together, these data support that defatted BSA proteins exhibit superior surface passivation performance on hydrophilic and hydrophobic surfaces.

We also tested the antifouling coating performance of BSA coatings on silica nanoparticles to inhibit nanoparticle-induced complement activation, which is an innate immune reaction (Fig. 5c). Using ELISA, we measured the degree of protection BSA coatings conferred against nanoparticle-induced complement activation in human serum, as assessed by the amount of SC5b-9 protein biomarker in solution (Fig. 5d and Supplementary Table 4–5, and Supplementary Note 4). Mechanistically, the enhanced conformational stability of defatted BSA proteins can be understood through the insertion of fatty acid molecules (via hydrophobic tails) into hydrophobic pockets on the molecular surface of BSA proteins, conferring a net stabilizing effect on the protein structure46,47 (Fig. 6a).

A less obvious but critically important effect of fatty acids on BSA relates to the protein adsorption process itself (Fig. 6b). Generally, a protein undergoes surface-induced denaturation upon adsorption, the extent of which depends on the protein’s conformational stability and strength of protein-surface interactions.35 More denaturation causes greater surface spreading and consequently, a larger protein adsorption footprint. Therefore, greater surface spreading—whether due to conformational stability48 or protein-surface interactions (e.g., as mediated by ionic strength effects20,49)—typically results in a smaller total number of adsorbed protein molecules on a target surface.

This scenario holds true for comparing defatted and fatted BSA proteins, however, the difference between the two BSA types is even more pronounced because the fatty acid molecules bound to BSA not only enhance conformational stability but also increase the negative surface charge of BSA protein molecules due to the exposed carboxylic acid headgroups. Thus, while fatted BSA proteins already have a smaller adsorption footprint due to greater conformational stability, the maximum surface density of adsorbed protein molecules in the fatted BSA case is further limited by more repulsive electrostatic interactions between nearest-neighbor protein molecules. These findings support that bound fatty acids enhance not only BSA conformational stability but also charge repulsion between protein molecules in the adsorbed state. As such, defatted BSA proteins are better suited to form passivation coatings since they have lower conformational stability, permitting greater denaturation and spreading in the adsorbed state, and the absence of fatty acids also permits more favorable protein-protein interactions that support the formation of tightly packed, adsorbed protein adlayers.

Taken together, our findings support that it is preferable to use fatty acid-free BSA proteins to form antifouling coatings on flat and nanostructured surfaces. In general, fatty acid-free BSA proteins outperformed fatty acid-stabilized BSA proteins regardless of the fractionation route used in the purification stage. Mechanistically, we show that defatted BSA proteins are superior because they have lower solution-phase conformational stability and reduced charge repulsion in the adsorbed state, which translates into greater surface-induced denaturation and adsorption uptake, resulting in tighter adlayer packing that yields superior passivation coatings.

Looking forward, these findings will be relevant for studying other fatty acid-binding proteins such as human serum albumin across various applications, including protein corona formation, and can also be further explored in the context of nanoparticle properties (i.e., surface chemistry, shape, and size)22,42,50,51. We anticipate that the rational selection of BSA protein options without or with fatty acid stabilizers can enable the fabrication of superior antifouling coatings for a wide range of applications, which can be readily implemented by researchers across different fields of materials science and nanobiotechnology.

Discussion

Our findings demonstrate that defatted BSA proteins exhibit distinct conformational and adsorption properties compared to fatted BSA proteins, and these differences lead to significant variations in antifouling coating performance. Overall, the data highlight the central role of fatty acid molecules in affecting BSA structure and function in the adsorbed monolayer state. To verify the effect of fatty acids, we doped defatted BSA with caprylic acid, thereby converting the defatted protein into a fatted protein. Caprylic acid addition led to markedly enhanced conformational stability, as well as decreased adsorption uptake and surface-induced denaturation (Supplementary Figs. 14–24, Supplementary Tables 4–5, and Supplementary Note 4). Mechanistically, the enhanced conformational stability of fatted BSA proteins can be understood through the insertion of fatty acid molecules (via hydrophobic tails) into hydrophobic pockets on the molecular surface of BSA proteins, conferring a net stabilizing effect on the protein structure46,47 (Fig. 6a).

Methods

Bovine serum albumin proteins. The six types of bovine serum albumin (BSA) proteins used in this study were selected based on three different fractionation methods or without with a fatty acid removal step, and were procured from Sigma-Aldrich (St. Louis, MO, USA). Lyophilized powders of fatty acid-containing BSA proteins purified by cold ethanol fractionation, heat-shock fractionation, and cold ethanol followed by heat-shock fractionation were selected and have catalog nos. A2153, A3059, and A7638, respectively. These three fatted BSA proteins were labeled BSA 1, 2, 3, respectively. The corresponding fatty acid-free versions were also obtained and have catalog nos. A6003, A7030 and A0281, respectively. These three defatted BSA proteins were labeled BSA 4, 5, 6, respectively. All six BSA proteins were used as provided and the fatty acid-free versions were confirmed to have ≤0.01% fatty acid residues by gas chromatography experiments according to the manufacturer (Supplementary Table 1).

Reagents. Sodium dodecyl sulfite (SDS, catalog no. L4390), sodium chloride (NaCl, catalog no. 746398), sodium hydroxide (catalog no. S5581) and octanoic acid (caprylic acid, catalog no. C2875) were also purchased from Sigma-Aldrich while Tris(hydroxymethyl)aminomethane (Tris, catalog no. 4977) was purchased from Fisher Scientific (Olah, OH, USA). Ethanol (95%) was purchased from Aik Moh Sin (Singapore), hydrochloric acid (HCl, catalog no. 100317) was purchased from Merck (Burlington, MA, USA) and fetal bovine serum (FBS, catalog no. BV30160.03, lot no. RC35980) was purchased from HyClone Laboratories (Logan, UT, USA) and stored at −80°C until experiment. Normal human serum (catalog no. NH-serum-38) was obtained from Complement Technology (Tyler, TX, USA) and stored at −80°C until experiment. 30% Acrylamide/Bis Solution 29:1 (catalog no. 1610156), ammonium persulfate (catalog no. 1610700), tetramethylthelylene diamine (TEMED, catalog no. 1610800), 4× Laemmli sample buffer (catalog no. 1610747), 2-mercaptoethanol (catalog no. 1610710), 10× Tris/glycine buffer (catalog no. 160734A), 10× Tris/glycine/SDS buffer (catalog no. 1610732), Tween 20 (catalog no. 1705631), nitrocellulose membranes (catalog no. 1620112), Precision Plus Protein Standards (catalog no. 1610375), Clarity Max Western enhanced chemiluminescent (ECL) substrate (catalog no. 1705062), and horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) antibody (catalog no. A21201L, batch no. 64109318) were all purchased from Bio-Rad Laboratories (Hercules, CA, USA). Complement C3b monoclonal antibody (catalog no. MA1-40155, lot no. SH2428445) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Methanol (catalog no. M/ ethanol) was obtained from Fisher Scientific (Loughborough, UK) and 100-nm diameter silica nanoparticles (catalog no. SIN100) were obtained from nanoComposix (San Diego, CA, USA).

Sample preparation. An aqueous buffer solution of 10 mM Tris, 150 mM NaCl, and pH 7.5 was prepared with Mill-Q-treated water (resistivity of >18.2 MΩ.cm at 25°C) and filtered through a 0.2 µm polyethersulfone (PES) membrane filter (Thermo Fisher Scientific, catalog no. 595–4520). BSA solutions were prepared by dissolving lyophilized BSA powder in this Tris buffer and then filtering through a 0.2 µm syringe filter (catalog no. PN–4612; Pall Corporation, Port Washington, NY, USA). The molar concentrations of BSA proteins in aqueous buffer solution were...
Circular dichroism spectroscopy. Circular dichroism (CD) spectroscopy experiments were conducted using an AVIV Model 420 CD spectrometer with the AVIV CDS software package (v3.36 MX) (AVIV Biomedical, Lakewood, NJ, USA). CD spectroscopy measurements were conducted using 400 µL solutions of 2.5 µM BSA samples in a 1 mm path length cuvette with a PTFE stopper (catalog no. 110-QS; Hellma, Müllheim, Germany). The measurements were recorded with a 1 nm spectral bandwidth, 0.5 nm step size, and an averaging time of 0.1 s. Elevated temperature experiments were conducted by increasing the temperature in the measurement chamber in 5 °C increments from 50 °C to 75 °C and recording the spectra at every temperature point after a 5 min equilibration time. All resulting spectra were processed by subtracting a background spectrum of equivalent solution conditions without BSA protein and the data were presented in mean residue molar ellipticity ([θ]) units based on the following equation:

$$\theta = \frac{n \times c \times l \times 10}{\pi \times n_c}$$  \tag{1}$$

where n is the number of amino acid residues, c is the protein molar concentration, and l is the cuvette path length in cm. All CD spectra were smoothed by using the Savitsky-Golay52 smoothing function with a smoothing window of 20 points and a polynomial order of 2 in the OriginPro 2019b (v9.6.5.169) software package (OriginLab, Northampton, MA, USA). The α-helical percentage of each BSA protein was calculated from the [θ] data at 222 nm ([θ]222) based on the following equation53:

$$\text{Helicity(%) = } \left( \frac{\text{[θ]222} - 3000}{-36000 - 3000} \right) \times 100\%$$  \tag{2}$$

Quartz crystal microbalance-dissipation monitoring. Quartz crystal microbalance-dissipation (QCM-D) measurements were conducted using a QSense E4 instrument (Biolin Scientific AB, Stockholm, Sweden). A silica-coated AT-cut quartz crystal sensor chip with a fundamental frequency of 5 MHz (QXS 303, Biolin Scientific) was used to characterize protein adsorption onto silica surfaces. The temperature was set to 25 °C for all experiments, and the measurement operation was controlled by the QSoft 401 (v2.5.13.664) (Biolin Scientific) software package. Prior to each experiment, the sensor chips were sequentially rinsed with 1% (wt/vol) aqueous SDS solution, water, and ethanol, and then dried under a gentle stream of nitrogen gas, followed by treatment with oxygen plasma (PDC-002, Harrick Plasma, Ithaca, NY, USA) for 3 min. A peristaltic pump was used to inject liquid samples into the measurement chamber at a volumetric flow rate of 100 µL min⁻¹. A stable baseline signal was first established in Tris buffer solution before a 100 µM BSA protein was introduced into the measurement chamber for 30 min, followed by a buffer washing step. The resonance frequency (ΔF) and energy dissipation (ΔD) shifts were recorded in real-time at multiple odd overtones, as previously described54, and the normalized data at the fifth overtone are reported.
The QCM-D technique was also employed to determine the passivation efficiency of BSA coatings against serum biofouling. For these experiments, 100 µM BSA solution was first (blank) on a bare silica surface under continuous flow of 60 min, followed by a 40 min buffer washing step. Undiluted fetal bovine serum (FBS) was then introduced under continuous flow for 80 min, followed by a final buffer washing step for 30 min. The passivation efficiency percentage was calculated by comparing the absolute difference between the frequency shift due to BSA adsorption alone (after first buffer washing step) and the subsequent frequency shift due to FBS biofouling (after second buffer washing step), which is denoted as $\Delta f_{\text{FBS-BSA}}$. A control experiment without BSA coating was also conducted in order to measure the absolute frequency shift due to FBS biofouling alone (after buffer washing step), which is denoted as $\Delta f_{\text{Control}}$. For each BSA coating, the passivation efficiency percentage was calculated by the following equation:

$$\text{Passivation efficiency} (\%) = 1 - \frac{\Delta f_{\text{FBS-BSA}}}{\Delta f_{\text{Control}}} \times 100\%$$

**Localised surface plasmon resonance.** Ensemble-averaged localised surface plasmon resonance (LSPR) measurements were conducted in optical transmission mode using an InSphero XNano instrument (InSphero AB, Gothenburg, Sweden), as previously described. A white light beam illuminating a circular area of ~4 mm$^2$ is transmitted through a silica-coated sensor chip (InSphero) consisting of well-separated gold nanodisk arrays (~8% surface coverage) deposited by hole-mask deposition. The sensor chip was then loaded into the measurement chamber and liquid samples were introduced under a peristaltic pump at a volumetric flow rate of 100 µL min$^{-1}$. A stable baseline signal was established in Tris buffer solution before 100 µM BSA was introduced into the measurement chamber under continuous flow for 30 min, followed by a buffer washing step. The InSphero software package (InSphero AB) was used to record the LSPR extinction spectra with a time resolution of 1 Hz and the centroid (peak) position ($\lambda$) in the extinction spectrum at each time point was calculated by using a high-order polynomial fitting. Thus, it was possible to determine the time-resolved $\Delta f$ shift due to protein adsorption and the time-derivative plot of the $\Delta f$ shift was also calculated using the OriginPro 2019b software package.

**ATR-FTIR spectroscopy.** Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy experiments were conducted using a Bruker Vertex 70 FTIR spectrometer with a liquid-nitrogen-cooled mercury cadmium telluride (MCT) detector (Bruker, Ettlingen, Germany) that was equipped with a MIRacle ATR accessory containing a three-reflection ZnSe ATR crystal (PIKE Technologies, Fitchburg, WI, USA). A 30 µL aliquot of 100 µM BSA solution was pipetted onto the ATR crystal to form a droplet, and an absorbance spectrum was immediately recorded to characterize the secondary structure of solution-phase BSA proteins. The BSA was then incubated with ATR crystal surface at room temperature for 30 min, followed by a series of ten buffer washing steps to remove weakly adsorbed proteins. During each washing step, 30 µL of extra buffer solution (without protein) was added to the sample droplet, followed by aspiration of a 30 µL total volume from the droplet. After the washing steps were completed, the second absorbance spectrum was recorded to characterize the secondary structure of BSA proteins in the adsorbed state. During measurements, the sample compartment was purged continuously with nitrogen gas to minimize ambient moisture. The OPUS 6.5 software package (Bruker) was used to collect the ATR-FTIR absorbance spectra at a 4 cm$^{-1}$ resolution and averaged over 128 scans. All recorded spectra were baseline-corrected and background subtracted. Each spectrum was then digitally filtered and a baseline was created by drawing a straight line between the two minima points on either side of each peak of interest. The Band tool was then used to determine the area bounded by the peak and the baseline. These values were defined as the intensity values for each selected non-specific band.

**Enzyme-linked immunosorbent assay.** The MicroVue SC5b-9 Plus Enzyme Immunoassay kit (catalog no. AI120; Quidel, San Diego, CA, USA) was used for enzyme-linked immunosorbent assay (ELISA) experiments. BSA-coated silica nanoparticle samples were prepared by mixing equal volumes of the appropriate 1 mg mL$^{-1}$ BSA solution with 2 mg mL$^{-1}$ silica nanoparticles in Tris buffer. The mixtures were then incubated at 37 °C for 2 h, followed by centrifugation at 14,000 × g for 30 min. The supernatants were removed and the nanoparticles were resuspended with fresh Tris buffer. Another round of centrifugation and resuspension was conducted to yield 1 mg mL$^{-1}$ BSA-coated silica nanoparticle samples. A 10 µL aliquot of each BSA-coated silica nanoparticle sample was then mixed with a 40 µL aliquot of freshly thawed normal human serum (NHS). A 50 µL aliquot of NHS was then used as the negative control (low-activation) and 10 µL of 1 mg mL$^{-1}$ uncoated silica nanoparticles plus 40 µL NHS was used as the positive control (highest level of complement activation). All test samples were incubated at 37 °C for 30 min.

The assay was then performed using the 96-well plate that were provided in the ELISA kit according to the manufacturer’s instructions. All serum samples, including controls, were diluted 40-times by using the provided sample diluent solution before addition to the wells. Standard solutions were added without dilution. The absorbance values from each well were determined by an InSphero XNano instrument (InSphero AB, Gothenburg, Sweden). Standard curves were calculated by using the known concentration of each sample to determine the respective absorbance values. The SC5b-9 protein concentration in each sample was then computed. The degree of protection afforded by each BSA coating was calculated by treating the SC5b-9 protein concentrations from the negative and positive control samples as 100% and 0% protection, respectively.

**Statistical analysis.** Statistical analyses were conducted using the GraphPad Prism (v8.0.1) software package from GraphPad Software (La Jolla, CA, USA). One-way or two-way analysis of variance (ANOVA) with the appropriate multiple comparisons test and the unpaired t-test were used to compute the statistical significance of experimental data as appropriate. All statistical analysis involved two-tailed tests. Unpaired t-tests results are reported as P values while multiple comparisons test results are reported as multiplicity-adjusted P values. P < 0.05, P < 0.01, P < 0.001 and P < 0.0001 indicate the levels of statistical significance. Additional information can be found in Supplementary Tables 6–14.
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Acknowledgements
The authors thank Mr. Tun Naw Sut for technical assistance with QCM-D experiments. This work was supported by the National Research Foundation of Singapore through a Competitive Research Programme grant (NRF-CRP10-2012-07) and a Proof-of-Concept grant (NRF2015NRF-POC0001-19), and by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (No. 2020R1C1C1004385).

Author contributions
G.J.M., A.R.F., J.A.J., and N.-J.C. planned the studies. G.J.M. and A.R.F. conducted experiments. G.J.M., A.R.F., J.A.J., and N.-J.C. interpreted the results. J.A.J. and N.-J.C. obtained funding. G.J.M., A.R.F., and J.A.J. wrote the first draft of the manuscript. All authors reviewed, edited and approved the paper.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s43246-020-0047-9.

Correspondence and requests for materials should be addressed to J.A.J. or N.-J.C.

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