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Albumin Removal from Human Serum using Surface Nanopockets on Silica-coated Magnetic Nanoparticles

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Abstract

Selective removal of albumin from human serum is an essential step prior to proteomic analyses, especially when using mass spectrometry. Here we report stable synthetic nanopockets on magnetic nanoparticle surfaces that bind to human serum albumin (HSA) with high affinity and specificity. The nanopockets are created by templating HSA on 200 nm silica-coated paramagnetic nanoparticles using mixed polymer layers of 4 organo-silane monomers. These monomers have amino acid-like side chains providing hydrophobic, hydrophilic and H-bonding interactions that closely mimic features of binding sites on antibodies. The binding capacity of the material was 21 mg HSA/g, and consistently removed ~88% albumin from human serum in multiple repeated use.

Graphical Abstract

Nanopockets were created on magnetic nanoparticles that bind human serum albumin (HSA) with high affinity. The nanopockets are templated with HSA using polymers made with organosilane monomers that mimic amino acids, and capped with PEG. The HSA-binding nanoparticles removed HSA from human serum at a fraction of the cost of commercial albumin removal systems.

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In protein biomarker discovery and other mass spectrometric proteomic efforts involving human serum, thousands of low abundance proteins and biomolecules may be associated with various disease states.^{1–3} However, HSA constitutes 50% to 70% of total protein in the serum (~40 mg/mL) and serves as a major source of non-specific noise. It must be depleted from the sample prior to proteomic analyses, particularly when targeting analyte proteins at pg/mL or lower concentration ranges.^{4–6} Antibody-based depletion strategies featuring affinity columns are widely used to remove HSA from serum.⁷ Non-antibody based methods, such as the ProteaPrep Albumin Depletion Sample Prep Kit (SP-200) using a recombinant protein as capture agent, also effectively remove HSA. However, these methods are relatively expensive and reusability is a challenge.

In this communication, we report a new robust, reusable, cost-effective magnetic nanoparticle featuring stable surface nanopockets for selectively removing HSA from serum. Nanopockets were synthesized on magnetic core-shell $\text{SiO}_2@\text{Fe}_2\text{O}_3$ nanoparticles (NP) in an optimized micro-scale method to mimic antibody binding sites for HSA. These HSA-binding nanoparticles have 17-fold larger binding capacity than pockets generated using only one monomer. Binding constants to HSA were 15 to 260-fold larger than those of non-template proteins. This optimized material consistently removed 85–88% of albumin from human serum over 15 use cycles. These new HSA-binding nanoparticles remove comparable HSA amounts at 2% of the cost of commercial single-use ProteoExtract HSA removal system chosen. Reuse of the HSA-binding NPs over multiple cycles enhances cost effectiveness compared to single-use HSA removal kits.

Chemical synthesis of artificial receptor sites mimicking antibodies is a major challenge, but promises large commercial paybacks in alternatives for expensive protein separation and purification processes.^{8–11} In preliminary studies, we developed a method for synthesizing antibody-like binding sites for proteins by molecularly imprinting surfaces of silica nanoparticles (SiNP) using multiple organosilane monomers with amino acid-like side chains. This approach provides binding sites that facilitate hydrophobic, hydrophilic and H-bonding interactions with target proteins similar to natural antibodies.¹² We demonstrated excellent selectivity and specificity of binding for HSA and glucose oxidase (GOx) on SiNPs, with binding constants 4 to 300-fold larger compared to a series of non-template proteins. Binding capacity increased 4-fold with combined use of the 4 different monomer types compared to using only a single monomer for templating. The new paramagnetic HSA binding nanoparticles reported in the present work have dramatically better binding capacity while retained selectivity similar to our earlier HSA-binding SiNPs.

In the present work, HSA-binding nanopockets were synthesized on surfaces of 200 nm diam. amine-functionalized silica-coated $\{\text{SiO}_2@\text{Fe}_2\text{O}_3\}$ paramagnetic nanoparticles (Full details in ESI[†]). Briefly, 2 mg $\text{SiO}_2@\text{Fe}_2\text{O}_3$ equipped with free surface amines were dispersed in 1 mL pH 7.4 buffer containing HSA, followed by crosslinking with homobifunctional glutaraldehyde via Schiff's base formation. Tetraethyl orthosilicate was then added to initiate polymerization, and silane monomers hydroxymethyltriethoxysilane,

[†]Electronic Supplementary Information (ESI) available: [Materials, details of experimental procedure, structure of monomers, gel-electrophoresis, other protein binding experimental results are discussed here]. See DOI: 10.1039/x0xx00000x

benzyltriethoxysilane, n-propyltriethoxysilane and 3-aminopropyltriethoxysilane (Figure 1a; Figure S1, ESI[†]) were added to synthesize binding sites surrounding the template protein. After 6 h, 50 mM sodium hydroxide and 0.01% Triton X-100 were added followed by ultrasonication to cleave the imine bonds and release HSA template. Monomers were chosen to provide protonated amine (NH₃⁺) and hydroxyl (OH) groups capable of H-bonding and ion-dipole interactions, and benzyl (C₆H₆) and propyl (CH₂CH₂CH₃) groups facilitating aromatic and aliphatic hydrophobic interactions central to natural protein-antibody binding.¹³ These combined binding motifs create high selectivity for the target protein, and are complemented by size and shape selectivity created by the template. Among the different monomer combinations employed, using all 4 of these hydrophobic and hydrophilic groups within the pockets provided highest HSA binding capacity, 20 mg HSA/g.

Binding constants were estimated using the Langmuir-Freundlich equation for protein binding to multiple sites on a particle^{14,15}

$$X/X_m = K_{LF} C^{1/n} / (1 + K_{LF} C^{1/n}) \quad (1)$$

where X is concentration of HSA bound/mg of NP, X_m is maximum concentration of HSA bound, **K_{LF}** is the binding constant, C is concentration of HSA in solution, and n is an empirical constant between 0.4 and 0.5 for protein adsorption onto solid surfaces. Expressing data as X' = X/X_m and C' = C^{1/n}, **K_{LF}** was estimated by non-linear regression of adsorption isotherm data, which gave good fits onto eq 1 (Figure 3a).

The HSA-binding SiO₂@Fe₂O₃ NPs provided higher specificity, affinity, and binding rates with the target compared to the nontemplate proteins (Figure 3; ESI, [†] Table S2, S3). The products were monitored for size, shape, and charge (Figure 1b; ESI, [†] Figure S8). Dynamic light scattering revealed dispersity indices for unreacted magnetic nanoparticles and nanopocket NP of 0.161 and 0.268, respectively.

Average hydrodynamic radii from DLS were 105 nm for the bare SiO₂@Fe₂O₃ NPs, 119 nm for the HSA-binding NPs without HSA, and 134 nm with bound HSA (ESI, [†] Figure S2). These data suggest a 14 nm polymer layer on the HSA-binding NPs that swells 15 nm when HSA is bound. These radii are roughly consistent with the 11x8.5 nm oblate ellipsoid shape HSA.¹⁶ Magnetic nanoparticles bring limitations for high resolution electron microscopy (EM, see ESI, [†] Figure S8). Thus, we prepared binding sites on silica nanoparticles for EM. HRTEM (high resolution TEM) images showed smooth surfaces for unreacted silica (Figure 2A); Figure 2B shows evidence of the coating. To confirm the binding pockets, we prepared ~3 nm diam. gold nanoparticles (AuNP)-HSA conjugates and bound them to NPs with nanopockets. The STEM (scanning TEM) images clearly shows the presence of polymer coating on the NPs (Figure 2C, no AuNPs) along with tiny bright spots (Figure 2D) represents the AuNPs, confirming the binding pockets.

The synthesis was highly reproducible for binding properties of NPs, shown by reproducible **K_{LF}** for different batches (Figure S3, ESI[†]). Nanopockets were also characterized for their

binding efficiency, protein binding capacity, specificity, reproducibility, and stability, as discussed below.

The effect of HSA concentration on templating was first evaluated. HSA was conjugated with the support nanoparticles at concentrations 0.5, 1.0, 5.0, 10, and 20 $\mu\text{g/mL}$, followed by surface nanopocket synthesis (see ESI[†]). In comparison to other template concentrations, we obtained maximum apparent binding constant (K_{LF} : $1.75 \times 10^5 \text{ (mL/mg)}^{1/n}$ and capacity (20 mg HSA/g) by using 1 $\mu\text{g/mL}$ HSA (ESI, [†] Figure S3, Table S1). To improve the selectivity further and minimize non-specific binding (NSB) possibly due to unreacted amine groups on the nanoparticle surface, we added 0.5 mM MeO-PEG₄-NHS to cap the amines just before removing of the template protein during synthesis (Details in ESI[†]). Therefore, we employed 1 $\mu\text{g/mL}$ HSA for templating the HSA-binding NPs and 0.5 mM MeO-PEG₄-NHS as described for the remainder of the data presented below, unless stated otherwise. K_{LF} for HSA binding to the HSA-binding NPs made under this condition was about 15–18 fold larger than for human IgG and BSA, and 260-fold larger that for lysozyme (Figure 3; ESI, [†] Table S2, Table S3, Figure S4).

Dissociation constants (K_{D}) are generally used to characterize natural antibodies assuming a one-to-one binding model, and thus $K_{\text{D}} = 1/K_{\text{B}}$, where K_{B} is the one-to-one binding constant. The K_{D} of 4.7 nM for monoclonal anti-HSA antibody, is in a typical range for selective monoclonal antibody.¹⁷

K_{LF} is a binding constant derived from a multivalent particle model, and has different units from K_{B} . To reconcile K_{LF} and K_{B} , we first estimated from binding capacity that there are an average of 250 nanopocket sites per HSA-binding SiO₂@Fe₂O₃ NP, so that in a 1 mg/mL dispersion there are 100 nM binding sites. Converting the determined K_{LF} to corresponding molarity units, $K_{\text{LF}} = 1.75 \times 10^5 \text{ (mL/mg)}^{2.22} = 1.80 \times 10^5 \text{ (1/100)}^{2.22} \text{ nM}^{-2.22}$. So is $K_{\text{LF}} = 6.53 \text{ nM}^{-2.22}$; $K_{\text{D}} \sim 1/K_{\text{LF}} = 0.15 \text{ nM}^{2.22}$ or $K_{\text{D}} \sim 0.43 \text{ nM}$, about 10 fold smaller than the dissociation constant of 4.7 nM for the native anti-HSA antibody. Clearly this is only an approximate conversion that is influenced by the multi-valency of the NPs, but is consistent with HSA-binding SiO₂@Fe₂O₃ NPs having comparable or better binding strength for HSA as native monoclonal anti-HSA.

Relative rates of binding to HSA were estimated by flowing a dispersion of HSA-binding NPs across a surface plasmon resonance (SPR) chip decorated with HSA molecules.⁰ Figure S5 (ESI[†]) shows that the SPR response increases while the particles are flowing and binding to the chip, then levels off when the effluent is switched to particle-free buffer. As found previously for multivalent particles with synthesized or antibody binding sites, the one-to-one binding model does not fit the SPR data.^{0,18} Thus, the initial rate of change of SPR signal vs. time (R/t) during association was plotted against [HSA-binding NP] (eq 2) to obtain association rate constant k_a from the initial slope (Figure 3b).

$$\Delta R / \Delta t = k_a [A_b] \quad (2)$$

Using this analysis, HSA-binding NPs had the fastest “on” rates of $52.8 \text{ (mg/mL)}^{-1} \text{sec}^{-1}$ for HSA with 7–10 fold lower rates for the other proteins tested (Figure 3b; ESI,[†] Fig S5, Table S2, S3).

SPR results also showed the absence of NP dissociation on the short time scale, 200 s (ESI,[†] Figure S5). We infer that strong multivalent cooperative interactions occur between multiple HSA molecules on the SPR chip with the multivalent NPs featuring multiple binding sites. This behavior is consistent with previous findings for magnetic beads decorated with multiple antibodies.¹⁸

We investigated the stability of the nanopockets in conjunction with the reproducibility of HSA removal (Figure 3). First, we observed very little leaching of HSA out of the NP-HSA complex over 20 days in physiological buffer (Figure 4a). The complex was highly stable for the first 10 days without no loss of HSA. Over the next 5 days leaching was 2.5%, and reached a maximum 5% on day 20. This behaviour is attributed to very slow decomposition or structural reorganization of binding sites in the NP polymer coating. Results are consistent with SPR, which indicated (Figure S5) very slow dissociation.

HSA-binding NPs were compared to the commercial ProteoExtract HSA-removal kit for effective removal from human serum. A maximum 88% albumin removal was achieved by using 10 mg HSA-binding NPs that decreased to ~85% at the end of the 15th reuse cycle. HSA removal from the NP for reuse was done by treating with 0.2 M glycine at pH 2.5 and 1 M TRIS-HCl at pH 9.0. The ProteoExtract kit removed ~84% albumin (Figure 4(b); number 16; ESI,[†] Figure S7) but is not reusable. HSA-binding NPs synthesized on day 0 gave HSA removal efficiency of 83–88% over 30 days storage with no activity loss (Figure S6, ESI[†]). Minimum activity loss was found for the NP suspensions in water, and was slightly larger for reused materials that had been treated with 0.2 M glycine (pH 2.5) multiple times with no protein contamination on the NPs after washing (ESI,[†] Figure S14). The high concentration of glycine at pH 2.5 disrupts major protein interactions without affecting structure, thus it was used to remove bound protein after each use (ESI[†], Figure S13). The relative intensity loss at ~65 kDa due to albumin in the SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) data (Figure 3c) also suggested efficient removal of albumin from human serum by the NPs and ProteoExtract. We further confirmed the removal efficiency by mass-spectrometry where time of flight (TOF) analysis indicated both the materials can remove ~82–90% HSA from human serum (details in ESI,[†] Figure S9). We also tested the specificity of the NP by spiking human serum with 10 ng mL⁻¹ of the protein prostate specific antigen (PSA) and found that NP didn't bind such a low abundant protein. The spiked PSA in human serum vs. the sample treated with NP gave a PSA assay signal 1.02X greater than controls whereas the ProteoExtract treated serum samples 1.25 X suggesting that removal of HSA may have slightly enriched the low abundant PSA in the sample but have not depleted it (see ESI,[†] Figure S10).

The optimized micro-synthesis gave nanopocket-decorated paramagnetic NPs highly specific for binding HSA, and was 30-fold cheaper for albumin removal than ProteoExtract. FT-IR spectra of protein-conjugated NPs confirmed the presence of bound proteins (ESI,[†] Figure S11). X-ray photoelectron spectroscopy showed the elements N, O, C, Si, Fe on the

surface from the amino-acid like groups of organosilane monomers used in the synthesis (ESI,[†] Figure S12). The NPs were stable, and average hydrated layer thickness of 15 nm from DLS reflects swelling upon binding of HSA. The NPs had an effective K_D lower than that of monoclonal anti-HSA antibody. The new NPs consistently removed ~88% HSA from human serum in multiple uses, while ProteoExtract removed ~84% but is single use. For the similar amount of HSA removed, the approximate cost for 10 mg HSA-binding NP synthesis is \$13.00 whereas the cost for single use 1 column ProteoExtract kit is \$29.00. Assuming that 15 reuses (Figure 4b) give acceptable results, cost of the HSA-binding $\text{SiO}_2@\text{Fe}_2\text{O}_3$ NPs per experiment is <\$1.00, about 2% of the cost of ProteoExtract. The synthesis cost of HSA-binding NPs is low compared to albumin removal kits such as ProteaPrep (\$465.00), AlbuSorb (\$295.00), PureProteome Human Albumin/Immunoglobulin (\$645.00). Here we compare cost of commercial product to cost of synthesis of our NPs, neglecting market value. While production and market cost of nanopocket NPs is uncertain, the synthesis is so cheap that cost advantages of our NPs are clear. The POROS® CaptureSelect® HSA Chromatography Column is promising and reusable, but very costly (\$1920.00).

In summary, synthesis of highly selective and functional HSA-binding NPs was achieved on paramagnetic core-shell nanoparticles. These new HSA-binding NPs effectively and selectively remove albumin from human serum at 2% of the cost of a competitive commercial material. The spiking experiment with PSA suggests that the materials can be applied to proteomics. Reusability is an important feature for cost effectiveness, since only one batch of template protein is required to produce a material that can be used many times. The paramagnetic nature of the support material makes for very convenient separation from solutions. The NPs here have been designed to selectively bind HSA, but the approach can be extended in principle to virtually any biologically important macromolecule.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. HSA-binding core-shell NPs: (a) Schematic representation of the monomers used during HSA-specific nanopockets synthesis on the surface of silica-coated magnetic nanoparticles. Figure (b) represents the size distribution of the support nanoparticles possessing nanopockets.

Figure 2.

Electron microscopy (EM) images for HSA-binding NPs. A. HRTEM image for uncoated silica nanoparticles shows smooth surface whereas B shows the presence of polymer coating for HSA-binding NPs on silica nanoparticles. Figure C represents the STEM image for coated nanopockets where thin layer of polymer is clearly visible. To understand the presence of nanopockets, AuNP-HSA was conjugated onto the NPs and the brighter spot in figure D represents the AuNPs, so as nanopockets.

Figure 3.

Binding data for HSA-binding NPs with surface nanopockets. (a) Fits of data to Langmuir-Freundlich mode to obtain K_{LF} of the HSA-specific nanopockets compared to BSA, lysozyme and IgG. (b) SPR analysis to obtain binding rates for each of the analytes employed in 'a' (see text). (c) Removal of HSA from human serum analysed by SDS-PAGE electrophoresis. A 40 μ L undiluted human serum sample was treated with the commercial ProteoExtract Albumin removal kit (lane 5) and with HSA-binding NP suspensions (lane 4) for comparison. From each fraction, depleted serum proteins were separated by SDS-PAGE and visualized by Coomassie brilliant blue staining. The others lanes are control lanes: (1) MW markers (2) human serum and (3) human serum albumin.

Figure 4.

Stability and reusability of the HSA-binding NPs. (a) Binding stability of an NP-HSA complex over 20 days. (b) Removal of HSA from 10-fold diluted human serum reusing a single batch of HSA-binding NPs for multiple cycles of binding and elution of HSA within a single day (1–15). Reuse involved treatment with 0.2 M glycine (pH 2.5), and washing with phosphate buffer. Number 16 represents amount of HSA removed by the ProteoExtract Albumin Removal Kit.