Improving Targeting of Metal-Phenolic Capsules by the Presence of Protein Coronas

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ABSTRACT Particles adsorb proteins when they enter a physiological environment; this results in a surface coating termed a “protein corona”. A protein corona can affect both the properties and functionalities of engineered particles. Here, we prepared hyaluronic acid (HA)-based capsules through the assembly of metal-phenolic networks (MPNs), and engineered their targeting ability in the absence and presence of protein coronas by varying the HA molecular weight. The targeting ability of the capsules was HA molecular weight-dependent, and a high HA molecular weight (>50 kDa) was required for efficient targeting. The specific interactions between high molecular weight HA capsules and receptor-expressing cancer cells were negligibly affected by the presence of protein coronas, whereas non-specific capsule-cell interactions were significantly reduced in the presence of a protein corona derived from human serum. Consequently, the targeting specificity of HA-based MPN

capsules was enhanced due to the formation of a protein corona. This study highlights the significant and complex roles of a protein corona in bio-interactions, and demonstrates how protein coronas can be used to improve the targeting specificity of engineered particles.

INTRODUCTION

Engineered drug delivery particles that can specifically bind to diseased cells have potential for improving therapeutic efficacy and reducing harmful side-effects to healthy cells and tissue.¹⁻⁴ A variety of biomolecules, such as monoclonal antibodies,^{5,6} folic acid,^{7,8} transferrin,^{9,10} and carbohydrates,¹¹⁻¹³ have been developed as targeting ligands for specific cellular recognition and internalization. Among them, hyaluronic acid (HA), a biodegradable, highly biocompatible, naturally existing polysaccharide, has been extensively combined with therapeutic carriers, including liposomes,^{14,15} micelles,^{16,17} and polymer nanoparticles,^{18,19} for targeted delivery of anti-cancer drugs. HA has a high binding affinity toward CD44 receptors that are over-expressed on many types of cancer cell membranes.^{20,21} HA-based drug carriers have therefore shown improved cancer-targeting efficiency and therapeutic efficacy.²² For example, coating HA on doxorubicin-loaded liposomes significantly enhanced their anti-tumor activity compared with non-HA-coated liposomes.²³ Moreover, HA-coated docetaxel-loaded micelles exhibited selective accumulation in tumors and higher cytotoxicity compared with commercial docetaxel-loaded micelles (i.e., Taxotere[®]) as a result of both passive and active targeting.²⁴ It was also reported that HA-coated liposomes exhibited different targeting ability depending on the molecular weight of the surface-bound HA.^{14,15} Increasing the HA molecular weight leads to increases in size and chain length, thereby allowing for multiple receptors to interact with a single HA molecule, resulting in enhanced HA-CD44 binding affinity and higher CD44-mediated cellular uptake.²⁵

When particles are dispersed in biological fluids (e.g., blood), proteins and other biomolecules adsorb onto their surfaces, forming a so-called “protein corona”.²⁶ This protein

adsorption is difficult to fully avoid despite the surface modification of particles with low-fouling materials (e.g., poly(ethylene glycol), PEG).^{27,28} It is widely reported that a protein corona can endow particles with a biological identity and affect the targeting ability by screening targeting ligands.^{29,30} For example, in a model targeting reaction, proteins adsorbed on alkyne-functionalized silica nanoparticles prevented the alkyne groups from coupling with azide groups on planar silicon substrates.³¹ Similarly, transferrin-conjugated silica nanoparticles lost their targeting specificity in serum-containing media.³² The formation of protein coronas can also lead to a significant reduction in the targeting specificity of single-domain antibody-functionalized silica nanoparticles.³³ However, monoclonal antibody-functionalized polymer particles fully retained their targeting ability after incubation in human serum (HS).³⁴ Additionally, the targeting specificity of affibody-functionalized polymer particles was inhibited by protein coronas derived from HS, but enhanced by protein coronas derived from human serum albumin solutions.³⁵ These studies highlight the complex roles of protein coronas in the targeting ability of various particle systems. It is likely that both the type of targeting ligands and the particle systems can influence the formation of protein coronas, as well as the impact of the protein corona on targeting specificity.

Recently, we developed a simple and rapid strategy to assemble films and capsules based on the coordination of metal ions and polyphenols.³⁶ The obtained metal-phenolic capsules exhibited pH-responsive disassembly and minimal cytotoxicity, allowing for intracellular drug release.^{37,38} PEG-polyphenol was synthesized by conjugating catechol groups onto PEG.³⁹ The conjugated catechol groups then facilitated the coating of PEG-polyphenol onto calcium carbonate (CaCO_3) templates, and also allowed for cross-linking through metal ion coordination to form metal-phenolic networks (MPNs). Following removal of the CaCO_3 templates, the resultant MPN_{PEG} capsules showed resistance to non-specific protein adsorption and cell adhesion.³⁹ Furthermore, HA-polyphenol, synthesized by conjugating

catechol groups to HA, was used as a building block to coordinate with metal ions and assembled into capsules, termed MPN_{HA} capsules, based on metal-phenolic coordination.⁴⁰ The incorporation of HA significantly enhanced the specific binding affinity of capsules toward CD44 overexpressing (CD44+) cells, compared with CD44 minimal expressing (CD44-) cells.⁴⁰ However, these binding studies were performed in the absence of HS, raising the important question of whether MPN_{HA} capsules maintain their targeting specificity in the presence of protein coronas. Therefore, it is of interest to investigate how protein coronas affect the targeting ability of MPN_{HA} capsules and if this is HA molecular weight dependent, as HA-CD44 binding affinity is molecular weight dependent.^{15,25} Herein, we engineered a library of MPN_{HA} capsules composed of HA ranging from 13 kDa to 230 kDa. The influence of protein coronas on the targeting ability of the MPN_{HA} capsules with different HA molecular weights was systematically compared using HS as the protein corona source (**Figure 1**). We demonstrated that a protein corona can increase the specificity of a targeting system, since it reduces nonspecific cell interactions while maintaining specific interactions with targeted cells.

Figure 1. Schematic illustration of the formation of protein coronas on MPN_{HA} capsules exposed to HS, and the association of corona-coated or non-corona-coated MPN_{HA} capsules with CD44 overexpressing (CD44+) cells (human breast cancer cells, MDA-MB-231 cells, blue) and CD44 minimal expressing (CD44-) cells (human breast cancer cells, BT-474 cells, purple). The protein corona improves the targeting specificity of MPN_{HA} capsules to CD44+ cells.

EXPERIMENTAL SECTION

Materials. Hyaluronic acid (HA) with average Mw of 13, 51, 120, and 230 kDa were purchased from Lifecore Co. (USA). Iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), dopamine hydrochloride, tris(hydroxymethyl)aminomethane (TRIS), calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$), sodium carbonate (Na_2CO_3), poly(sodium 4-styrenesulfonate) (PSS, 70 kDa), sodium phosphate dibasic (Na_2HPO_4), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), ethylenediaminetetraacetic acid (EDTA), anhydrous dimethyl sulfoxide (DMSO), deuterium oxide (D_2O), and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (USA). Dulbecco's phosphate-buffered saline (DPBS), Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAXTM supplement, Roswell Park Memorial Institute (RPMI) 1640 medium with L-Glutamine, Alexa Fluor 488 succinimidyl ester (AF488-NHS), wheat germ agglutinin Alexa Fluor 594 conjugate (WGA-594), Hoechst 33342, NuPAGE Bis-Tris precast gel 4-12%, NuPAGE MOPS SDS running buffer, NuPAGE LDS sample loading buffer, and NuPAGE sample reducing agent were obtained from Life Technologies (USA). All of the chemicals were used as received without further purification. The pH of the solutions was measured by a Mettler-Toledo MP220 pH meter. The Milli-Q water used in all experiments was obtained from a three-stage Millipore Milli-Q plus 185 purification system (Millipore

Corporation, USA) with a resistivity greater than 18.2 M Ω cm. All aqueous solutions were filtered with 220 nm diameter membranes before use.

Synthesis of HA-polyphenol. To synthesize HA-polyphenol, dopamine was conjugated with the carboxylic acids of HA through a carbodiimide coupling reaction using EDC and NHS, following a previously reported protocol.⁴⁰ 500 mg of HA was fully dissolved in 25 mL of degassed phosphate buffered solution (50 mM, pH 5.5). Next, 239 mg of EDC and 143 mg of NHS were slowly dissolved into the HA solution and incubated at 24 °C for 40 min. After incubation, 283 mg of dopamine hydrochloride was added and dissolved into the above aqueous solution. The final molar ratio between carboxylic acid of HA, EDC, NHS, and dopamine was set to 1:1:1:1.2. The mixed solution was stirred at 24 °C with argon flow for 20 h. After reaction, the product was purified by dialysis (7 kDa regenerated cellulose membrane, Sigma, USA) for three days against 5 L of degassed Milli-Q water with acidic pH (pH 3.7). Subsequently, the product was lyophilized to obtain HA-polyphenol as a white powder. To synthesize HA-polyphenol with different molecular weights, HA with different averaged molecular weights (13, 51, 120, and 230 kDa) was employed to conjugate with dopamine under the same conditions as above. The efficiency of catechol modification on HA was determined using nuclear magnetic resonance (¹H NMR) spectroscopy with a 400 MHz Varian INOVA system in D₂O at 25 °C.

Synthesis of Calcium Carbonate (CaCO₃) Templates. CaCO₃ particles, with an average diameter of 1.4 ± 0.3 μ m, were synthesized according to a previously published method.^{40,41} Briefly, 200 mg of PSS (70 kDa) was completely dissolved in 200 mL of Ca(NO₃)₂ solution (20 mM, pH adjusted to above 7). Next, 40 mg of PSS (70 kDa) was completely dissolved in 40 mL of Na₂CO₃ solution (20 mM, pH was adjusted to above 7). Subsequently, the two solutions were rapidly mixed and vigorously stirred for 40 s. Then, the mixed solution stand still at 24 °C for 1 h, followed by vigorous stirring for 30 s. The resulting CaCO₃ particles

were washed three times with Milli-Q water by centrifugation (2000 g, 2 min), dried in an oven and calcined under air flow at 450 °C for 6.5 h.

Assembly of MPN_{HA} Capsules. MPN_{HA} capsules were assembled following a previously reported procedure with slight modification.⁴⁰ To assemble MPN_{HA-230} capsules, 130 µL of FeCl₃·6H₂O solution (5 mg mL⁻¹) was added to 100 µL of CaCO₃ suspension (12 mg mL⁻¹), followed by vigorous stirring. Subsequently, 400 µL of HA-polyphenol solution (230 kDa, 5 mg mL⁻¹) and 500 µL of TRIS buffer (20 mM, pH 8.5) were successively added into the above suspension, followed by vigorous stirring and brief sonication after each step. The obtained MPN_{HA}-coated particles were washed three times with Milli-Q water by centrifugation (1000 g, 1 min), and incubated with EDTA solution (100 mM, pH 7.5) at 24 °C for 1 min to dissolve the CaCO₃ templates. The resulting MPN_{HA-230} capsules were washed three times with Milli-Q water to remove EDTA and excess coating materials, dispersed in phosphate buffer (10 mM, pH 7.4), and refrigerated at 4 °C before use. To assemble MPN_{HA-120}, MPN_{HA-51}, and MPN_{HA-13} capsules, HA-polyphenol with molecular weight of 120 kDa, 51 kDa, or 13 kDa was employed for the capsule fabrication. All other steps were the same as for the synthesis of MPN_{HA-230} capsules. To fluorescently label the MPN_{HA} capsules, 50 µL of AF488-NHS (1 mg mL⁻¹ in DMSO) was incubated with MPN_{HA}-coated CaCO₃ particle suspension (concentrated to 50 µL in DPBS) for 8 h at 24 °C, followed by washing three times with Milli-Q water and core removal by EDTA solution.

Formation of Protein Corona-Coated Capsules. Equivalent numbers of MPN_{HA-230}, MPN_{HA-120}, MPN_{HA-51}, and MPN_{HA-13} capsules (5×10^7) were incubated in 300 µL of 100% human serum (HS) with constant shaking (600 rpm) in an Eppendorf Thermomixer Comfort (Eppendorf, Germany) at 37 °C for 1 h. The capsules were then washed twice with Milli-Q water to obtain the protein corona-coated capsules. These protein corona-coated capsules

were dispersed in phosphate buffer (10 mM, pH 7.4) and used directly for cell targeting study or further washed with Milli-Q water for characterization.

Capsule Characterization. Capsules with or without protein coronas were characterized by the following methods. Differential interference contrast (DIC) and fluorescence microscopy images were obtained using an inverted Olympus IX71 microscope (Olympus, Japan). Transmission electron microscopy (TEM) images were taken by a FEI Tecnai TF20 instrument (FEI, USA) with an operation voltage of 200 kV. Capsule suspensions were dropped and air-dried on formvar carbon-coated gold grids before TEM measurement. The number of capsules was counted using an Apogee A50-Micro flow cytometer (Apogee Flow Systems, UK). The ζ -potential of the capsules was measured by microelectrophoresis using a Zetasizer Nano-ZS (Malvern Instruments, UK). The capsules were dispersed in phosphate buffer (10 mM, pH 7.4) before measuring.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). For SDS-PAGE analysis, protein corona-coated capsules were obtained using the protocol described above. As previously described,^{30,34} the adsorbed proteins were eluted from capsules by incubating with NuPAGE LDS sample loading buffer and heated at 70 °C for 10 min. The disulfide bonds of the eluted proteins were then cleaved by adding NuPAGE sample reducing agent and heated at 70 °C for 10 min. Subsequently, the samples were loaded on NuPAGE Bis-Tris precast gel 4-12% and separated by electrophoresis at 200 V for 50 min. The gel was then stained with Coomassie G-250 (SimplyBlue SafeStain, Invitrogen). Each gel contained one lane of a standard molecular weight ladder (Life Technologies, USA).

Cell Culture. Human mammary gland cancer cell line, MDA-MB-231 and BT-474 were purchased from ATCC (USA). MDA-MB-231 cells were maintained in complete DMEM media containing 10% FBS and GlutaMAXTM, whilst BT-474 cells were cultured in complete

RPIM-1640 media containing 10% FBS and L-Glutamine. Both cell lines were maintained at 37 °C in a cell culture incubator with 5% CO₂ and 95% relative humidity.

Cell Targeting Studies via Flow Cytometry. As previously described,⁴⁰ MDA-MB-231 cells and BT-474 cells were seeded into a 24-well plate at a cell density of 8×10^4 cells per well. The seeded MDA-MB-231 cells and BT-474 cells were then cultured in complete media at 37 °C for 24 h and 40 h, respectively, to allow cellular adhesion on substrates. Before adding the sample, the cell culture media was changed to serum-free media. Subsequently, AF488-labeled MPN_{HA-230}, MPN_{HA-120}, MPN_{HA-51}, and MPN_{HA-13} capsules with or without protein corona were added into cells at a capsule-to-cell ratio of 50:1 and incubated in serum-free media for 1 h at 4 °C or 37 °C for cell binding or association studies, respectively. After incubation, the cells were gently washed three times with DPBS, lifted by a trypsin solution, and analyzed with an Apogee A50-Micro flow cytometer (Apogee Flow Systems, UK). The degree of cell binding or association of the capsules was evaluated by using the percentage of cells that exhibited stronger fluorescence intensity than control cells (untreated cells).

Cell Targeting Studies via Confocal Microscopy. As previously described,⁴⁰ MDA-MB-231 cells and BT-474 cells were separately seeded into 8-well Lab-Tek chambered coverglass slides (Thermo Fisher Scientific, USA) at a cell density of 4×10^4 cells per well. The seeded MDA-MB-231 cells and BT-474 cells were then cultured in complete media at 37 °C for 24 h and 40 h, respectively, to allow cellular adhesion on substrates. After that, AF488-labeled MPN_{HA-230}, MPN_{HA-120}, MPN_{HA-51}, and MPN_{HA-13} capsules with or without protein corona were added into the cells at a capsule-to-cell ratio of 50:1 and incubated in serum-free media for 1 h at 37 °C. After incubation, the cells were gently washed three times with DPBS, fixed with paraformaldehyde (4% in DPBS) for 30 min at 37 °C, then stained with WGA-594 (5 µg mL⁻¹) for 5 min at 4 °C, and stained with Hoechst 33342 (0.1 mg mL⁻¹) for 10 min at 24 °C.

For the cell mixture experiments, MDA-MB-231 and BT-474 cells were mixed at a ratio of 1:2 and seeded into 8-well Lab-Tek chambered coverglass slides at a total cell density of 4.5×10^4 cells per well. Before seeding the cells, MDA-MB-231 cells were labeled with CellTracker Deep Red dye (1 μ M in DPBS), and BT-474 cells were labeled with Hoechst 33342 NucBlue Live Cell Stain ReadyProbes reagent (2 drops per mL in complete media) and CellTracker Deep Red dye (1 μ M in DPBS). The seeded MDA-MB-231 and BT-474 cells were then incubated in complete media at 37 °C for 24 h to allow cell adhesion. Following this, AF488-labeled MPN_{HA-230} capsules with or without a protein corona were added into the cells at a capsule-to-cell ratio of 50:1 and incubated in serum-free media for 1 h at 37 °C, followed by washing with DBPS three times.

Imaging to monitor the cell association of the capsules was performed using a Nikon A1R+ laser scanning confocal microscope (Nikon Corporation, Japan) with a Plan Apo λ 60 $\times$ 1.4 NA oil immersion objective, 405, 488, 561, and 640 nm lasers and 450/50, 525/50, 595/50, and 700/75 nm bandpass emission filters. The obtained images were further processed by Fiji software using the maximum intensity projection.⁴²

Statistical Analysis. Statistical significance of the data obtained from flow cytometry were analyzed by the GraphPad software Prism (Version 6.0) using the model of two-way ANOVA.

RESULTS AND DISCUSSION

Assembly of MPN_{HA} Capsules. HA of different molecular weight (13, 51, 120, and 230 kDa) was reacted with dopamine through a carbodiimide coupling reaction to conjugate catechol groups onto HA, resulting in HA-polyphenol. The degree of catechol group conjugation was controlled to be similar, ranging from 6% to 7%, as determined by ¹H-NMR spectroscopy (**Figure S1-4**). To assemble MPN_{HA} capsules, iron(III) chloride (FeCl₃) and HA-polyphenol solution were successively added into a suspension of CaCO₃ templates.

Subsequently, increasing the pH (to 8.5) led to the instantaneous formation of MPNs due to the cross-linking of bis- and tris-catechol-iron coordination complexes.⁴³ Following removal of the CaCO_3 templates by EDTA solution (100 mM, pH 7.5), MPN_{HA} capsules were obtained. $\text{MPN}_{\text{HA-13}}$, $\text{MPN}_{\text{HA-51}}$, $\text{MPN}_{\text{HA-120}}$, and $\text{MPN}_{\text{HA-230}}$ capsules were prepared by employing HA-polyphenol with different molecular weights (i.e., 13, 51, 120, and 230 kDa, respectively) of HA. The obtained capsules were well dispersed in aqueous solution (**Figure 2a,e,i,m**), and their structures were characterized by TEM (**Figure 2b,f,j,n**). The morphology of the capsules reproduced the shape of CaCO_3 , which is consistent with previous reports on the fabrication of multilayer capsules using CaCO_3 particles as templates.⁴⁴⁻⁴⁶

Figure 2. Fluorescence microscopy images (a,c,e,g,i,k,m,o, scale bars are 5 μm) and TEM images (b,d,f,h,j,l,n,p, scale bars are 0.5 μm) of MPN_{HA-13}, MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules without (top row) or with (bottom row) a protein corona derived from HS. Arrows in the TEM images (d,h,l,p) indicate protein coronas around the capsules.

Preparation of Protein Corona-Coated MPN_{HA} Capsules. To obtain protein corona-coated capsules, MPN_{HA-13}, MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules were incubated in HS at 37 °C for 1 h, followed by extensive washing with water to remove unbound proteins. HS was employed as the source to form protein coronas because it is a multi-component biologically relevant fluid. The capsules did not aggregate after the formation of protein coronas (**Figure 2c,g,k,o**), and the protein coronas could be observed on the capsule surfaces in TEM images (**Figure 2d,h,l,p**). These tightly bound proteins, termed the “hard” protein corona, are generally considered as a relatively stable layer on particles due to their extremely slow desorption or exchange rates.^{47,48} Subsequently, these proteins were eluted and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (**Figure 3a**). As observed, various serum proteins were eluted from the MPN_{HA-13}, MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules, which further confirmed the formation of protein coronas on the MPN_{HA} capsules regardless of the incorporated HA molecular weight. Despite the difference in HA molecular weight, bare MPN_{HA-13}, MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules had similar ζ -potentials, around -32 to -34 mV in 10 mM phosphate buffer at pH 7.4 (**Figure 3b**). However, in the presence of protein coronas, the ζ -potentials of the MPN_{HA-13}, MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules changed to -20, -26, -27, and -29 mV, respectively. This neutralization of the surface charge is likely due to the shielding effect of the protein corona, which is consistent with previous reports on other charged particle systems.^{49,50} Moreover, the surface charge of MPN_{HA-13} capsules was slightly more affected

by the protein corona (~5 mV higher) than the other MPN_{HA} capsules, which may be because the low molecular weight HA is more easily shielded by proteins due to its smaller size.

Figure 3. (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) image of separated corona proteins of MPN_{HA-13}, MPN_{HA-51}, MPN_{HA-120}, MPN_{HA-230} capsules after 1 h of incubation with HS at 37 °C. Reference bands associated with particular molecular weights are displayed in the image (as indicated by an arrow). (b) ζ-potential of MPN_{HA-13}, MPN_{HA-51}, MPN_{HA-120}, MPN_{HA-230} capsules without or with a protein corona derived from HS. Data are shown as the mean ± standard error of at least three independent experiments. ***p<0.001.

Effect of Protein Coronas on Specific Cell Binding. To investigate the effects of protein coronas on the targeting ability of MPN_{HA} capsules, cell binding studies were performed using two human breast cancer cell lines, CD44⁺ MDA-MB-231 cells and CD44[−] BT-474 cells.⁵¹ The specificity of HA-based capsules with CD44 receptors was previously demonstrated by a competition study where pre-blocking CD44 receptors with free HA led to significantly reduced binding and association of the capsules with MDA-MB-231 cells, while nonspecific interactions with BT-474 cells were not affected by the receptor blocking.⁴⁰ Thus, MDA-MB-231 cells and BT-474 cells are considered as valid cell lines to investigate the targeting specificity of MPN_{HA} capsules by comparing the degree of binding to the two cell lines. Identical numbers of MPN_{HA-13}, MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules with or without protein coronas derived from HS were separately incubated with MDA-MB-231 cells and BT-474 cells in a serum-free medium with a capsule-to-cell ratio of 50:1 for 1 h at 4 °C. The cell binding degree of the capsules was quantitatively measured with flow cytometry. In the absence of protein coronas, a significantly higher percentage of CD44⁺ cells bound with MPN_{HA} capsules when compared with CD44[−] cells (**Figure 4**), demonstrating a high targeting specificity of the MPN_{HA} capsules. Moreover, it was found that the targeting ability of the MPN_{HA} capsules toward CD44⁺ cells was dependent on the molecular weight of the incorporated HA. Compared with MPN_{HA-13} capsules, MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules had 2.6, 3.0 and 3.1 times higher cell binding to MDA-MB-231 cells, respectively. These results are further supported by previous reports that HA-CD44 binding affinity increased with increasing sizes of HA.^{15,25} In the presence of protein coronas, MPN_{HA} capsules still have significantly higher binding to CD44⁺ cells compared with CD44[−] cells, except for MPN_{HA-13} capsules which lost their targeting specificity. With a protein corona, the binding degree of MPN_{HA-13} and MPN_{HA-51} capsules was reduced by 16% and 19%, whilst MPN_{HA-120} and MPN_{HA-230} capsules retained most of their binding toward CD44⁺ cells with

only a 5% reduction in the presence of a protein corona. These results indicate that the affinity of MPN_{HA} capsules toward CD44⁺ cells could be affected by protein coronas to varying extents depending on the molecular weight of HA. When HA with a higher molecular weight was incorporated into MPN_{HA} capsules, the binding affinity to CD44⁺ cells was less affected by the formation of protein coronas. It was previously reported that the reduction and loss of targeting can be attributed to the shielding of targeting ligands by protein coronas.³² When the size of HA is larger, the HA incorporated into the capsules may extend further out from the protein corona and bind to receptors, thereby preserving the targeting ability.

Figure 4. Cell binding of MPN_{HA-13}, MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules to MDA-MB-231 cells (CD44⁺) and BT-474 cells (CD44⁻) in the absence or presence of a protein corona derived from HS after 1 h incubation in serum-free media at 4 °C analyzed by flow cytometry. The capsule-to-cell ratio was set to 50:1 for all cell experiments. Data are shown as the mean $\pm$ standard error of at least four independent experiments (two-way ANOVA, ***p<0.001). “PC” denotes the capsules with a protein corona derived from HS.

Effect of Protein Coronas on Targeting Specificity. Cell association experiments at 37 °C were then carried out to further verify the targeting specificity of protein corona-coated capsules, considering that both specific and non-specific particle-cell interactions are more active at physiologically relevant temperatures (37 °C). An equivalent number of MPN_{HA-13}, MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules with or without a protein corona were separately incubated with MDA-MB-231 cells (CD44+) and BT-474 cells (CD44-) in serum-free medium with a capsule-to-cell ratio of 50:1 for 1 h at 37 °C. MPN_{HA-13} capsules did not exhibit targeting specificity in either the absence or the presence of protein coronas, as a result of the increased non-specific interactions at 37 °C (**Figure 5a**). Furthermore, in the absence of protein coronas, 47%, 33%, and 30% of BT-474 cells were associated with MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules, respectively, demonstrating significantly enhanced non-specific interactions at 37 °C compared with 4 °C. However, in the presence of protein coronas, the non-specific capsule-cell interactions were dramatically reduced to about 9% for MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules. The formation of a protein corona is known to decrease the surface energy of particles, thereby reducing non-specific interactions between the particle-corona complex and the cell membrane.⁵² Moreover, the phenolic groups of MPN_{HA} capsules are known to exhibit non-specific interactions with biological systems due to their high binding ability.⁵³⁻⁵⁵ Coating of serum proteins on the capsules may reduce the exposure of phenolic groups to cells, therefore reducing non-specific capsule-cell interactions. Different from non-specific capsule-cell interactions, the specific association of MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules to CD44+ MDA-MB-231 cells was negligibly affected by the presence of protein coronas at 37 °C. Specifically, with a protein corona, the association of capsules toward MDA-MB-231 cells slightly reduced by about 10% for MPN_{HA-51} capsules, and around 5% for MPN_{HA-120} and MPN_{HA-230} capsules. This is probably because the long HA chains may extend outward from the protein corona to interact

with cell receptors and also because the binding affinity of HA-CD44 is sufficiently high to overcome blocking of the protein coronas. As a result, the difference between specific capsule-targeted cell association and non-specific capsule-cell association increased in the presence of protein coronas (**Figure 5a**). Therefore, the targeting specificity of these MPN_{HA} capsules (assessed by the ratio of their association with CD44⁺ and CD44⁻ cells) was significantly enhanced by the presence of protein coronas: 4.7 times for MPN_{HA-51} capsules; 3.1 times for MPN_{HA-120} capsules; and 3.2 times for MPN_{HA-230} capsules (**Figure 5b**). Our previous study found that protein coronas derived from a single model protein solution (i.e., human serum albumin) could facilitate the binding of polymer particles to targeted cells by increasing the accessibility of targeting ligands,³⁵ whereas herein we demonstrate that protein coronas derived from biologically relevant HS could enhance the targeting specificity of polymer capsules by maintaining specific interactions but reducing non-specific capsule-cell interactions.

Figure 5. (a) Cell association of MPN_{HA-13}, MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules to MDA-MB-231 cells (CD44+) and BT-474 cells (CD44-) in the absence or presence of a protein corona derived from HS after 1 h incubation in serum-free media at 37 °C analyzed by flow cytometry. The capsule-to-cell ratio was set to 50:1 for all cell experiments. Data are shown as the mean $\pm$ standard error of at least four independent experiments (two-way ANOVA, *** p <0.001). (b) Ratio of cell association with MDA-MB-231 cells (CD44+) and BT-474 cells (CD44-) after 1 h of incubation in serum-free media at 37 °C with MPN_{HA-13}, MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules with or without a protein corona derived from HS. “PC” denotes the capsules with a protein corona derived from HS.

Confocal microscopy was then employed to confirm the targeting ability of MPN_{HA} capsules in the presence of protein coronas. As expected, MDA-MB-231 cells associated with a large number of protein corona-coated MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules, which was similar to what was observed for these capsules without a protein corona (**Figure 6**). However, fewer MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules were associated with BT-474 cells, especially for those with protein coronas. The targeting ability of MPN_{HA-230} capsules was further confirmed by incubating the capsules with the mixture of two cell lines at a ratio of 1:2 (MDA-MB-231:BT-474) (**Figure S5**). The MPN_{HA-230} capsules with or without protein coronas were mainly associated with MDA-MB-231 cells. In contrast, a similar number of MPN_{HA-13} capsules were associated with MDA-MB-231 cells and BT-474 cells in the absence or presence of protein coronas (**Figure 6**), consistent with the results obtained from flow cytometry.

Figure 6. Confocal microscopy images of MDA-MB-231 cells (a,c,e,g,i,k,m,o, CD44+) and BT-474 cells (b,d,f,h,j,l,n,p, CD44-) incubated with MPN_{HA-13}, MPN_{HA-51}, MPN_{HA-120}, and MPN_{HA-230} capsules in the absence (top rows) or presence (bottom rows) of a protein corona derived from HS. Cells were incubated with the capsules at 37 °C for 1 h at a capsule-to-cell ratio of 50:1 in serum-free medium and imaged using confocal microscopy after extensive washing with DPBS. The cell membrane and nuclei were stained with Alexa Fluor 594-conjugated wheat germ agglutinin (WGA-594, red) and Hoechst 33342 (blue), respectively. The capsules were fluorescently labeled with AF488 (labeled to HA, green). Scale bars are 20 μm .

CONCLUSIONS

Overall, the present study examined the formation of protein coronas on MPN_{HA} capsules consisting of HA of different molecular weight and the targeting ability of such capsules in the presence of protein coronas. The layer of proteins coating the surface of MPN_{HA} capsules was clearly visible from TEM images. Various proteins were eluted from the capsule-corona complex and analyzed on SDS-PAGE images. To examine the targeting ability of the capsules, two types of human breast cancer cells, MDA-MB-231 cells (CD44+) and BT-474 cells (CD44-) were employed. As observed, the molecular weight of HA above 50 kDa is required for MPN_{HA} capsules to exhibit significant targeting specificity. The incorporation of HA with high molecular weight (51 kDa, 120 kDa, or 230 kDa) enhanced the binding affinity of capsules toward CD44+ cells. This enhanced affinity was negligibly affected by the presence of protein coronas. In contrast, the non-specific interactions of capsules with CD44- cells were significantly reduced by protein coronas at 37 °C. As a result, the targeting specificity of the capsules was enhanced. Taken together, our data demonstrate that a protein corona is not detrimental, but rather beneficial, to the targeting specificity of MPN_{HA} capsules. Given the versatility of metal-phenolic coatings, the formation of protein coronas on MPN films can potentially be used as a biological surface engineering tool and applied to other particle systems for enhanced targeted drug delivery. Additionally, as MPNs can be used to easily engineer planar surfaces, this method could allow for the selective culturing of specific cells out of complex biological media containing multiple cell types.

ASSOCIATED CONTENT

Supporting Information. ^1H NMR Spectrum, confocal microscopy image of a cell mixture with associated capsules. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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