Link between Low-Fouling and Stealth – A Whole Blood Biomolecular Corona and Cellular Association Analysis on Nanoengineered Particles

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ABSTRACT

Upon exposure to human blood, nanoengineered particles interact with a multitude of plasma components, resulting in the formation of a biomolecular corona. This corona modulates downstream biological responses, including recognition by and association with human immune cells. Considerable research effort has been directed toward the design of materials that can demonstrate a low affinity for various proteins (low-fouling materials) and materials that can exhibit low association with human immune cells (stealth materials). An implicit assumption common to bio–nano research is that nanoengineered particles that are low-fouling will also exhibit stealth properties. Herein, we investigated the link between the low-fouling property of a material and its propensity for stealth in whole human blood. High-fouling mesoporous silica (MS) particles and low-fouling zwitterionic poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) particles were synthesized and their interaction with blood components was assessed before and after precoating with serum albumin, immunoglobulin G, or complement protein C1q. We performed an in-depth proteomics characterization of the biomolecular corona that both identifies present species and measures their relative abundance. This was compared with observations from a whole blood association assay that identified with which cell type each particle system associates. PMPC-based particles displayed reduced association both with cells and with serum proteins compared with MS-based particles. Furthermore, the enrichment of specific proteins within the biomolecular corona was found to correlate with association with specific cell types. This study demonstrates how the low-fouling properties of a material are indicative of its stealth with respect to immune cell association.

KEYWORDS: biomolecular corona, human whole blood assay, low-fouling, stealth, cellular association

Understanding and controlling the interactions of nanoengineered particles with specific cell types is necessary to unlock their medicinal utility. For particles with potential uses in drug delivery, a long circulation half-life, and thus reduced cellular association (*i.e.* stealthy), is desirable.^{1,2} One challenge in the design of stealth materials is the multitude of biomacromolecules that particles are exposed to upon their introduction to a complex biological fluid such as blood. Particles interact with proteins, sugars, and lipids, forming a biomolecular corona on their surface.³⁻⁵ This biomolecular corona is conventionally described as a two-component system consisting of an inner tightly bound layer, or “hard” biomolecular corona, and an outer layer of loosely associated proteins (which can be removed through washing or centrifugation), or “soft” biomolecular corona.^{6,7} The biomolecular corona significantly influences the physiochemical properties of a given particle, including its size, shape, surface charge, and surface chemistry.^{8,9} Furthermore, the biomolecular corona influences physiological interactions *in vivo*, thereby modulating the biodistribution, cellular association, cytotoxicity, and targeting ability of the particle.⁸

Several strategies have emerged to control and exploit the biomolecular corona to modulate downstream cellular reactions to particles. One strategy is through the use of “low-fouling” materials, which exhibit low affinity for a wide variety of protein species.¹⁰⁻¹² Low-fouling materials are commonly assumed to exhibit stealth, so much so that these terms are used interchangeably.^{13,14} Methods to engineer low-fouling particles include the use of polyethylene glycol or zwitterionic polymers—a tightly bound water layer forms around the polymer chains that act as a physical and energetic barrier to protein adsorption.¹⁵ Another strategy to modulate corona

formation is through the controlled preadsorption of specific plasma proteins such as immunoglobulins or albumin, which have the potential to enhance or limit protein deposition onto a particle surface.¹⁶⁻²¹ However, understanding the relationships between particle design, biomolecular corona composition, and human immune cell interactions remains limited, thus preventing the accurate prediction of biological interactions for nanomaterials.

The complexity of human blood plasma is a major barrier to studying the link between corona composition and immune cell response, with more than 3700 distinct proteins identified to date,²² including factors that enhance phagocytic uptake of particles (termed opsonins), and alternative factors that limit this process (dysopsonins). Concurrent with this exposure to a rich milieu of proteins, particles are exposed to a variety of immune cell types within the blood.

In the present work, we combine particle engineering, comprehensive proteomics analysis, and whole human blood immune assays to study the links between particle design (encompassing surface chemistry and protein precoating), biomolecular corona formation, and the association of particles with human immune cells. Specifically, we sought to understand the influence of (a) particle surface chemistry, (b) protein preadsorption, and (c) the biomolecular corona on interactions between particles and human immune cells.

Mesoporous silica (MS) particles and zwitterionic poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) particles were used as representative examples of high- and low-fouling particle systems, respectively. Each particle system was precoated with an opsonin (immunoglobulin G (IgG) and complement protein C1q), a dysopsonin (human serum albumin (HSA)), or used without coating (blank) prior to incubation in whole human blood, human blood plasma, or washed human blood. Corona formation was then characterized both in terms of absolute absorption of proteins *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE) and through identification and relative abundance determination of all protein species present (mass spectrometry, proteomics). Finally, the degree to which each system associated with particular human immune cells was determined through a previously established *ex vivo* whole human blood assay.²³

Overall, our results demonstrate how low-fouling materials can achieve stealth and elucidate the specific plasma opsonins that modulate this process. Additionally, protein precoating of MS and PMPC particles was not found to significantly alter the composition of the biomolecular corona or the dynamics of immune cell association. In contrast, removal of all serum proteins from the cell media reduced immune cell association, highlighting the central role of the biomolecular environment in immune recognition. Taken together, this study presents a comprehensive analysis of bio–nano interactions.

RESULTS AND DISCUSSIONS

MS and PMPC Particle Syntheses, Characterization, and Precoating. We derived two monodisperse particle systems: MS particles and zwitterionic PMPC replica particles, representing high- and low-fouling particle systems, respectively. MS particles were synthesized from templated materials according to a modified literature method.^{24,25} Zwitterionic PMPC replica particles were synthesized *via* surface initiated-atom transfer radical polymerization (SI-ATRP) based on an altered literature method.²⁶ Both particle systems were fluorescently labeled by the attachment of *N*-hydroxysuccinimide (NHS)-activated Alexa Fluor (AF488-NHS).

Fluorescence microscopy, transmission electron microscopy (TEM), and scanning electron microscopy (SEM) analyses revealed the spherical morphology and monodispersity of both the MS and PMPC particles (Figure 1), as well as the porous structure of the MS particles (Figure 1b). The average particle sizes calculated from the electron microscopy analyses were very similar, at

1012 $\pm$ 15 nm for the MS particles, and 1030 $\pm$ 30 nm for the PMPC particles (Table S1). Zeta potential measurements showed that the fluorescently labeled MS particles assessed in Dulbecco's phosphate-buffered saline (DPBS) had a zeta potential near zero, indicating successful attachment of AF488-NHS. The PMPC particles also exhibited a zeta potential of $\sim$ 0 mV, which is characteristic for zwitterionic materials. The particle count was determined by flow cytometry and kept constant at 2×10^8 particles for all proteomics analyses and at 1×10^6 particles for all human whole blood assays (Figure S1, Table S2).

Figure 1. (a–c) Characterization of MS particles and (d–f) PMPC particles using (a,d) fluorescence microscopy, (b,e) TEM, and (c,f) SEM. Sizes are compared in Table S1.

The MS and PMPC particles were precoated with dysopsonins (HSA) and opsonins (IgG, C1q). Precoating particles with HSA has been reported to reduce phagocytic uptake *in vivo* and thus prolong the blood circulating time.²⁷⁻²⁹ In contrast, precoating particles with immunoglobulins or

complement proteins greatly enhances phagocytic uptake.³⁰⁻³³ Successful adsorption of proteins on MS particles was confirmed by SDS-PAGE (Figure 2) and a shift in zeta potential toward the isoelectric point of the adsorbed protein (Table 1). In contrast to the MS particles, minimal evidence of protein adsorption on the PMPC particles was detected by SDS-PAGE or zeta potential measurements, and crude densitometry of the SDS-PAGE gel suggests that the PMPC particles absorb 50–1000-fold less protein than the MS particles. This confirms the low-fouling nature of the zwitterionic PMPC polymer.

Figure 2. Characterization of protein pre-coating of the MS and PMPC particles using HSA, IgG, and C1q: cropped Coomassie blue-stained SDS-PAGE gels of the single proteins (HSA, IgG, and C1q), and after incubation of the MS particles or the PMPC particles in the protein solutions. The reference band associated with specific molecular weights is displayed on the left.

Table 1. Zeta Potential of the MS Particles and PMPC Particles Immersed in DPBS (pH 7.3) before Incubation (Blank) and after Incubation with HSA, IgG, or C1q^a

	Zeta potential [mV]		Isoelectric point ^b	Protein net charge
	MS particles	PMPC particles		
blank	-11 ± 1	0		
HSA	-14 ± 1	-2 ± 0	6.0	Slightly negative

IgG	-5 ± 0	-2 ± 0	8.1	Slightly positive
C1q	-2 ± 1	-2 ± 0	8.6	Positive

^aThe isoelectric points of HSA, IgG, and C1q at physiological pH (pH 7.3) indicate the resulting net charges of the proteins. ^bData taken from reference.³⁴

Design of Human Blood Assays. Human whole blood assays provide a highly complex, biologically relevant microenvironment, wherein particle–cell interactions occur simultaneously with biomolecular corona formation.^{23,35} We modified previously developed methods²³ to explore the cellular interactions with human immune cells and biomolecular corona formation of a panel of blank (particles that are not precoated with proteins) and protein-precoated MS and PMPC particles. Four different conditions were examined, as depicted in Scheme 1. Blank and protein-precoated (HSA, IgG, C1q) MS and PMPC particles were incubated in either whole blood (Scheme 1a) or washed blood (Scheme 1b–d). Based on the incubation conditions (whole blood or washed blood) and washing steps, either a “hard” and “soft” corona (Scheme 1a, c), “no” corona (Scheme 1b), or “hard” corona only (Scheme 1d) was formed.

Scheme 1. Schematic illustration of the experimental setup: blank and protein-precoated (HSA, IgG, C1q) MS and PMPC particles were incubated in (a) whole blood ("hard" and "soft" corona), (b) washed blood ("no" corona), (c) washed blood ("hard" and "soft" corona), or (d) washed blood ("hard" corona). For (b–d), whole blood was separated in its components, plasma proteins, and blood cells. The blood cells were resuspended in DPBS and are referred to as "washed blood". Based on the incubation conditions (whole blood or washed blood) and washing steps, either (a,c) a "hard" and "soft" corona, (b) "no" corona, or (d) "hard" corona only was formed. The schematic

representation of the blank PMPC particles was adapted from ref. 26. Copyright 2018 American Chemical Society.

In the first condition—whole blood “hard” and “soft” corona (Scheme 1a)—particles were incubated directly in human whole blood. The three other conditions (Scheme 1b–d) required separation of whole blood into plasma and blood cells *via* repeated centrifugation and washing steps in DPBS.²³ Removal of plasma proteins was confirmed by SDS-PAGE (Figure S2) and mass spectrometry (Table S3). In the second condition—washed blood “no” corona (Scheme 1b)—particles were incubated with blood cells alone in the absence of plasma proteins. In the third condition—washed blood “hard” and “soft” corona (Scheme 1c)—particles were preincubated with plasma for 1 h before being reconstituted with isolated blood cells. In the fourth condition—washed blood “hard” corona (Scheme 1d)—particles were incubated in human plasma for 1 h, followed by repeated washing steps to remove any loosely bound (“soft” corona) proteins. The removal of “soft” corona proteins by repeated washing steps is widely described in the literature.^{7,36} The particles coated with a “hard” corona only were then incubated with isolated blood cells. Under each of the aforementioned conditions, the association of blank and precoated MS or PMPC particles was assessed, with six separate human immune cells (granulocytes, monocytes, dendritic cells, B cells, T cells and natural killer (NK) cells) by flow cytometry (Figure 3, Figure S3, Table S4, Table S5) using a previously published gating strategy (Figure S4).³⁵

Figure 3. Cellular association of blank and protein-precoated (HSA, IgG, C1q) MS and PMPC particles with granulocytes, monocytes, and B cells after incubation in (a) whole blood and (b–d) washed blood. Based on the incubation conditions (whole blood or washed blood) and washing steps, either (a,c) a “hard” and “soft” corona, (b) “no” corona, or (d) “hard” corona only was formed. “Proportion with particle association” refers to the proportion of each cell type with positive fluorescence of AF488-NHS attached to the particles above background (see gating strategy in Figure S4). A proportion of cells with particle association equaling 1 corresponds to

100% of cells positive for AF488. Data are shown as the mean $\pm$ standard error of three independent experiments, with at least 100,000 leukocytes analyzed in each experimental condition. Control shows the respective cell populations without particles present in the incubation medium. Statistical data are presented in Figure S5 and Figure S6. Cellular association data of the other three cell populations studied, dendritic cells, T cells, and NK cells, were uniformly low and are displayed in Figure S3.

Surface Chemistry Dictates the Degree of Cellular Association. Consistent with our previous observations,²⁴ MS particles consistently displayed significant association with granulocytes, monocytes, and B cells when incubated in “whole blood”, irrespective of protein precoating with HSA, IgG, or C1q (Figure 3a). In comparison, zwitterionic PMPC particles (both blank and precoated with either HSA, IgG, or C1q) displayed detectable, but comparatively lower association with phagocytic cells (granulocytes, monocytes) and B cells (Figure 3a). Surprisingly, precoating did not significantly influence cell association. (To aid readability, full statistical analyses are presented in Figure S5 and Figure S6.) The observed influence of the IgG coating of MS particles is discussed in detail in the following sections.

Plasma Proteins are Required for Particle Association with Human Immune Cells. The incubation of both MS and PMPC particles in “washed blood” substantially reduced or eliminated association with both phagocytes and B cells (Figure 3b) in comparison to incubation in whole blood (Figure 3a) (Table S4, Table S5), suggesting that plasma proteins are a requisite for cellular association and/or uptake. This observation was confirmed when both MS and PMPC particles were preincubated in plasma prior to the addition of washed blood (Figure 3c), which restored cellular association to levels observed in whole blood. The extent to which loosely bound or residual plasma proteins influenced the cellular association of a given particle was then studied by

performing multiple washing steps to remove loosely bound proteins (“soft” corona) prior to incubation with cells (Figure 3d). This resulted in decreased association with the phagocytic populations of monocytes and neutrophils, whereas the degree of B cell association was relatively unchanged. Overall, we observed that plasma proteins depositing onto the particle surface modulate cellular association with both phagocytic cells and B cells, although the candidate plasma proteins and mechanism may differ between cell types. We note that our cell assay only detects particle association, which can encompass both surface-bound and internalized particles, and that assays that differentiate between surface-bound and internalized particles may provide additional insight into the link between low-fouling and stealth behavior.

Proteomics Analysis of the Biomolecular Corona. To examine the composition of the biomolecular corona, proteomics analysis was performed using mass spectrometry. Three replicates of each particle system (blank and protein-precoated MS and PMPC particles) were analyzed following incubation in each of the four blood assay conditions, as well as the respective incubation media alone. For each of the identified proteins in the biomolecular coronas or in the incubation media, a normalized spectral abundance factor (NSAF) was determined (<https://figshare.com/s/cc5de286c313f426fd71>). The NSAF describes the relative abundance of each protein in a complex protein solution.³⁷ Being devoid of plasma, particles incubated in washed blood yielded no proteomic information (only two plasma proteins were identified) (Table S3). Therefore, analyses primarily focused on particles recovered from human whole blood or human blood plasma. Mass spectrometry sample preparation following incubation in whole blood necessitated lysing of red blood cells (RBC), resulting in the detection of additional intercellular proteins. As intracellular proteins are naturally not present in plasma, and thus may lead to a misinterpretation of the proteomics data, we primarily focused on proteomics data obtained from

incubation in human blood plasma. All proteomics data and analyses regarding incubation in human whole blood are presented (<https://figshare.com/s/986f430d27b21af5547c>). Note that all intracellular proteins were excluded from the “raw data” for further downstream analyses. We refer to this dataset as “processed protein abundance” dataset (<https://figshare.com/s/84e751bf91c8601bee5d>).

PMPC Particles Display Low Plasma Protein Adsorption. It remains an open question how to best quantify material stealth in the context of biomolecular corona research. One metric of stealth that has been commonly used is the number of identified proteins in the biomolecular corona.^{26,38,39} A smaller number of proteins was identified in the coronas of blank and precoated PMPC particles compared with that observed for blank and precoated MS particles following incubation in human blood plasma (Table S6), with blank PMPC particles consistently demonstrating the smallest number of identified proteins. The same trends were confirmed by SDS-PAGE (Figure S7).

However, analysis based solely on the number of identified proteins (Table S6) does not account for relative protein abundance within the corona, which is likely to be an important determinant of stealth. As an alternative to the number of identified proteins, we suggest that correlation of the abundance of proteins within a corona to the abundance of proteins in an incubation medium may be a more appropriate metric of stealth. A stealth material is expected to have a corona that is similar in composition to the incubation media, as there is no tendency of specific proteins to adsorb. We therefore compared the relative abundance of proteins within the biomolecular coronas of blank and protein-precoated (HSA, IgG, C1q) MS and PMPC particles following incubation in human blood plasma using Pearson correlation and principal component analysis (PCA), as presented in Figure 4. Both Pearson correlation and PCA of the first two principal components

revealed that the biomolecular coronas of MS- and PMPC-based particles were different from each other. However, for a given material (MS or PMPC) the biomolecular coronas of the blank and protein-precoated particles were similar. Furthermore, both analyses demonstrated that the PMPC-based particles exhibited a corona that was similar to the composition of the incubation media (human blood plasma), whereas the coronas obtained for the MS-based particles were different from the composition of the incubation media. Therefore, there was a higher enrichment of certain proteins within the MS-based particle coronas compared with the low-fouling PMPC-based particles.

Figure 4. Pearson correlation and principal component analysis (PCA) with identified clusters (dashed lines) of the relative abundance (NSAF) of identified secreted proteins in the biomolecular coronas of blank and protein-precoated (HSA, IgG, C1q) MS and PMPC particles following incubation in human blood plasma. Data are presented as averages across all replicates. For the Pearson correlation, lighter colors correlate to more similar biomolecular coronas. For the PCA, data are compared across the first two principal components, which cover ~76% of the combined Eigenvalues.

Comparison of the corona is one methodology to evaluate potential stealth. In addition, we investigated the impact of the abundance of individual corona proteins. We performed an enrichment analysis to identify proteins that were overexpressed in a particular corona when compared to the incubation medium human blood plasma (Figure 5) (<https://figshare.com/s/6969eac7d8e9153d6177>). The low-fouling properties of PMPC particles were confirmed using this analysis, with markedly lower expression observed across a range of proteins implicated in complement and inflammation responses compared with the results of the MS particles.

Figure 5. Enrichment analysis for incubation of blank and precoated (HSA, IgG, C1q) MS and PMPC particles in human blood plasma. Top 50 secreted proteins (ordered by maximum enrichment) are displayed. Proteins not detected in plasma control sample were excluded.

Overall, these findings confirmed the low-fouling character of the zwitterionic PMPC particles. In addition, the results highlighted the dominant role of surface chemistry over protein precoating for biological interactions—though protein precoating affected the composition of the biomolecular coronas, it did not affect cellular association of the particles. We note that the assays used to assess the biomolecular corona within this work do not absolutely quantify the amount of absorbed protein or the absolute amount of each absorbed species, both of which may prove to be interesting areas of future research.

Immunoglobulins Influenced the Association of MS Particles with Phagocytes in the Absence of Plasma. Of interest was the finding that the extent of association of blank, and HSA- and C1q-precoated MS particles with monocytes and granulocytes was diminished in the absence of plasma proteins (Figure 3b, Table S4). Interestingly, IgG precoating of MS particles restored association with phagocytes (Figure 3d). However, despite a clearly visible overabundance of IgG in the IgG-precoated MS particle system compared with other particle systems in the simpler “washed blood” condition (Figure S8), there is no obvious enrichment of IgG in the more complex serum condition (Figure 5). We hypothesize that IgG coating resulted in either the formation of a more stable “hard” corona, compared with HSA and C1q coating, or that IgG proteins “attracted” other immunoglobulins, leading to their enrichment.

This enrichment promoted association with phagocytes. We hypothesized that this association might be mediated by Fc γ R on the surface of monocytes binding to exposed Fc regions of adsorbed IgG.⁴⁰ To investigate this further, we employed a LALA variant of an IgG monoclonal antibody, comparing it to the wild type. The LALA variant contains two mutations in the Fc region that inhibit binding to Fc γ receptors that might be expected to mediate monocyte uptake of particles.⁴¹ The effect of precoating MS particles with a monoclonal human IgG1 (specific for an irrelevant,

viral protein) compared to its LALA equivalent was evaluated (Figure 6). Although the association of MS particles with monocytes was generally higher in whole blood compared with the experiment shown in Figure 3, the difference between blank and polyclonal IgG-coated MS particles in washed blood was maintained. In contrast, we observed minimal uptake of particles associated with the monoclonal IgG1 or its LALA variant. This suggests factors associated with polyclonal IgG (as compared with monoclonal IgG) are important in phagocytic uptake of MS particles.

Figure 6. Cellular association of blank and protein-precoated (IgG, IgG1, and IgG LALA) MS particles with monocytes after incubation in human whole blood and human washed blood. Proportion with particle association refers to the proportion of each cell type with positive fluorescence of AF488-NHS attached to the particles above background. Data are shown as the mean $\pm$ standard error of three independent experiments, with at least 100,000 leukocytes analyzed in each experimental condition. Control shows the respective cell populations without particles present in the incubation medium. Full statistical analysis is presented in Figure S9.

Complement Proteins Are Linked to Particle–B Cell Association. Of additional interest was the finding that both MS and PMPC particles (blank and precoated) demonstrated high levels of

association with B cells when incubated in human whole blood (Figure 3a, Table S4, Table S5), a finding that we have also observed with other particle systems.^{35,42} We hypothesized that B cells capture opsonized particles *via* complement receptors. This hypothesis was consistent with our protein corona characterization, in which a number of complement proteins were enriched on particles following incubation in blood plasma (Figure 5). These results showed more pronounced enrichment occurring on MS particles, which also displayed higher association to B cells (Figure 3), when compared with PMPC particles. To further study the effect of complement proteins on B cell association, the plasma used to coat MS particles was heated to 56 °C for 30 min, a standard method that is used to destroy complement activity (Figure 7a).⁴³ This almost completely stopped B cell association with particles. As shown in Figure 3d, a high level of B cell association with MS particles remained in the presence of only a “hard” corona. We next examined the kinetics of the “hard” corona formation on B cell association with particles. MS particles were incubated in plasma for 5, 30, or 120 min before being added to washed blood directly or after being washed to retain only a “hard” corona (Figure 7b). “Hard” corona formation required 120 min to reach the equivalent level of association of B cells with MS particles observed for incubation in reconstructed human blood where the particles were surrounded by a “hard” and “soft” corona. Taken together, we conclude that complement formed a “hard” corona on MS particles over time and this was required for B cell association with MS particles. This suggested that longer blood circulation times might result in increased complement deposition and association with B cells. Activation of plasma complement by addition of particles of varied physiochemical properties has been observed.^{44,45} As B cells make up 10–15% of human blood leukocytes, particle systems that prevent complement opsonization need to be developed for effective biodistribution.

Figure 7. Cellular association of blank MS particles with B cells after incubation in washed blood where the particles were surrounded by both a “hard” and “soft” corona or a “hard” corona only. (a) Complement proteins were inactivated to assess their influence on B cell associations. (b) Time evolution of the corona formation studied over 120 min. Proportion with particle association refers to the proportion of each cell type with positive fluorescence of AF488-NHS attached to the particles above background. Data are shown as the mean $\pm$ standard error of three independent experiments, with at least 100,000 leukocytes analyzed in each experiment. Control shows the respective cell populations without particles present in the incubation medium. Full statistical analysis is presented in Figure S10.

CONCLUSIONS

We have presented a comprehensive investigation on the protein corona that forms around representative low-fouling (PMPC) and high-fouling (MS) particles in human blood, quantifying both the proteins present and the relative abundance of each protein species. We have linked this protein corona characterization to immune cell response using an *ex vivo* whole human blood assay. Taken together, this work demonstrates the mechanistic link between the fouling of a

particle and its immune cell association. However, we caution against the assumption that any low-fouling material (which exhibits low association to a variety of protein species) will exhibit stealth behavior. Rather, our results show that “not all proteins are created equal” in the protein corona and that particular plasma opsonins correlate with immune cell recognition. Thus, to achieve stealth, engineered particles must demonstrate low association to *specific* proteins, rather than proteins “in general”. Additionally, our results demonstrate that, in whole human blood, protein precoating has little effect on the resulting protein corona and subsequent particle association to immune cells. Furthermore, complete removal of the biomolecular corona is shown to severely limit immune cell association. Taken together, this suggests, for our studied particle system, that (a) protein surface chemistry is the dominant factor in determining biological responses in whole human blood and (b) protein surface chemistry affects biological responses by modulating the formation of the protein corona, which is subsequently recognized by immune cells. Ultimately, this improved mechanistic understanding of the interactions between human blood cells and nanoengineered particles, modulated by the biomolecular corona, is anticipated to help unlock the potential of human particle-based medicines and vaccines.

METHODS

Materials. All chemicals were of analytical grade and used as received without further purification except for copper(I) bromide (CuBr), which was purified by washing sequentially with glacial acetic acid, absolute ethanol (EtOH), and diethyl ether, and dried under vacuum. Furthermore, all liquid monomers were passed through an aluminum oxide column prior to polymerization to remove the inhibitor. High-purity water (Milli-Q water) with a resistivity of $>18.2 \text{ M}\Omega \text{ cm}$ was obtained from an inline Millipore RiOs/Origin water purification system (Millipore Corporation, MA, USA). Piranha cleaning was performed using sulfuric acid (H_2SO_4)

and hydrogen peroxide (H_2O_2), both purchased from Aldrich (MO, USA). Synthesis of MS particles was carried out using cetyltrimethylammonium bromide (CTAB) from Amresco (TX, USA), ammonium hydroxide solution (NH_3 , 28–30%) and tetraethyl orthosilicate (TEOS, 98%) from Aldrich, and poly(acrylic acid) (PAA, average molecular weight ~240 kDa, 35 wt.% solution in water) from Alfa Aesar (MA, USA). Silica particle functionalization was carried out using (3-aminopropyl)triethoxysilane (APTES, 98%), pyridine (anhydrous, 99.8%), tetrahydrofuran (THF, anhydrous, 99.9%), and α -bromoisobutyryl bromide (98%), which were all purchased from Aldrich. For the SI-ATRP, ethylene glycol dimethacrylate (EGDMA, 95%), 2-hydroxyethyl methacrylate (HEMA, 97%), *N,N,N',N'',N'''*-pentamethyldiethylenetriamine (PMDETA, 99%), 2-methacryloyloxyethyl phosphorylcholine (MPC), CuBr (98%), and nitric acid (70%) from Aldrich were used. Template removal was performed using hydrofluoric acid (HF, 48%) and ammonium fluoride (NH_4F , 98%) from Aldrich. Fluorescence labeling was carried out using AF488-NHS, purchased from Thermo Fisher (Victoria, Australia), and dimethyl sulfoxide (DMSO, anhydrous, >99%) obtained from Aldrich. Biomolecular corona characterization was carried out using DPBS, ammonium bicarbonate (NH_4HCO_3 , 99.0%), acetonitrile (CH_3CN , 99.5%), DL-dithiothreitol (99.0%), iodoacetamide, trypsin from porcine pancreas (proteomics grade), trifluoroacetic acid (99.0%), and formic acid (95.0%) were purchased from Aldrich. BioRad 4–20% Mini-PROTEAN TGX Stain-Free protein Bis-Tris gels and Tris/glycine/sodium dodecyl sulfate running buffer were purchased from BioRad (CA, USA). LDS sample buffer, NuPAGE sample reducing agent, SeeBlue prestained protein standard, and SimplyBlue safe stain were purchased from Thermo Fisher. Lysis of RBC was carried out using Pharm Lyse buffer from BD Bioscience (Victoria, Australia). For particle precoating albumin from human serum lyophilized (>97%), IgG from human serum lyophilized (>95%) and complement component C1q from human serum (>95%)

were used, both purchased from Aldrich. Viral-specific human monoclonal IgG1 and IgG1 LALA were prepared in house. For cell phenotyping, antibodies against CD3 AF700 (SP34-2), CD14 APC-H7 (MΦP9), CD66b BV421 (G10F5), CD45 V500 (HI30), CD56 PE (B159), lineage-1 cocktail APC, HLA-DR PerCP-Cy5.5 (G46–6), and CD19 BV650 (HIB19) were used, all purchased from BD Biosciences, except for APC and CD19 that were obtained from BioLegend (CA, USA). Purification following phenotyping was carried out using DPBS buffer containing 0.5% w/v bovine serum albumin (BSA) purchased from Sigma and 2 mM ethylenediaminetetraacetic acid (EDTA) from Thermo Fisher. Cell fixation was carried out using formaldehyde (1% w/v) purchased from Aldrich diluted in DPBS.

Synthesis of MS Particles. MS particles were synthesized according to a recently published method.²⁴⁻²⁵ After dissolving CTAB (1.1 g, 3 mmol) in 50 mL Milli-Q water, PAA (4.3 g, 0.02 mmol) and NH₃ (3.5 mL, 0.8 mmol) were added under vigorous stirring. The solution was left to react for 20 min before TEOS (4.46 mL, 20 mmol) was added. Following further stirring for 15 min, the mixture was transferred into a Teflon-sealed autoclave, which was heated at 100 °C for 48 h. The synthesized MS particles were purified by repeated centrifugation (10000 g, 5 min) and washing (Milli-Q water (3×), EtOH (3×)) followed by drying at 80 °C. The organic templates were removed by calcination at 550 °C for 6 h. To fluorescently label the particles, the MS particles were functionalized with amine groups followed by the attachment of AF488-NHS to the amine groups. The particles (50 mg) were dispersed in EtOH (1000 µL, 20 v/v), and APTES (50 µL, 1 v/v) and NH₃ (50 µL, 1 v/v) were added. After 24 h at 20 °C under constant shaking (Eppendorf Thermomixer Comfort, Germany) and repeated centrifugation (10000 g, 5 min) and washing in EtOH (2×) and Milli-Q water (2×), the particles were dispersed in DMSO (anhydrous, 200 µL), and AF488-NHS (5 µL, 1 mg mL⁻¹ in anhydrous DMSO) was added. A scheme for this reaction

is given in Figure S11. After 2 h at 20 °C under constant shaking, unreacted dye was removed by repeated centrifugation (10000 g, 5 min) and resuspension steps in Milli-Q water (3×). The particles were stored in EtOH in the fridge (~8 °C) prior to use.

Synthesis of PMPC Particles. Zwitterionic particles were synthesized using a recently published method.²⁶ Briefly, MS particles were functionalized with amine groups, prior to attachment of ATRP initiators and subsequently used as sacrificial templates for SI-ATRP. The monomers, MPC, HEMA, and EGDMA, were infiltrated into the pores of the MS particles and polymerized to form a polymer crosslinked polymer network. Controlled etching of the silica templates resulted in the preparation of PMPC particles. To fluorescently label the particles, AF488-NHS was reacted with the hydroxyl group of the HEMA repeating units. The particles were dispersed in DMSO (anhydrous, 200 μ L), and AF488-NHS (5 μ L, 1 mg mL⁻¹ in anhydrous DMSO) was added. After 2 h at 20 °C under constant shaking, unreacted dye was removed by repeated centrifugation (10000 g, 5 min) and resuspension steps in Milli-Q water (3×). The particles were stored in EtOH in the fridge (~8 °C) prior to use.

Particle Characterization. Fluorescence microscopy images were taken with an Olympus IX71 inverted fluorescence microscope (10× objective, Olympus) equipped with a differential interference contrast (DIC) slider (U-DICT, Olympus), the corresponding filter sets, and a 100× oil immersion objective (Olympus UPFL20/0.5NA, W.D. 1.6). ImageJ was used for image processing. Zeta potential measurements of the particles were conducted on a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) equipped with a He-Ne ion laser (λ = 633 nm). TEM images were acquired using a FEI Tecnai TF20 instrument at an operation voltage of 120 kV under liquid nitrogen cooling. Samples were prepared from a Milli-Q water-based solution by placing the particles onto Formvar-coated copper grids (plasma-treated; low oxygen plasma, 30 s) and

allowing them to air dry. All images were analyzed using ImageJ processing software. SEM images were obtained using a Philips XL30 scanning electron microscope operating at 10 kV (high vacuum). SEM samples were prepared on piranha-cleaned (3:7 v/v 30% H₂O₂/98% H₂SO₄), plasma-treated (high oxygen plasma, 3 min), nitrogen-dried silicon wafers. *Caution! Piranha solution is extremely corrosive and reacts violently with organic materials. It should be handled with great care.* The wafers were then attached onto a sample holder using a double-sided adhesive, electrically conductive carbon tape. The wafers were then sputter-coated with gold. Image analysis was performed using ImageJ software. Particle counting was carried out using flow cytometry (Apogee Flow), and data analysis and gating were performed using FlowJo_V10 software. Differences in particle fluorescence, as measured by flow cytometry, were found to be less than an order of magnitude (Figure S12).

Protein Precoating of MS and PMPC Particles with HSA, IgG, and C1q. Protein precoating of the MS and PMPC particles was performed by immersing the particles (2×10^8 particles dispersed in DPBS) in the protein solutions (HSA, polyclonal IgG, C1q, monoclonal IgG1, IgG1 mAb, and IgG1 LALA mAb) for 1 h at 37 °C under constant shaking. A protein concentration of 1 mg mL⁻¹ in DPBS was used. Purification was done by isolating the particles through centrifugation (10000 g, 5 min) and resuspending them in DPBS. This step was repeated thrice to remove all unbound proteins.

Assays Using Human Blood and Derivatives. Fresh blood was collected from a healthy human volunteer into sodium heparin vacuettes (Greiner Bio-One) after obtaining informed consent in accordance with the University of Melbourne Human ethics approval 1443420 and the Australian National Health and Medical Research Council Statement on Ethical Conduct in Human Research. Cell counts were obtained using a CELL-DYN Emerald analyzer (Abbott) (Table S7, Table S8).

Plasma was prepared by collecting the top layer following centrifugation of blood (900 g, 15 min, without brake). The centrifugation step was repeated to remove any remaining cells. Inactivation of plasma was carried out by heating plasma at 56 °C for 30 min. To prepare washed blood, the blood cells with plasma removed were first topped up with DPBS (40 mL) and centrifuged at 950 g for 10 min, slow brake. The supernatant was removed, and this wash step was repeated four times. The absence of plasma proteins was confirmed by the lack of absorbance of the supernatant at 280 nm (Nanodrop 2000, Thermo Fisher Scientific, Australia), the absence of bands in the SDS-PAGE gels (Figure S2), and by subjecting washed samples to a comprehensive proteomics analysis (Table S3). Within (before and after blood separation and washing steps) and across all different sets, the cell count was kept constant within the limits of the experimental conditions and a natural day-to-day variation for human blood (Table S7, Table S8).

Blood Assay to Measure Association of Particles with Human Immune Cells. The particles (1×10^6) were preincubated in plasma (50 μ L) or DPBS (50 μ L) for 1 h (or other specified time) at 37 °C. To remove loosely bound proteins that belong to the “soft” biomolecular corona, particles were washed three times with DPBS (8000 g, 5 min) and resuspended in DPBS (50 μ L), leaving only a “hard” biomolecular corona. The particles (1×10^6) with either no corona, a “hard”, or a “soft” biomolecular corona were then added to whole blood (100 μ L) or washed blood (100 μ L) for 1 h at 37 °C. RBC were lysed by adding Pharm Lyse buffer at 40 $\times$ blood volume and washed with DPBS (4 mL, 2 $\times$) (500 g, 7 min). Cells were phenotyped on ice for 1 h using titrated concentrations of antibodies against CD3 AF700 (SP34-2, BD), CD14 APC-H7 (M Φ P9, BD), CD66b BV421 (G10F5, BD), CD45 V500 (HI30, BD), CD56 PE (B159, BD), lineage-1 cocktail APC (BioLegend), HLA-DR PerCP-Cy5.5 (G46–6, BD), and CD19 BV650 (HIB19, BioLegend). Unbound antibodies were removed by washing and centrifugation (500 g, 7 min) with a cold (4

°C) DPBS buffer containing 0.5% w/v BSA and 2 mM EDTA at pH 8. Cells were fixed in 1% w/v formaldehyde in DPBS. The samples were directly used for cell association analysis by flow cytometry (LSRFortessa, BD Biosciences) and analyzed using FlowJo V10.

Incubation of Particles in Human Whole Blood for Proteomics Analysis. Blank and protein-precoated MS and PMPC particles (2×10^8 particles dispersed in DPBS) were immersed in whole human blood (500 μ L) for 1 h at 37 °C. RBC were lysed with Pharm Lyse buffer at 20 $\times$ blood volume and topped up to 4 mL using 1 $\times$ DPBS before pelleting the white blood cells (WBC) and particles (500 g, 40 min). To remove unbound and loosely bound proteins, the particles and WBC were washed by multiple centrifugation (300 g, 3 min) and resuspension steps (DPBS, 3 $\times$). To remove the WBC, the particles were pelleted and washed in DPBS (3 $\times$) without redispersion. Washing steps were carried out under ice cooling to prevent potential re-activation of the incubation process. The obtained biomolecular corona-coated particles were directly used for proteomics analysis.

SDS-PAGE and Sample Preparation. Adsorbed proteins were eluted off the particles by adding NuPAGE LDS sample buffer (10 μ L) and NuPAGE sample reducing agent (3 μ L) to the sample and heating to 70 °C for 10 min. The particles were separated from the eluted proteins by centrifugation (10000 g, 5 min). The supernatant was transferred to a LoBIND Eppendorf tube and diluted with DPBS (20 μ L). An aliquot of 15 μ L per sample was loaded on the gel to run at 120 V. Gel staining was carried out using Coomassie blue. Data analysis was done using ImageJ. The gels were cropped and presented with their respective standard molecular weight ladder. For the characterization of single proteins, SDS lysis buffer and reducing agent were added to the protein solutions to keep the sample treatment identical to the samples with particles present in the incubation solution.

Mass Spectrometry and Protein Identification. Sample preparation was carried out following a recently published protocol.²⁶ Samples were analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using Q-Exactive plus mass spectrometer (Thermo Fisher Scientific) fitted with nanoflow reversed-phase-HPLC (Ultimate 3000 RSLC, Dionex). The nano-LC system was equipped with an Acclaim PepMap nano-trap column (Dionex – C18, 100 Å, 75 µm × 2 cm) and an Acclaim PepMap RSLC analytical column (Dionex – C18, 100 Å, 75 µm × 50 cm). Typically, for each LC-MS/MS experiment, the peptide mix (1 µL) was loaded onto the enrichment (trap) column at an isocratic flow of 5 µL min⁻¹ of 3% CH₃CN containing 0.1% formic acid for 6 min before the enrichment column is switched in-line with the analytical column. The eluents used for the LC were 0.1% v/v formic acid (solvent A) and 100% CH₃CN/0.1% formic acid (v/v). The gradient used was 3% B to 20% B for 95 min, 20% B to 40% B in 10 min, 40% B to 80% B in 5 min and maintained at 80% B for the final 5 min before equilibration for 10 min at 3% B prior to the next analysis. All spectra were acquired in positive mode with full scan MS spectra scanning from *m/z* 375 to *m/z* 1400 at 70,000 resolution with AGC target of 3e⁶ with maximum accumulation time of 50 ms. A lock mass of 445.120024 was used. The 15 most intense peptide ions with charge states ≥2–5 were isolated using an isolation window of 1.2 *m/z* and fragmented with normalized collision energy of 30 at 17,500 resolution with AGC target of 1e⁵ with a maximum accumulation time of 100 ms. The underfill threshold was set to 2% for triggering of precursor for MS. Dynamic exclusion was activated for 30 s. Data were analyzed using the PatternLab for Proteomics 4.0 suite containing the Comet search engine.⁴⁶ Searches were performed against the Uniprot *Homo Sapiens* target-decoy database. Search parameters were as follows: precursor mass tolerance of 40 ppm, fragment bin tolerance of 0.02, carbamidomethyl of cysteine as fixed modification, and oxidation of methionine as variable modifications. Trypsin

with a maximum of one missed cleavage was used as the cleavage enzyme. Peptide spectrum matches were filtered using PatternLab's Search Engine Processor (SEPro) module to obtain a list of identifications with less than 1% false discovery rate at both the peptide and protein level. Protein quantification was achieved by spectral counting. Proteins were first grouped by maximum parsimony and on the unique peptides followed by normalizing the spectral counting data using the NSAF.

Proteomics Data Processing. Protein NSAF abundance data were further screened by only including proteins that were secreted, identified using the UniProt database. Abundance readings were then renormalized. Enrichment analysis for each sample was performed according to the following steps. First, the relative abundance of each protein (including the control samples) was determined by taking the arithmetic mean of the relative abundance in the replicates of each sample. If a protein was only detected in a subset of a sample's replicates, its abundance was assumed to be zero for replicates in which it was not detected. Next, enrichment for each protein in the sample was determined according to the following equation:

$$\text{enrichment} = \frac{\text{abundance}_{\text{sample}}}{\text{abundance}_{\text{incubation media}}} \quad (1)$$

Thus, a protein that is ten times more abundant in the protein corona of a sample than in the control sample would have an enrichment of ten. For Figure 5, we included only proteins that occurred both in the incubation media (plasma) and sample. The top 50 proteins were chosen according to their maximum enrichment across all samples examined; they were ordered from top to bottom. The software used to perform the protein processing and enrichment analysis is freely available at <https://bitbucket.org/mwfcomp/corona-analysis/src/master/>.

PCA Analysis. PCA is a powerful method for providing an overview of complex multivariate data such as the levels of adsorbed proteins on different particle systems. This type of analysis is

commonly used for revealing relations between samples (*e.g.*, clustering) that might not otherwise be obvious due to the dimensionality of the data. We use PCA to project our multidimensional data for adsorbed proteins on particles onto a reduced set of orthogonal axes (Eigenfunctions) with minimal loss of information. The Eigenfunctions are linear combinations of the original data orthogonal by construction, each representing an independent aspect of the dataset.

PCA analysis was performed on the 50 most abundant proteins for each system. The principal components that account for the most and the second most variances in the data are termed the first and second principal components, respectively. These two are shown in Figure 4. The higher the principal component Eigenvalue, the more variances that are explained. As the first two principal components in Figure 4 cover about 76% of the total Eigenvalues combined, the 50 dimensional dataset can be reduced into a two-dimensional subspace while still capturing 76% of the variance in the data.

Minimum Information Reporting in Bio-Nano Experimental Literature (MIRIBEL). The studies conducted herein, including material characterization, biological characterization, and experimental details, conform to the MIRIBEL reporting standard for bio–nano research,⁴⁷ which is provided in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information. Experimental details of particle characterization, SDS-PAGE data, mass spectrometry data, and MIRIBEL checklist for reporting research in bio–nano science. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

#These authors contributed equally. A.C.G.W. synthesized and characterized the particles, performed the protein preincubation studies and mass spectrometry studies. H.G.K. performed the human whole blood assays. The design of the experiments, data analysis and interpretation, and preparation of the paper was performed equally by A.C.G.W and H.G.K. M.F. assisted with analysis of the mass spectrometry data and preparation of the manuscript. Q.A.B. performed statistical and principal component analysis of the blood assay and proteomics data. C.-S.A. performed mass spectrometry experiments and protein identification. A.K.W., E.J.C, F.C., and S.K. supervised the studies, interpreted data, and edited the paper.

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Notes

The authors declare no competing financial interest.

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