Engineered Metal-Phenolic Capsules Show Tunable Targeted Delivery to Cancer Cells

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ABSTRACT We engineered metal-phenolic capsules with both high targeting and low non-specific cell binding properties. The capsules were prepared by coating phenol-functionalized hyaluronic acid (HA) and poly(ethylene glycol) (PEG) on calcium carbonate templates, followed by cross-linking the phenol groups with metal ions and removing the templates. The incorporation of HA significantly enhanced binding and association with a CD44 overexpressing (CD44+) cancer cell line, while the incorporation of PEG reduced nonspecific interactions with a CD44 minimal-expressing (CD44-) cell line. Moreover, high specific targeting to CD44+ cells can be balanced with low nonspecific binding to CD44- cells simply by using an optimized feed-ratio of HA and PEG to vary the content of HA and PEG incorporated into the capsules. Loading an anticancer drug (i.e., doxorubicin) into the

obtained capsules resulted in significantly higher cytotoxicity to CD44+ cells but lower cytotoxicity to CD44– cells.

KEYWORDS polymer capsules, cell targeting, low fouling, poly(ethylene glycol), hyaluronic acid, polyphenols

INTRODUCTION

Conventional chemotherapy can lead to the indiscriminate distribution of anti-cancer drugs, which can cause harmful side-effects in healthy cells and tissue.¹⁻³ Hence, it is critical to develop drug delivery systems that can specifically bind to diseased cells, while avoiding interactions with normal, healthy cells.^{4,5} A variety of biomacromolecules, including hyaluronic acid (HA),⁶⁻⁸ folic acid,⁹ peptides,¹⁰ and monoclonal antibodies¹¹ have been developed as targeting ligands for selective receptors on carcinoma cells. HA, a benign, biodegradable, naturally occurring polysaccharide, has been extensively combined with drug carriers because it has a high binding affinity toward its primary receptor, CD44, which is over-expressed on different types of cancer cells (e.g., breast, ovarian, and lung cells).¹²⁻¹⁵ However, HA-based carriers can also have non-specific interactions with normal cells; this reduces the targeting specificity toward cancers. For example, the immobilization of HA on liposomes compromises the *in vivo* circulation time, leading to lower tumor-site accumulation.¹⁶ One way to address this issue is to incorporate low-fouling materials, such as poly(ethylene glycol) (PEG),^{17,18} into targeted carriers to avoid non-specific interactions with healthy cells, and thereby reduce immune responses and prolong *in vivo* circulation. However, the low-fouling nature of PEG can also hinder cellular recognition and active targeting by limiting the interactions between HA and CD44 receptors.¹⁹ Therefore, a balance between reducing non-specific interactions and promoting active targeting needs to be considered when designing targeted drug delivery carriers.

Capsules are a versatile drug delivery system due to their tunable composition and structure, and the high degree of control over their degradation and cargo release properties.^{20,21} Recently, we reported a simple and rapid strategy to assemble metal-phenolic network (MPN) capsules based on the coordination between polyphenols and metal ions.²² By varying the incorporated metal ions, the MPN capsules were endowed with diverse properties and were applied in drug delivery, diagnostic imaging, and catalysis.^{23,24} Furthermore, by using a synthetic PEG-polyphenol (PEGp) as a building block, MPN capsules resistant to non-specific protein adsorption and cell adhesion were engineered.²⁵ Although MPN capsules represent a promising platform for drug delivery, the development of MPN capsules showing high targeting efficacy remains unexplored.

Herein, we engineer MPN_{HA-PEG} capsules, consisting of targeting ligands (HA) and low-fouling PEG, both functionalized with phenolic moieties, for tunable cancer cell targeting. The assembly of the MPN_{HA-PEG} capsules is driven by metal-phenol coordination, which provides an easy and rapid approach to incorporate multiple components into one capsule (**Figure 1**). HA-polyphenol (HAp) and PEGp were synthesized by conjugating dopamine onto HA and PEG, respectively. The introduced phenolic groups act as both an anchoring ligand, to facilitate the coating of HA and PEG on calcium carbonate (CaCO₃) templates, and a cross-linking ligand, to coordinate with iron(III) ions (Fe^{III}). Following formation of the coordination complexes and removal of the templates, MPN_{HA-PEG} capsules are obtained. In this study we demonstrate that: (i) the coordination-driven assembly of MPNs of diverse phenolic-functionalized components allows their simultaneous and rapid assembly on substrates; (ii) the incorporation of HA into MPN capsules significantly enhances their targeting ability to a CD44 positive (CD44+) cell line (MDA-MB-231 cells), compared with a CD44 negative (CD44-) cell line (BT-474 cells); (iii) non-specific interaction with CD44- cells is largely reduced by the incorporation of PEG in the capsules; (iv) targeting and non-

specific cell binding can be controlled by varying the HA to PEG ratio in the capsules; and (v) encapsulation of an anticancer drug (e.g., doxorubicin, Dox) into the MPN_{HA-PEG} capsules resulted in significantly higher cytotoxicity to CD44⁺ cells compared with CD44[−] cells.

Figure 1. Schematic illustration of a) Metal-phenolic assembly of MPN_{HA-PEG} capsules. MPN_{HA-PEG} capsules with three different HA:PEG ratios were prepared by simultaneously adding HA-polyphenol (HAp, orange) and PEG-polyphenol (PEGp, blue) at three different ratios to a suspension of CaCO₃ particles, followed by adding Fe^{III}, increasing the pH, and dissolving the templates. b) Targeting to CD44⁺ (MDA-MB-231, blue) cells and non-specific binding to CD44[−] (BT-474, red) cells can be controlled by the ratio of HAp and PEGp incorporated into the MPN_{HA-PEG} capsules.

EXPERIMENTAL SECTION

Materials. Hyaluronic acid (HA, M_w 289 kDa) was purchased from Lifecore Co. (USA). 8-arm-poly(ethylene glycol) succinimidyl succinate (8-arm-PEG-NHS, hexaglycerol core, M_w 10 kDa) was purchased from JenKem Technology (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), sodium acetate (NaOAc), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM), ethylenediaminetetraacetic acid (EDTA), anhydrous *N,N*-dimethylformamide (DMF), triethylamine (TEA), anhydrous dimethyl sulfoxide (DMSO), deuterium oxide (D_2O), fetal bovine serum (FBS), tannic acid (TA), 3-(*N*-morpholino)-propanesulfonic acid (MOPS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). Doxorubicin hydrochloride (Dox, purity ~99%) was purchased from OChem Inc. (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), Alexa Fluor 488 cadaverine (AF488 cadaverine), Alexa Fluor 488 hydrazide (AF488 hydrazide), Alexa Fluor 633 hydrazide (AF633 hydrazide), wheat germ agglutinin Alexa Fluor 594 conjugate (WGA-594) and Hoechst 33342 were obtained from Life Technologies (USA). All of the chemicals were used as received without further purification. The pH of the solutions was monitored by a Mettler-Toledo MP220 pH meter. The 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 HAp. HAp was synthesized by conjugating dopamine to the carboxylic acids of HA via a carbodiimide coupling reaction, as previously reported.²⁶ HA (0.5 g) was fully dissolved in 25 mL of degassed phosphate buffered solution (50 mM, pH 5.5). EDC (239 mg) and NHS (143 mg) were slowly added into the HA solution and stirred at room temperature for 40 min. Dopamine hydrochloride (283 mg) was then added to the solution. The final molar ratio of carboxylic acid of HA/EDC/NHS/dopamine was set to 1:1:1:1.2. The mixture was stirred at room temperature under argon atmosphere for 20 h. After reaction, the product was purified by dialysis (14 kDa regenerated cellulose membrane, Sigma, USA) for three days against 5 L of degassed water with acidic pH (pH 3.7). Subsequently, the product was lyophilized to obtain HAp as a white powder.

To fluorescently label the HA, HA (100 mg) and DMTMM (110 mg) were dissolved in 6 mL of phosphate buffered solution (50 mM, pH 7.2) and incubated for 30 min. Then, 0.3 mg of AF488 hydrazide or AF633 hydrazide in anhydrous DMSO (1 mg mL⁻¹) was added to the above solution. The mixture was incubated at room temperature for 48 h. After reaction, the product was purified by dialysis and collected by lyophilization. AF488- or AF633-labeled HA was then conjugated with dopamine, as described above, to produce AF488- or AF633-labeled HAp. The efficiency of catechol modification was determined by measuring the absorbance peak of HAp solution at 280 nm using a Carian Cary 4000 ultraviolet to visible (UV-Vis) spectrophotometer (Varian, USA). A calibration curve of absorbance of dopamine solution at 280 nm was used to quantify the catechol groups on HA. In addition, nuclear magnetic resonance (¹H NMR) spectroscopy was measured in D₂O at 25 °C using a 400 MHz Varian INOVA system to confirm the conjugation of catechol groups on HA.

Synthesis of PEGp. PEGp was synthesized by conjugating dopamine to 8-arm-PEG-NHS via succinimidyl succinate and amine coupling, as previously described.²⁵ Briefly, 8-arm-PEG-NHS (200 mg) and dopamine hydrochloride (152 mg, molar ratio of PEG

terminal/dopamine 1:5) were dissolved separately and then mixed in 1.7 mL of degassed anhydrous DMF. Subsequently, anhydrous TEA (111 μ L, molar ratio of TEA/dopamine 1:1) was added. The mixture was stirred at room temperature under argon atmosphere for 20 h. The resultant product was purified by dialysis (3.5 kDa regenerated cellulose membrane; Thermo Fisher Scientific, USA) for three days against 5 L of degassed acidic water (pH 3.5), and then collected by lyophilization as a white powder.

To fluorescently label the PEGp, AF488 cadaverine (0.64 mg, molar ratio of PEG/AF488-cadaverine 20:1) in anhydrous DMSO (1 mg mL⁻¹) was added into 8-arm-PEG-NHS solution. Subsequently, dopamine and TEA were added and incubated for 20 h as described above. The efficiency of catechol modification was determined by comparing the integral value of methylene protons of PEG at ~2.5 ppm to the aromatic protons of catechol at 6.6 to 7.0 ppm in the ¹H NMR spectrum measured by a 400 MHz Varian INOVA system in D₂O at 25 °C.

Synthesis of Calcium Carbonate (CaCO₃) Templates. CaCO₃ particles with an average diameter of 1.2 $\pm$ 0.3 μ m were synthesized via an amorphous intermediate with PSS, as previously described.²⁷ PSS (200 mg, 70 kDa) was dissolved in 200 mL of Ca(NO₃)₂ solution (20 mM, adjusted to pH > 7). Then, PSS (40 mg, 70 kDa) was dissolved in 40 mL of Na₂CO₃ solution (20 mM, adjusted to pH > 7). The two solutions were rapidly mixed and vigorously stirred for 30 s. After that, the mixture was allowed to stand still for 1 h, followed by vigorous stirring for 30 s. The resultant CaCO₃ particles were washed three times with water with centrifugation (2000 g, 2 min)/redispersion steps, dried in an oven and calcined in air at 450 °C for 6.5 h. PSS-doped CaCO₃ particles were synthesized with the same protocols as above without calcination.

Fabrication of MPN_{HA-PEG} Capsules. To assemble MPN_{HA-PEG-I} capsules, 350 μ L of an aqueous solution of HAp (2 mg) and PEGp (2 mg) was added to 100 μ L of an aqueous CaCO₃ (2 mg) suspension at room temperature (~22 °C), followed by vigorous stirring for 2

min. Subsequently, 180 μL of aqueous $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution (10 mg mL^{-1}) and 500 μL of aqueous TRIS buffer solution (20 mM, pH 8.5) were successively added to the above solution, followed by vigorous stirring and brief sonication after each addition. The particles were then washed three times with water with centrifugation (1000 g, 2 min)/redispersion steps, and incubated with aqueous EDTA solution (100 mM, pH 7.5) for 1 min at room temperature to remove the CaCO_3 core. The resultant capsules were washed three times with water to remove EDTA and excess coating material, dispersed in aqueous phosphate buffered solution (10 mM, pH 7.4), and refrigerated at 4 $^{\circ}\text{C}$ before use. To assemble $\text{MPN}_{\text{HA-PEG-II}}$ capsules, 350 μL of a mixed solution of HAp (0.4 mg) and PEGp (2 mg) was incubated with 100 μL of a CaCO_3 suspension (2 mg) for 2 min, followed by the addition of 180 μL of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution (5 mg mL^{-1}) and 500 μL of TRIS buffer (20 mM, pH 8.5). To assemble $\text{MPN}_{\text{HA-PEG-III}}$ capsules, 350 μL of a mixed solution of HAp (0.16 mg) and PEGp (2 mg) was incubated with 100 μL of a CaCO_3 suspension (2 mg) for 2 min, followed by the addition of 180 μL of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution (3 mg mL^{-1}) and 500 μL of TRIS buffer (20 mM, pH 8.5). All other steps were the same as for the synthesis of $\text{MPN}_{\text{HA-PEG-I}}$ capsules. The distribution of pore sizes and surface areas in the CaCO_3 sample can give slightly different coating amounts, but the data are comparable as a single CaCO_3 batch was used for capsule synthesis. To fluorescently label the capsules, 20 μL of AF488-NHS (1 mg mL^{-1}) was incubated with $\text{MPN}_{\text{HA-PEG}}$ -coated CaCO_3 particles (concentrated to 100 μL) in DPBS for 4 h at room temperature, followed by washing with water and core removal by EDTA. To further increase the fluorescence intensity of the capsules, AF488-labeled PEGp was used for capsule synthesis.

Fabrication of MPN_{HA} Capsules. To assemble MPN_{HA} capsules, 1 mg of CaCO_3 particles was suspended in 100 μL water. 130 μL of aqueous $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution (5 mg mL^{-1}), 200 μL of aqueous HAp solution (8 mg mL^{-1}), and 500 μL of aqueous TRIS buffer

solution (20 mM, pH 8.5) were successively added into the CaCO_3 particle suspension at room temperature, followed by vigorous stirring and brief sonication after each step. Subsequently, the particles were washed three times with water with centrifugation (1000 g, 1 min)/redispersion steps, and incubated with aqueous EDTA (100 mM, pH 7.5) solution for 1 min at room temperature to remove the templates. The resultant capsules were washed three times with water, dispersed in phosphate buffer (10 mM, pH 7.4), and refrigerated at 4 °C before use. To label the MPN_{HA} capsules, 40 μL of AF488-NHS was incubated with the capsule suspension (concentrated to 70 μL) in DPBS for 8 h at room temperature, followed by washing three times with water.

Fabrication of MPN_{PEG} Capsules. MPN_{PEG} capsules were assembled following the previously reported protocols.²⁵ 8 mg CaCO_3 particles were suspended in 800 μL of water. 800 μL of aqueous PEGp solution (10 mg mL^{-1}), 1.72 mL of aqueous $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution (1 mg mL^{-1}), and 4 mL of aqueous TRIS buffer solution (20 mM, pH 8.5) were successively added to the aqueous CaCO_3 particle suspension at room temperature, followed by vigorous stirring for 2 min after each addition. Subsequently, the particles were washed three times with water with centrifugation (3000 g, 3 min)/redispersion steps, and incubated with aqueous EDTA solution (100 mM, pH 7.5) for 1 min at room temperature to dissolve the CaCO_3 templates. The resultant capsules were washed with water with centrifugation (3000 g, 7 min)/redispersion steps, and stored at 4 °C before use. To fluorescently label the capsules, AF488-labeled PEGp was used to synthesize MPN_{PEG} capsules.

Fabrication of Dox-Loaded $\text{MPN}_{\text{HA-PEG}}$ Capsules. To assemble Dox-loaded $\text{MPN}_{\text{HA-PEG}}$ capsules, 350 μL of a mixed solution of HAp (0.4 mg) and PEGp (2 mg) was incubated with 100 μL of an aqueous CaCO_3 suspension (2 mg) for 2 min at room temperature, followed by the addition of 180 μL of aqueous $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution (5 mg mL^{-1}) and 500 μL of aqueous TRIS buffer solution (20 mM, pH 8.5) with vigorous stirring and brief sonication

after each addition. The particles were then washed three times with water with centrifugation (1500 g, 2 min)/redispersion steps, and incubated with aqueous Dox solution (1.3 mg mL⁻¹) at room temperature overnight. After incubation, the particles were washed five times with water and incubated with aqueous EDTA solution (100 mM, pH 7.5) for 2 min at room temperature to remove the CaCO₃ templates. The resultant capsules were washed three times with water to remove EDTA and excess Dox and coating material. The capsules were dispersed in water for three days, and the supernatant was changed with fresh water every 24 h to eliminate free Dox and refrigerated at 4 °C before use.

Fabrication of Dox-Loaded MPN_{TA} Capsules. Dox-loaded MPN_{TA} capsules were assembled following a previously published protocol.²⁴ 4 mg mL⁻¹ of aqueous Dox solution was incubated with PSS-doped CaCO₃ for 12 h at room temperature, followed by extensive washing with water to remove excess Dox. Next, 1 mg of Dox-loaded CaCO₃ particles was suspended in 500 μL of water. 5 μL of aqueous TA solution (40 mg mL⁻¹), 5 μL of aqueous FeCl₃·6H₂O solution (10 mg mL⁻¹), and 500 μL of aqueous MOPS buffer solution (50 mM, pH 8.0) were successively added into the CaCO₃ particle suspension at room temperature, followed by vigorous stirring and sonication after each step. Subsequently, the particles were washed three times with water with centrifugation (1000 g, 1 min)/redispersion steps, and incubated with aqueous NaOAc solution (100 mM, pH 5.0) for 3 min at room temperature to remove the templates. The resultant capsules were washed three times with water, dispersed in water, and refrigerated at 4 °C before use.

Capsule Characterization. Differential interference contrast (DIC) and fluorescence microscopy images were obtained using an inverted Olympus IX71 microscope equipped with several combinations of optical filters (excitation at 460-495 nm or 530-550 nm, and emission at 510-550 nm or 575-625 nm, respectively) (Olympus, Japan). The structure and metal content of the capsules were analyzed by transmission electron microscopy (TEM) and

energy dispersive X-ray spectroscopy (EDX) by using 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 amount of HAp and PEGp incorporated into the capsules was quantified by measuring the fluorescence intensities at an excitation wavelength of 488 nm and an emission wavelength of 521 nm using an Infinite M200 microplate reader (Tecan, Switzerland). Specifically, two sets of MPN_{HA-PEG} capsules, each consisting of three different HA/PEG assembly ratios (1, 0.2, and 0.1), were prepared using AF488-labeled HAp and non-labeled PEGp or non-labeled HAp and AF488-labeled PEGp. The capsules were counted using an Apogee A50-Micro flow cytometer (Apogee Flow Systems, UK). The capsules were disassembled by incubation in NaOAc buffer (50 mM, pH 4) in an Eppendorf Thermomixer Comfort (Eppendorf, Germany) at 37 °C with constant shaking (600 rpm) overnight and intensive sonication. Following this, the fluorescence intensity of the resultant solution was measured. The amount of HAp or PEGp incorporated in the capsules was calculated based on a standard curve of fluorescence intensity versus concentration calibrated with a set of AF488-labeled HAp or PEGp solutions with different concentrations at pH 4. The amount of HAp or PEGp incorporated into the capsules was expressed as femtograms (fg) per capsule.

Dox Loading Capacity. The number of capsules were counted using an Apogee A50-Micro flow cytometer (Apogee Flow Systems, UK). A certain number of Dox-loaded MPN_{HA-PEG-II} capsules were disassembled at pH 2 and diluted in NaOAc buffer (100 mM, pH 5). The UV absorbance at 480 nm of the above solution was then measured by an Infinite M200 microplate reader (Tecan, Switzerland). The UV absorbance at 480 nm of disassembled MPN_{HA-PEG-II} capsules without Dox loading was also measured under the same condition and used as a background absorbance. The amount of Dox was then calculated based on a standard curve of UV-absorbance at 480 nm versus concentration calibrated with a

set of Dox solutions at different concentrations in NaOAc buffer (100 mM, pH 5). When measuring Dox solutions, the UV absorbance at 480 nm of NaOAc buffer (100 mM, pH 5) was used as a background absorbance. The amount of Dox loaded in the capsules was expressed as picograms (pg) per capsule.

Dox Release from MPN_{HA-PEG} Capsules. Dox-loaded MPN_{HA-PEG-II} capsules were separately incubated in 20 mM NaOAc buffer (20 mM, pH 5) and 20 mM HEPES buffer (20 mM, pH 7.4) solutions, respectively. Time-dependent release of Dox from the capsules was studied at room temperature for 48 h. The supernatant was then collected by centrifugation and the fluorescence intensity was measured by an Infinite M200 microplate reader (Tecan, Switzerland) at an excitation wavelength of 480 nm and an emission wavelength of 575 nm at the allocated time points.

Cell Culture. Human breast adenocarcinoma cell line, MDA-MB-231, and human breast ductal carcinoma cell line, BT-474, were purchased from ATCC (USA). MDA-MB-231 was cultured in completed DMEM media (supplied with 10% FBS and GlutaMAXTM), while BT-474 was cultured in completed RPMI-1640 media (with 10% FBS and L-Glutamine added). Both cells were cultured in a cell culture incubator maintained at 37 °C, 5% CO₂ and 95% relative humidity.

Binding Affinity of HAp. Three sets of AF633-labeled HAp solutions with different concentrations (1, 0.1, 0.02 mg mL⁻¹) in DMEM in the absence of FBS were incubated with MDA-MB-231 cells for 1 h at 37 °C. After incubation, the cells were gently washed three times with DPBS, lifted with trypsin, and suspended in DPBS. The fluorescence intensity of cells was measured using an Apogee A50-Micro flow cytometer (Apogee Flow Systems, UK) with an excitation at 638 nm.

Binding Affinity of MPN_{HA} Capsules. To assess the receptor-mediated cell binding of engineered MPN_{HA} capsules, MPN_{HA} capsules were incubated with MDA-MB-231 cells and

BT-474 cells for 1 h at 4 °C and 37 °C in serum-free media. In parallel, a competition study (receptor-blocking study) was performed. To block the CD44 receptors, 10 mg mL⁻¹ HA solution in serum-free media (DMEM or RPMI-1640) was incubated with MDA-MB-231 cells and BT-474 cells for 2 h at 37 °C. After blocking, the HA solution was replaced by fresh serum-free media (DMEM for MDA-MB-231 cells and RPMI-1640 for BT-474 cells). Subsequently, AF488-labeled MPN_{HA} capsules were added to the cells at a capsule-to-cell ratio of 100:1, followed by 1 h incubation in serum-free media at 4 °C or 37 °C. After incubation, the cells were washed three times with DPBS and analyzed with an Apogee A50-Micro flow cytometer (Apogee Flow Systems, UK). The fluorescence of AF488-labeled capsules was monitored using an excitation wavelength of 488 nm. The degree of cell binding of the capsules was evaluated by determining the percentage of cells that exhibited stronger fluorescence intensity than control cells (untreated cells). The gating strategy used for flow cytometry analysis is shown in Figure S1. Minimal number of untreated cells were placed in the gating region. The binding of capsules shifted the cell population into the gating region due to the increase of fluorescence intensity.

Targeting Efficacy Studies via Flow Cytometry. 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. Subsequently, the seeded MDA-MB-231 cells and BT-474 cells were incubated for 24 h and 40 h, respectively, to allow cellular adhesion on substrates. AF488-labeled MPN_{HA-PEG} capsules, MPN_{HA} capsules, and MPN_{PEG} capsules were incubated with cells at a capsule-to-cell ratio of 100:1 in completed media (with 10% FBS) for 8 h. After incubation, the cells were gently washed three times with DPBS, lifted with trypsin, and analyzed with an Apogee A50-Micro flow cytometer (Apogee Flow Systems, UK). The degree of cell association of the capsules was evaluated by using the percentage of cells that exhibited stronger fluorescence intensity than

control cells (untreated cells). The gating strategy is the same as that used for binding affinity study (Figure S1).

Targeting Efficacy Studies via Deconvolution Microscopy. MDA-MB-231 cells and BT-474 cells were seeded into an 8-well Lab-Tek chambered coverglass slides (Thermo Fisher Scientific, USA) at a cell density of 3×10^4 cells per well. MPN_{HA-PEG-III} capsules were incubated with the cells at a capsule-to-cell ratio of 100:1 in completed media (with 10% FBS) for 1, 4, 8, and 12 h at 37 °C. Following incubation, the cells were washed three times with DPBS, fixed with 3% paraformaldehyde in DPBS for 30 min at 37 °C, then stained with WGA-594 ($5 \mu\text{g mL}^{-1}$) for 5 min at room temperature, and stained with Hoechst 33342 (0.1 mg mL^{-1}) for 10 min at room temperature. Imaging to monitor the cell association of the capsules was performed using a DeltaVision deconvolution fluorescence microscope (Applied Precision, Slovakia) with a 60× 1.42 NA oil objective and a standard FITC/TRITC/DAPI filter set. The images were processed by Imaris (Bitplane, Switzerland) software.

Cell Viability Assay. MDA-MB-231 and BT-474 cells were plated on a 96-well plate (Costar 3596, Corning, USA) at a cell density of 1×10^4 cells per well in 100 μL of cell culture media. Dox-loaded MPN_{HA-PEG-II} capsules and Dox-loaded MPN_{TA} capsules were added at various concentrations of Dox. After 3 h incubation at 37 °C in completed media (with 10% FBS), the media was aspirated and replaced with fresh media to remove unbound capsules. The cells were then incubated in the fresh completed media (with 10% FBS) for another 45 h at 37 °C. After that, the media was replaced with 100 μL of media containing MTT (0.5 mg mL^{-1}), and the cells were incubated for another 4 h at 37 °C. The resultant formazan was dissolved in 100 μL DMSO per well and the absorbance at 570 nm was measured by an Infinite M200 microplate reader (Tecan, Switzerland).

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

RESULTS AND DISCUSSION

Synthesis of HAp and PEGp. To synthesize HAp, dopamine was conjugated with the carboxylic acid groups of HA (289 kDa) through a carbodiimide coupling reaction using *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). The degree of modification was 3-4%, as determined by UV-Vis spectroscopy using the characteristic catechol absorption peak at 280 nm (Figure S2), which is consistent with previous reports.^{26,28} The catechol content was further confirmed with ¹H-NMR spectroscopy (Figure S3), by comparing the integral value of the methyl protons of HA with the aromatic protons of the catechol moieties. PEGp was similarly synthesized by reacting dopamine with 8-arm-PEG-NHS (10 kDa).²⁵ All eight PEG end-groups were coupled with catechol groups, as determined by ¹H-NMR spectroscopy (Figure S4).

Figure 2. Characterization of MPN_{HA-PEG} capsules. a) Transmission electron microscopy (TEM) images of the CaCO₃ templates; b) fluorescence microscopy image, c) TEM image,

and d) high-angle annular dark-field (HAADF) microscopy image and corresponding energy-dispersive X-ray (EDX) elemental mapping of MPN_{HA-PEG-II} capsules; e) Amount of HAp and PEGp per capsule, the feed and resultant mass ratio of HAp to PEGp per capsule (from left to right are MPN_{HA-PEG-I}, MPN_{HA-PEG-II}, and MPN_{HA-PEG-III} capsules) (mean $\pm$ SD, n=3). Scale bars are a,d) 500 nm, b) 3 μ m, and c) 1 μ m.

Fabrication of MPN_{HA-PEG} Capsules. For the assembly of MPN_{HA-PEG} capsules, CaCO₃ particles with an average diameter of 1.2 μ m were synthesized and used as templates (**Figure 2a**). HAp and PEGp solutions were pre-mixed at desired HAp:PEGp mass ratios (1, 0.2, or 0.1), and were then added into the suspension of CaCO₃ templates. The three different feed ratios of HAp to PEGp (1, 0.2, or 0.1) were expected to produce capsules with different HA and PEG content, and are denoted MPN_{HA-PEG-I}, MPN_{HA-PEG-II}, and MPN_{HA-PEG-III} capsules, respectively. Due to the formation of bis- and tris-catechol-Fe^{III} coordination complexes above pH 7,²⁹ the addition of Fe^{III}, followed by increasing the pH of the solution, resulted in the cross-linking of HAp and PEGp to form MPN coatings. Following template removal with EDTA (100 mM, pH 7.5), MPN_{HA-PEG} capsules were obtained (Figure 2b,c, Figure S5d-l). The presence of Fe in the MPN_{HA-PEG-II} capsules was verified by energy-dispersive X-ray (EDX) spectroscopy (Figure S6) and elemental mapping (Figure 2d). The Fe, O, and N signals matched well with the capsule configuration in the dark fields, indicating the uniform distribution of iron, oxygen, and nitrogen in the film structure (the magnified EDX mapping image shown in Figure S7). The nitrogen and oxygen were present in both HAp and PEGp polymers. The amount of HAp and PEGp incorporated into the capsules was quantified by fluorescence spectrophotometry. The amount of HAp incorporated into the MPN_{HA-PEG-I}, MPN_{HA-PEG-II}, and MPN_{HA-PEG-III} capsules were 10, 1.7, and 0.7 fg per capsule, respectively, while the amount of PEGp incorporated into the MPN_{HA-PEG-I}, MPN_{HA-PEG-II}, and MPN_{HA-PEG-III} capsules were 8, 6.8, and 4.3 fg per capsule, respectively (Figure 2e, Table S1). The

resultant ratios of HAp to PEGp were determined to be 1.3, 0.3, and 0.2 for the MPN_{HA}-PEG-I, MPN_{HA}-PEG-II, MPN_{HA}-PEG-III capsules, respectively (Figure 2e, Table S1). The higher resultant ratio (than feed ratio) suggests that HAp incorporates into the MPN capsules to a greater extent, probably due to its larger molecular weight and negative charge. Capsules composed solely of HAp (MPN_{HA} capsules) and PEGp (MPN_{PEG} capsules) were also synthesized and characterized (Figure S5a-c, m-o). There were 10 fg of HAp and 9 fg of PEGp incorporated into a single MPN_{HA} and MPN_{PEG} capsule, respectively (Table S1).

Figure 3. Flow cytometry analysis of AF488-labeled MPN_{HA} capsule binding to MDA-MB-231 (CD44+) and BT-474 cells (CD44-) with and without blocking with free HA, after 1 h incubation in serum-free media at a) 4 °C or b) 37 °C (mean $\pm$ SD, n=6, ****p<0.0001). The capsule-to-cell ratio was set to 100:1 for all cell experiments.

Tunable Targeting of MPN_{HA-PEG} Capsules to Cancer Cells. As the carboxylic acid groups of HA were conjugated with catechols, the binding efficacy of HAp and MPN_{HA} capsules to CD44 was first investigated. Solutions of Alexa Fluor 633 (AF633)-labeled HAp with different concentrations (1, 0.1, 0.02 mg mL⁻¹) were incubated with MDA-MB-231 cells, a CD44 overexpressing human breast cancer cell line,³⁰ for 1 h at 37 °C in serum-free media. Serum-free media was employed to eliminate the influence of serum proteins on HA-CD44 interactions. After incubation, the fluorescence intensity of the cells was significantly higher than that of untreated cells due to the binding of the AF633-labeled HAp (Figure S8). Moreover, the higher the concentration of the HAp in solution, the higher the eventual fluorescence intensity of the cells, illustrating that the cell binding efficacy is dependent on the HA concentration. To investigate the targeting specificity of the capsules containing HAp, MPN_{HA} capsules were incubated with MDA-MB-231 cells (CD44+ cell line) and BT-474 cells (CD44- cell line) in serum-free media. BT-474 cells are human breast cancer cells with minimal CD44 expression.³⁰ After 1 h incubation at 4 °C, more than 65% of MDA-MB-231 cells had MPN_{HA} capsule binding was facilitated by CD44-mediated surface binding (**Figure 3a**). In contrast, only about 5% of BT-474 cells had capsules bound, likely due to the absence of CD44 on the surfaces of these cells. A competition study (receptor-blocking study) was performed by incubating the cells with free HA to block the CD44 receptors before the addition of the MPN_{HA} capsules. After blocking, five times fewer MPN_{HA} capsules bound to the MDA-MB-231 cells, while there was no significant difference for BT-474 cells. A similar trend was also observed at 37 °C, where the cell association of the MPN_{HA} capsules with MDA-MB-231 cells was about six times more than that for the BT-474 cells, and about two times more than that for CD44-blocked MDA-MB-231 cells (Figure 3b). Little difference was observed for cell association with BT-474 cells with or without blocking with free HA, despite around two-fold increase on cell association at 37 °C versus 4 °C (Figure 3). These

results suggest that the association between MPN_{HA} capsules and MDA-MB-231 cells is due to specific HA-CD44 interactions, whilst the association of MPN_{HA} capsules with BT-474 cells is due to non-specific interactions that were enhanced at 37 °C after the short incubation time of 1 h.

Figure 4. Effect of HA:PEG ratio on cell association of MPN_{HA-PEG} capsules to MDA-MB-231 and BT-474 cells analyzed by flow cytometry. a) Percentage of cells associated with MPN_{HA}, MPN_{HA-PEG-I}, MPN_{HA-PEG-II}, MPN_{HA-PEG-III}, and MPN_{PEG} capsules after 8 h of incubation in completed media with 10% FBS at 37 °C (mean $\pm$ SD, n=6, ****p<0.0001, **p<0.01, *p<0.05). b) Percentage of cells associated with MPN_{HA-PEG-III} capsules after

different incubation times (1, 4, 8, and 12 h) in completed media with 10% FBS at 37 °C (mean $\pm$ SD, n=6). The capsule-to-cell ratio was set to 100:1 for all cell experiments.

We next sought to incorporate low-fouling materials into these capsules to reduce non-specific interactions with CD44[−] cells under long-term incubation (8 h) at 37 °C in serum-containing media. We studied the effects of PEG incorporation as well as the HA:PEG ratio on the targeting specificity and non-specific binding of the hybrid MPN_{HA-PEG} capsules. AF488-labeled MPN_{HA-PEG-I}, MPN_{HA-PEG-II}, and MPN_{HA-PEG-III} capsules with different HA:PEG ratio (1.3, 0.3, and 0.2) were incubated with MDA-MB-231 cells (CD44⁺) and BT-474 cells (CD44[−]) at a capsule-to-cell ratio of 100:1 for 8 h at 37 °C in completed media with 10% FBS, and the percentage of cell association was evaluated by flow cytometry (**Figure 4a**). For comparison, AF488-labeled MPN_{HA} capsules and MPN_{PEG} capsules were also investigated under the same conditions. Approximately 90% of MDA-MB-231 cells associated with MPN_{HA} capsules after 8 h; however, there was also a large amount (41%) of BT-474 cells associated with MPN_{HA} capsules. This high association with CD44[−] cells suggests that the targeting specificity of these capsules could be compromised after long-term incubation at 37 °C. In contrast to the MPN_{HA} capsules, the cell association of the MPN_{PEG} capsules with both MDA-MB-231 and BT-474 cells was low (about 8%), indicating the high resistance of PEG to non-specific interactions. When PEGp was incorporated into the MPN_{HA} capsules, cell association with BT-474 cells was dramatically reduced. Compared with the MPN_{HA} capsules, the cell association with BT-474 cells decreased from 41% to only 18% for MPN_{HA-PEG-I} capsules which can be attributed to the shielding effect of PEG.^{31,32} In contrast to the reduced association with BT-474 cells, MPN_{HA-PEG-I} capsules still had 88% cell association with MDA-MB-231 cells. Moreover, as the HA:PEG ratio further decreased, fewer BT-474 cells associated with MPN_{HA-PEG} capsules (15% for MPN_{HA-PEG-II} and 12% for MPN_{HA-PEG-III}), while MDA-MB-231 cells maintained high cell association (81% for

MPN_{HA-PEG-II} and 74% for MPN_{HA-PEG-III}). Thus, the targeting specificity of MPN_{HA-PEG} capsules assessed by the ratio of their cell association with CD44⁺ and CD44⁻ cells increased from 2.2 for MPN_{HA} capsules to 4.8 for MPN_{HA-PEG-I} and further to a ratio of 6 for MPN_{HA-PEG-III} capsules (Figure S9). There was no significant difference of MDA-MB-231 cell association between the MPN_{HA} capsules and the MPN_{HA-PEG-I} capsules (91% versus 88%, **Figure 4a**), likely because comparable amounts (10 fg capsule⁻¹, Table S1) of HAp were incorporated into the two capsules, and because the affinity of HA-CD44 binding is high (high binding avidity for the multivalent interaction of high molecular weight HA to CD44 receptors on cell surfaces).^{33,34} Nevertheless, further decreasing the HA:PEG ratio led to reduced cell associations with MDA-MB-231 cells. The targeting ability of the MPN_{HA-PEG-II} capsules and MPN_{HA-PEG-III} capsules with MDA-MB-231 cells was compromised (10% and 17% reduction, respectively) as the relative amount of PEG was significantly more than HA (3.3 and 5 times higher, respectively, Figure 2e, Table S1). We hypothesize the contact between HA and CD44 receptors was inhibited by the hydration layer that is likely to form around PEG.³⁵ Therefore, when designing HA-based carriers for effective targeted drug delivery, the HA:PEG ratio should be carefully considered and tuned.

Figure 5. Deconvolution microscopy images of MPN_{HA-PEG-III} capsules incubated with MDA-MB-231 cells (top row) and BT-474 cells (bottom row) in completed media with 10% FBS at 37 °C for 1, 4, 8, and 12 h. Cell nuclei and membrane were stained with Hoechst 33342 (blue) and WGA-594 (red), respectively. MPN_{HA-PEG-III} capsules were fluorescently labeled with AF488 (green). Scale bars are 10 μm. The capsule-to-cell ratio was set to 100:1 for all cell experiments.

To further investigate the targeting efficacy of the MPN_{HA-PEG} capsules, the cell association kinetics were evaluated by incubating MPN_{HA-PEG-III} capsules with MDA-MB-231 cells and BT-474 cells for different periods of time at 37 °C in completed media with 10% FBS. After incubation, the cell association was assessed by flow cytometry. The cell association of the MPN_{HA-PEG-III} capsules with MDA-MB-231 cells went above 50% after 1 h incubation and gradually increased with longer incubation time to 80% at 12 h (Figure 4b). In contrast, for the CD44[−] cell line, the association of the MPN_{HA-PEG-III} capsules with BT-474 cells was minimal, at less than 5% within the first 4 h with a gradual increase to about 22% after 12 h incubation. This is less than a third of the association of the same capsules with MDA-MB-231 cells (80%) at 12 h. Deconvolution microscopy was then employed to corroborate the targeting ability of the MPN_{HA-PEG-III} capsules (**Figure 5**). Consistent with the flow cytometry data, an increased number of MDA-MB-231 cells associated with the capsules as the incubation time increased, while fewer BT-474 cells had associated capsules, even after 12 h of incubation.

Figure 6. a) Differential interference contrast (DIC) and b) fluorescence microscopy images of Dox-loaded $\text{MPN}_{\text{HA-PEG-II}}$ capsules. Scale bars are 3 μm . c) Cytotoxicity of Dox-loaded $\text{MPN}_{\text{HA-PEG-II}}$ capsules as a function of Dox concentration after 48 h incubation in completed media with 10% FBS at 37 °C against MDA-MB-231 and BT-474 cells. Cell viability was evaluated by MTT assay (mean $\pm$ SD, $n=4$). The cell viability of untreated cells was normalized to 100%.

Cytotoxicity of Dox-loaded $\text{MPN}_{\text{HA-PEG}}$ Capsules. Encapsulation and targeted delivery of anticancer drugs is of interest for next-generation drug delivery.³⁶ After balancing the high targeting specificity and low non-specific association, $\text{MPN}_{\text{HA-PEG-II}}$ capsules was chosen to load a model drug, Dox, for cytotoxicity study. As shown in **Figure 6a,b**, Dox-loaded $\text{MPN}_{\text{HA-PEG-II}}$ capsules were dispersed in aqueous solution with the red fluorescence originating from the loaded Dox. The Dox loaded amount is 0.31 ± 0.03 pg per capsule. The loading of Dox into $\text{MPN}_{\text{HA-PEG-II}}$ capsules is possibly due to electrostatic interactions

between HA and Dox and π - π stacking between benzyl groups of the MPN and Dox. The release of Dox was faster at pH 5.0 compared with pH 7.4 (82% versus 23% at 48 h, Figure S10), possibly due to the reduced stability of MPNs and increased hydrophilicity of Dox at low pH.^{25,37} This implies that Dox can be readily released from the internalized capsules in the intercellular compartments with acidic pH (e.g., endosomes and lysosomes). The cytotoxicity of Dox-loaded MPN_{HA-PEG-II} capsules against MDA-MB-231 and BT-474 cells was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MDA-MB-231 and BT-474 cells were incubated with Dox-loaded MPN_{HA-PEG-II} capsules at various Dox concentrations for 3 h at 37 °C in completed media with 10% FBS, followed by removal of non-associated capsules and a further 45 h incubation. The MTT assay showed that the MDA-MB-231 cell viability was lower than that of BT-474 cells at all corresponding Dox concentrations (Figure 6c). At high Dox concentrations (2.5 to 10 $\mu\text{g mL}^{-1}$), the cell viability of MDA-MB-231 cells was more than two times lower than that of BT-474 cells. This reduced viability of MDA-MB-231 cells was likely caused by the increased uptake of Dox-loaded MPN_{HA-PEG-II} capsules due to CD44 receptor-mediated endocytosis,¹⁹ and further release of Dox from capsules in the intercellular compartments. As a control, Dox-loaded tannic acid (TA)-based MPN (MPN_{TA}) capsules without targeting ligands were also incubated with both cells under the same conditions. The cell viability between the two cell lines was similar after 48 h incubation of Dox-loaded MPN_{TA} capsules (Figure S11). Moreover, the cytotoxicity of Dox-loaded MPN_{HA-PEG-II} capsules to MDA-MB-231 cells was higher than that of Dox-loaded MPN_{TA} capsules, while the cytotoxicity of Dox-loaded MPN_{HA-PEG-II} capsules against BT-474 cells was much lower than MPN_{TA} capsules. This further indicates the high targeting of MPN_{HA-PEG-II} capsules to MDA-MB-231 cells and low non-specific interactions to BT-474 cells.

CONCLUSIONS

In conclusion, we have reported a facile strategy to prepare MPN capsules consisting of HA and PEG via rapid assembly for cancer cell targeting. The conjugation of phenolic groups to HA and PEG allows for the rapid deposition of HAp and PEGp on CaCO_3 templates, and the instantaneous chelation of Fe^{III} leads to the rapid formation of robust films based on metal-phenol coordination. The incorporation of HA enhanced the binding and association of the capsules with CD44+ cell line (MDA-MB-231 cells), whilst the incorporation of PEG reduced the non-specific interactions of the capsules with CD44– cell line (BT-474 cells). Moreover, the ratio of HAp and PEGp incorporated into the capsules can be readily tuned by changing the feed ratio during assembly. As a result, $\text{MPN}_{\text{HA-PEG}}$ capsules assembled using a lower HA:PEG ratio show higher targeting specificity but associate at a lower level with MDA-MB-231 cells, exhibiting tunable targeting. Dox-loaded $\text{MPN}_{\text{HA-PEG-II}}$ capsules with high targeting specificity, but low levels of non-specific association exhibited significantly higher cytotoxicity to CD44+ compared with CD44– cell lines. This study provides a simple way to maximize the targeting specificity of MPN-based carriers, while minimizing their non-specific cellular interactions.

ASSOCIATED CONTENT

Supporting Information

UV-Vis absorption spectra, ^1H -NMR spectrum, structural characterization of capsules, EDX spectrum, fluorescence intensity of cells, ratio of specific and non-specific association with cells, cell viability assay, and fluorescence calibration curve. 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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