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Human Serum Lipoproteins Influence Protein Deposition Patterns on Nanoparticle Surfaces

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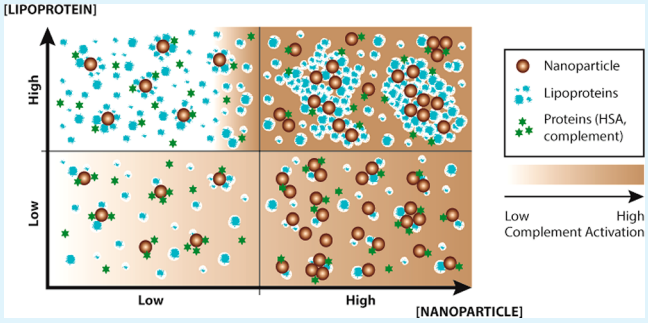
Supporting Information

ABSTRACT: We report the concentration-dependent adsorption of serum lipoproteins onto silica nanoparticles, wherein elevated lipid levels deter complement activation. Two clinically relevant serum lipid levels – corresponding to low and borderline high levels in normal, healthy adults – were used to examine the influence of lipoprotein concentration on nanoparticle complement activation. Human serum albumin was used to study protein adsorption in the presence of lipoproteins. Preferential adsorption of high affinity lipoproteins led to greater lipid fractions in the protein corona, shielding particles from complement activation. These findings have significant implications for the design of intravenously administered carriers with biocompatible surface chemistries.



KEYWORDS: *complement activation, silica, serum lipids, lipoproteins, opsonization, human serum albumin*

The dynamics of nanoparticle–protein interactions are complex and evolve over time: the varying surface affinities, dissociations, and plasma concentrations of constituents in the blood influence the pattern of the proteome adsorbed onto the nanoparticle (NP) surface.[1−3] Previous research has focused on characterizing the composition of the external protein sheath or corona that develops around nanoparticles in the bloodstream.[4−6] Here, we have identified for the first time that the pattern of adsorption, in addition to the composition of the protein corona, is crucial for a complete understanding of nanoparticle–protein interactions and sets a precedent for future protein adsorption studies. We demonstrate the use of the complement hemolysis 50% (CH_{50}) assay for the visualization of this holistic approach by quantifying the degree of complement activation elicited by a pattern of antibodies bound to a surface – a characteristic pattern that is otherwise unobservable using currently prevalent techniques.

Traditionally, studies of nanoparticle–protein interactions have examined the makeup of the protein corona using methods such as two-dimensional gel electrophoresis, enzyme-linked immunosorbent assays (ELISA), mass spectrometry, and the bicinchoninic acid (BCA) and Lowry total protein assays.[4−7] Although this compositional information is important, these methods function on the basis of the assumption that the adsorption of a specific subset of proteins leads to an increase in the immunogenic potential of nanoparticles and neglect the importance of the pattern of adsorption. Thus, they rely upon the quantification or identification of proteins, whether general or specific, and are not able to differentiate between different types of nanoparticles that may have adsorbed the same quantities of proteins, but in different patterns or configurations that can induce a difference in particle biocompatibility and immunogenicity. Therefore, the aforementioned methods are not sufficient for a complete analysis of nanoparticle–protein interactions as they do not provide a comprehensive picture of these interactions—specifically, they are unable to detect the resulting differences in bioactivity of the protein corona.

We propose the CH_{50} method as a holistic approach to quantifying the bioactivity of the absorbed protein pattern on nanoparticle surfaces. The CH_{50} assay measures the activation of the classical complement pathway, wherein immunoglobulins (IgM, IgG) initiate a biochemical cascade by binding to an antigen, changing conformation, and subsequently triggering the cleavage of complement proteins.[7] It is well-established in literature that antibodies deposit onto foreign surfaces as part of immune activation processes; moreover, the protein corona formed around nanoparticles has been shown to include many components, including albumin, immunoglobulins, complement proteins, apolipoproteins, acute-phase proteins, coagulation factors, and lipoproteins.[1−3] Immunoglobulins or antibodies have been shown to bind with high affinity to many surfaces, including polystyrene and polylactic acid (PLA).[5] Thus, the amount and pattern of antibody binding onto a nanoparticle can be directly linked to nanoparticle immunogenicity and, as such, the CH_{50} technique has great potential as a

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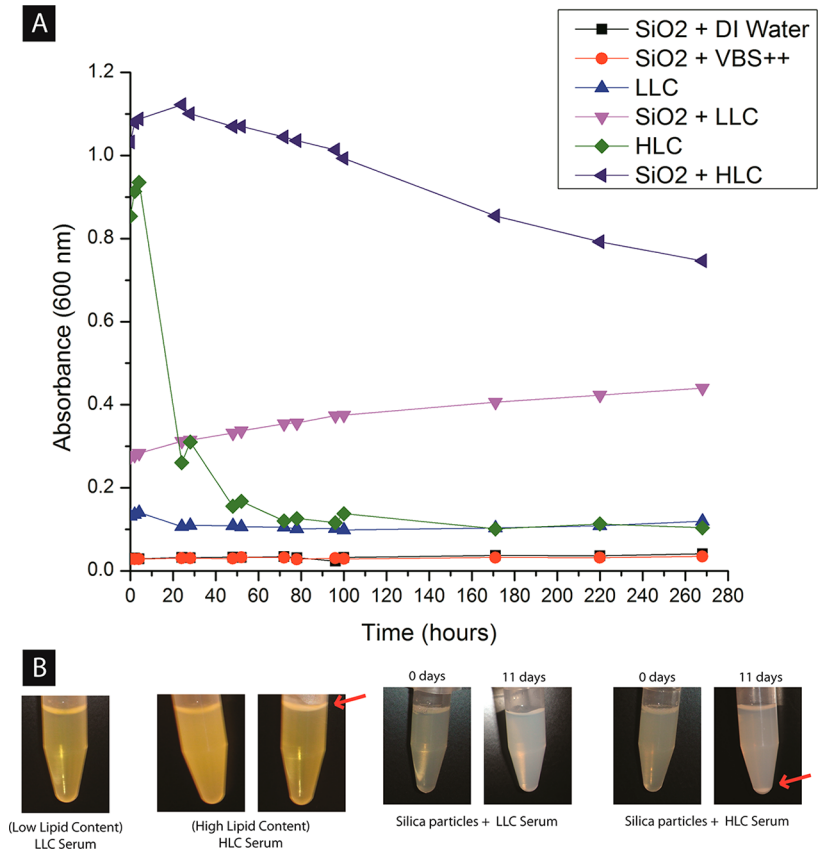

Figure 1. Kinetic stability of silica in VBS^{2+} buffer, silica in water, LLC serum, silica in LLC serum, HLC serum, and silica in HLC serum as shown by (A) absorbance at 600 nm over a 12 day time period and (B) pictures taken at 0 and 11 days.

tool for assessing both the pattern of antibody deposition on nanoparticle surfaces and the overall tendency of these nanoparticles to elicit an immune response.

The study of protein adsorption to various surfaces may involve subtle differences in the protein corona, both in vitro and in vivo, but widely available methods are largely unable to detect such minor changes in binding pattern or configuration. The proposed method addresses this shortcoming by using the body's own amplification system (i.e., the C3 to C3b positive feedback loop) to magnify the effects of these subtle changes into a measurable quantity.[7] In addition, the method is also known to be sensitive to the different types of antibodies, where IgM results in more rapid complement activation than IgG.[9] The CH_{50} method proves to be advantageous to current methods as it amplifies and provides a quantitative comparison of these subtle differences in the antibody deposition pattern in the form of degree of complement activation, which can be used for the screening of nanoparticles by their ability to elicit biological and immunogenic responses.

We report the ability to quantify differences in the composition and pattern of the adsorbed protein corona onto silica nanoparticles by measuring the complement response of the particles incubated with varying serum lipoprotein concentrations. Recent studies have demonstrated that lipoproteins and apolipoproteins exhibit preferential binding to surfaces by way of their high affinity and slow exchanging properties,[1,2,10,11] much like immunoglobulins.[5] An active exchange of proteins occurs at the nanoparticle−protein interface, where the initial adsorption of low affinity and fast exchanging proteins is gradually replaced irreversibly over time by higher affinity and slower exchanging proteins.[1−3] In addition, Moghimi et al. have shown that abnormal or elevated lipid profiles at two clinically relevant concentrations resulted in the decreased complement activation of poloxamer particles.[12] Other studies also indicate that the presence of the high density lipoprotein has an inhibitory effect upon complement activation.[13,14]

The binding of serum lipoproteins to silica nanoparticles at two different concentrations was used to evaluate the ability of the CH_{50} method to quantify a differential pattern of antibody adsorption (see the Supporting Information). High lipid content (HLC) serum was cleared by a centrifugation process to produce low lipid content (LLC) serum. This processing step was performed for the purpose of keeping all proteins and other components the same while varying only the lipid content, in order to inherently change the affinity of the nanoparticle surface presented to antibodies and measure the change in surface bioactivity. It was found that the triacylglycerides (TAG) and total-cholesterol levels in LLC and HLC serum fell within the range that corresponded to those of normal, desirable, and borderline high physiological levels in healthy adults.[15] The TAG and total-cholesterol levels respectively in LLC serum were 1.27 ± 0.0115 mmol/L and 5.13 ± 0.0300 mmol/L, and in HLC serum were 1.82 ± 0.00577 mmol/L and 5.42 ± 0.0208 mmol/L.

To confirm the formation of a protein corona around the silica nanoparticles, the particles were incubated in both types of serum and their kinetic stability was studied over a period of 2 weeks. In HLC serum without nanoparticles, the serum rapidly separated to form a floating, white and foamy lipid layer

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above a clear supernatant. In comparison, with the addition of silica nanoparticles, this layer was not observed; rather, the particles were held in solution for a longer period of time and slowly precipitated to the bottom of the tube over the 2 weeks, as shown in panels A and B in Figure 1, respectively, suggesting that the particles were being emulsified by the elevated level of serum lipoproteins which improved their stability in solution. A similar trend was observed for LLC serum through spectrophotometric absorbance, as the absence of any significant change was likely due to the relatively low lipid concentration. We concluded that the two types of serum result in silica surfaces that are both compositionally and configurationally different—an elevated presence of lipoproteins with high surface affinities will alter the protein corona formed around the nanoparticle due to preferential adsorption. These lipoproteins will also compete with immunoglobulin binding, resulting in an altered antibody deposition profile upon the surface and subsequent change in complement activation behavior as measured using CH_{50}.

The ability of the CH_{50} assay to differentiate between the protein coronas of the silica nanoparticles in LLC and HLC serum is illustrated in Figure 2A, as silica nanoparticles in the two types of serum show different complement activation behaviors over a range of nanoparticle surface areas. While the two serum treatments elicit similar responses at relatively low surface areas as well as high surface areas, an intermediate surface area range from 2500 to 5000 cm^2 represents a statistically significant divergence in behavior between the serum types: the HLC curve seems to exhibit a delayed response with respect to LLC over the first portion of this range, but also saturates much more quickly as particle concentration increases. This shift may be attributed to the increased lipid fraction of the protein corona at elevated lipoprotein levels, which both shield the surface from and compete with antibody binding for classical activation of the complement system. We also examined the extent of complement activation in both LLC and HLC serum with added human serum albumin (HSA). Although human serum naturally contains high concentrations of HSA, albumin was added in order to study whether this excess might offset the differences between LLC and HLC serum in terms of lipid and lipoprotein binding to nanoparticle surfaces, as measured by the CH_{50} assay. As shown in Figure 2B, it was found that both HLC and LLC serum behaved similarly due to increased adsorption of HSA in both cases. Although HSA has a low surface affinity and is likely to be replaced by higher affinity proteins over time,[1,2] this dissociation is not expected to occur within the length of the assay.

To determine whether differences in aggregation behavior between LLC serum and HLC serum are responsible for discrepancies in the activation of complement by the silica nanoparticles, we determined mean particle sizes after incubation using dynamic light scattering (DLS), as illustrated in Figure 3. On the basis of the measurement of sizable complexes even in the absence of NPs, the entities characterized using DLS are largely lipid and lipid-NP complexes, as supported by predominantly unimodal behavior of volume-weighted particle sizes. Although the most drastic size increases are observed at large surface areas (above 4800 cm^2) in LLC serum, there does not appear to be large amounts of aggregation in the surface area ranges corresponding to the statistically significant divergence in complement activation behavior between the two types of serum. Thus, it is expected

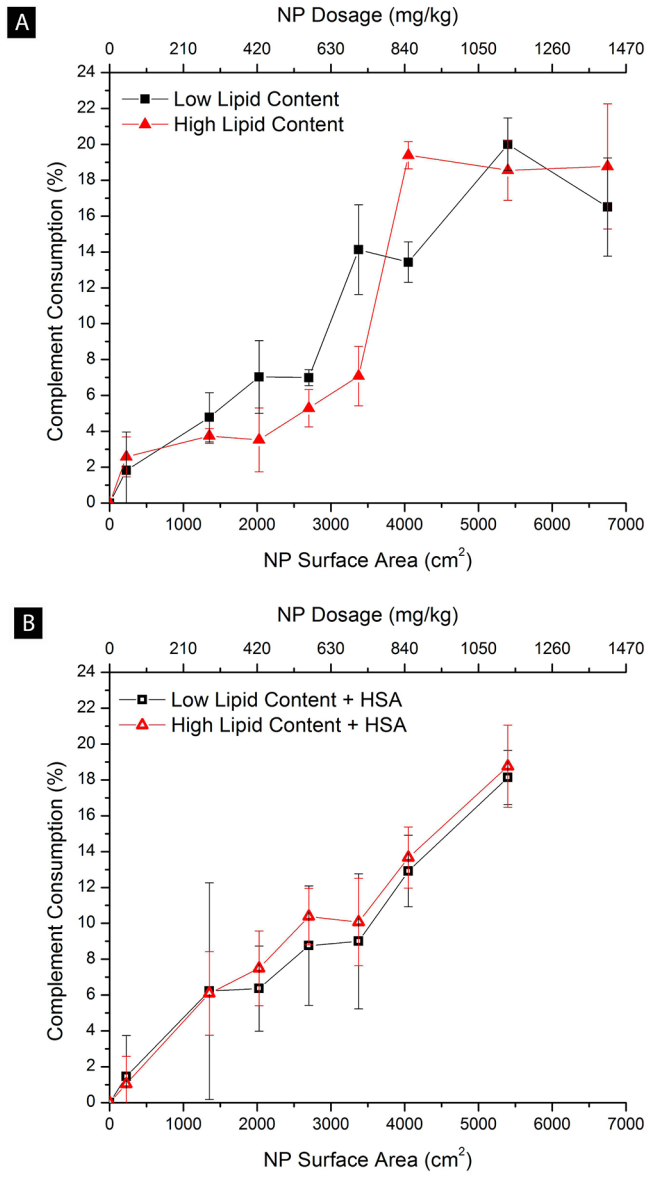

Figure 2. Complement activation of 24 nm silica nanoparticles as a function of nanoparticle surface area (cm^2/400 μL serum) and dosage (mg/kg) for sera with relatively low lipid content (LLC) and high lipid content (HLC), both (A) without HSA and (B) with 5 mg/mL HSA coadsorption. Calculations for the dose were normalized assuming a 69.40 kg adult, with 5L total blood and 55% serum volume.

that these behaviors are not entirely dependent upon aggregation.

It was found that the CH_{50} assay is capable of assessing the bioactivity of nanoparticle−protein surfaces with minute differences − measurable changes in complement activation behavior were produced from differences of 0.54 ± 0.017 mmol/L (42.7%) TAG and 0.29 ± 0.051 mmol/L (5.59%) total-cholesterol between the HLC and LLC serum used in our experiments. As such, we propose that the CH_{50} assay may provide insight, albeit in an indirect manner, into the nature of the protein corona in the presence of elevated lipid levels via complement activation measurements, similar to that previously demonstrated by Moghimi et al. based on measurement of the terminal complement complex SC5b-9.[12] Figure 4 shows transmission electron microscopy (TEM) images taken for both types of serum, with and without silica nanoparticles. The

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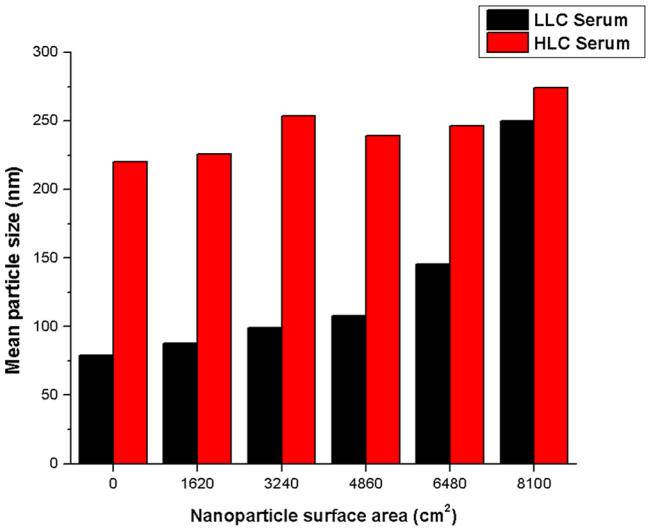

Figure 3. Comparison of NP aggregation behavior in LLC and HLC serum using DLS. Mean particle sizes reported as mean values with n = 3.

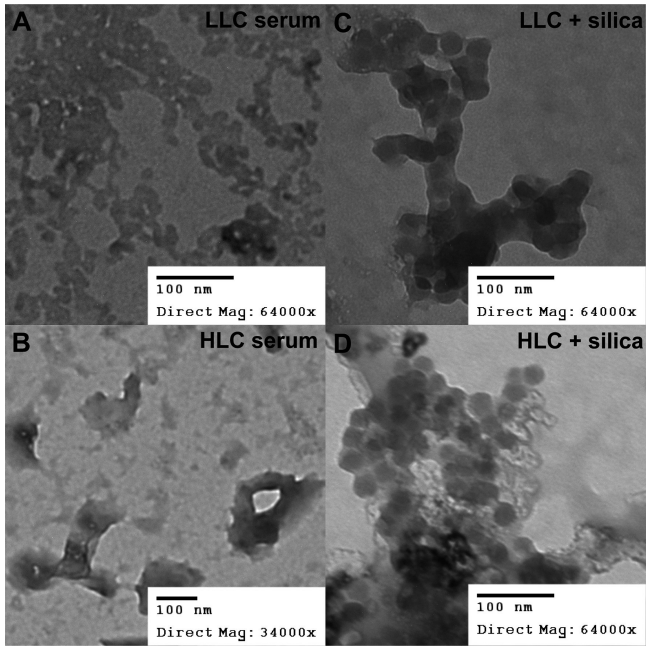

Figure 4. Transmission electron microscopy images of (A) LLC serum, (B) HLC serum, (C) LLC serum with silica NPs, and (D) HLC serum with silica NPs.

images do not show a significant difference between HLC and LLC serum, and the particles seem to associate with serum components and agglomerate in both cases. In contrast, the CH_{50} assay was able to quantify subtle differences in complement response that may reflect changes in protein corona compositions and patterns that, despite being biologically significant, were not apparent on TEM.

In conclusion, we have demonstrated the ability of the CH_{50} assay to quantify minute differences in protein adsorption to nanoparticles. By varying the lipid content of the serum used in the assay, we showed that silica nanoparticles with protein coronas of different compositions and configurations were obtained. The assay was able to differentiate between the particles treated with each serum type, whereas TEM characterization did not show a marked difference in nanoparticle−protein interactions. We emphasize the importance of establishing a more comprehensive understanding of nanoparticle−protein interactions, which includes both protein corona composition and pattern of adsorption: future work will explore in detail the presence of specific classes of antibodies and include the controlled variation of lipid and lipoprotein levels. The CH_{50} assay is presented as a model for the detection of these subtle changes in surface bioactivity by measuring the pattern of antibody deposition onto nanoparticle surfaces.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and materials used for serum treatment and testing, complement activation measurements, particle stability tests, and transmission electron microscopy. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CH_{50}, 50% hemolytic complement
HLC, high lipid content
HSA, human serum albumin
LLC, low lipid content
NP, nanoparticle
PLA, polylactic acid
TAG, triacylglycerides

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