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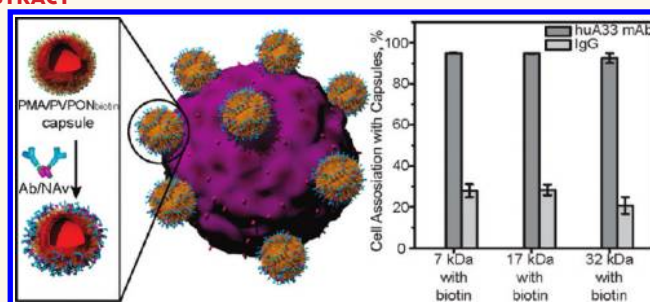
Macromolecule Functionalization of Disulfide-Bonded Polymer Hydrogel Capsules and Cancer Cell Targeting

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For more than a decade, multilayered polymer capsules have attracted interest as versatile candidates for a diverse range of biomedical applications, including drug delivery vehicles,^{1–3} microreactors,⁴ and as hierarchical components of artificial cells.^{5,6} Significant effort has focused on engineering multilayered, responsive, and functional capsules with control over their physicochemical properties, such as size, permeability, and stability. Recently, we developed a redox-responsive multilayered hydrogel capsule system based on thiolated poly(methacrylic acid) (PMA_{SH}).⁷ An approach to stabilize such capsules is based on reversible disulfide linkages, as these bonds are stable in the extracellular milieu, but then can degrade in simulated intracellular conditions.⁸ These PMA hydrogel capsules (HCs) are prepared by the sequential adsorption of PMA_{SH} and poly(*N*-vinyl pyrrolidone) (PVPON) on silica particles, mediated through hydrogen bonding. Following assembly, the PMA_{SH} multilayers are stabilized by oxidation of the thiol groups to form disulfide bonds. The silica templates are then dissolved, and the PVPON is released from the multilayers, resulting in PMA HCs. Several studies have shown that this assembly procedure has the potential to controllably tailor key properties of the PMA HCs, including size, permeability,⁹ cargo loading,¹⁰ and triggered release.¹¹ Various therapeutics, including nucleic acids,¹² peptide vaccines,¹⁰ and hydrophobic chemotherapeutic drugs,^{13,14} have been encapsulated in these capsules and effectively delivered to human cells *in vitro* and *in vivo*.^{15,16} More recently, subcompartmentalized PMA HCs containing intact liposomes^{17,18} or smaller PMA HCs¹⁹ have been reported, suggesting the

ABSTRACT



We present a generic and versatile method for functionalization of disulfide-stabilized PMA hydrogel capsules (HCs) with macromolecules, including a number of specific antibodies to cancer cells. Functionalization was achieved by reversible addition–fragmentation chain transfer (RAFT) polymerization of poly(*N*-vinyl pyrrolidone) (PVPON), which introduced biorelevant heterotelechelic end groups (thiol and amine) to the polymer chain. The PVPON with heterotelechelic end groups was conjugated to the outermost layer of PMA HCs through the thiol groups and reacted with biotin *via* the amine groups to generate PMA/PVPON_{biotin} HCs. On the basis of the high specific interaction and high affinity between biotin and avidin, and its derivatives, such as NeutrAvidin (NAv), we functionalized the PMA HCs with biotinylated antibodies. We demonstrate significantly enhanced cellular binding and internalization of the antibody (Ab)-functionalized capsules compared with control human immunoglobulin (IgG)-functionalized capsules, suggesting these capsules can specifically interact with cells through antibody/antigen recognition. We anticipate that the versatility of the functionalization approach reported in this study will assist in targeted therapeutic delivery applications.

KEYWORDS: PMA hydrogel capsules · biofunctionalization · RAFT polymerization · antibodies · biotin–avidin interaction · targeting

potential of PMA HCs for microencapsulated catalysis and advanced drug delivery.²⁰

Given the complexity of biological environments, it is often desirable to functionalize capsules with biomolecular binding partners to achieve specific interactions.²¹ For example, cells can be specifically targeted by functionalizing the surface of capsules with appropriate antibodies, or specific cell signaling pathways can be activated

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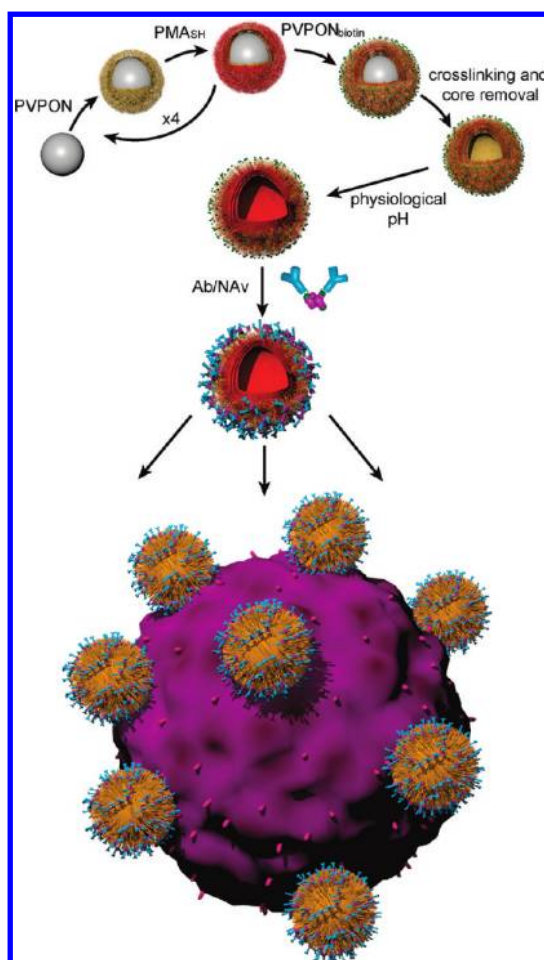
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by conjugating capsules with activating ligands. In cancer drug delivery, the specific and targeted delivery of a drug is a crucial goal in the development of drug delivery carriers, as it can potentially reduce the drug toxicity to healthy tissues, while delivering a high dosage to the targeted cancer cells.^{22,23} A number of covalent coupling chemistries, including click and thiol/maleimide reactions, have been used to covalently conjugate biomolecules to a range of surfaces.²⁴ For instance, we recently reported the functionalization of PVPON-click capsules with specific antibodies and *in vitro* targeting to cancer cells.²⁵ Additionally, functionalization of multilayered capsules with specific antibodies can also be achieved through electrostatic interactions.²⁶

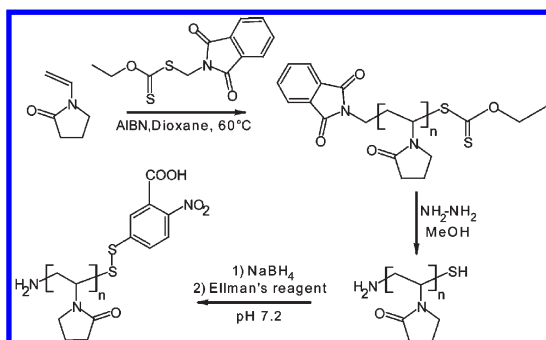
The strong noncovalent interaction between biotin and avidin ($K_d \sim 10^{-15}$ M), which forms a stable complex over a wide pH, ionic strength, and temperature range,²⁷ represents a versatile strategy to tether biomolecules. In addition, the routine biotinylation of a variety of macromolecules, as has been demonstrated for proteins,²⁸ peptides,²⁹ and polymers,³⁰ enables such materials to interact and/or be immobilized through biotin–avidin interactions, providing a facile coupling and functionalization strategy for developing functional biomaterials.

Herein, we report a generic approach based on biotin/avidin interactions to functionalize PMA HCs with antibodies and illustrate their potential by targeting human colorectal cancer cells *in vitro* (Scheme 1). First, we synthesized poly(*N*-vinyl pyrrolidone) (PVPON) via reversible addition–fragmentation chain transfer (RAFT) polymerization using *O*-ethyl *S*-(phthalimidomethyl) xanthate as a RAFT agent to introduce a thiol group at one end of the polymer chain and an amine group at the other (Scheme 2). This heterotelechelic polymer can also be used for various bioconjugation methods, which utilize amine- and thiol-reactive residues. Next, the PVPON with heterotelechelic end groups was conjugated to the outermost layer of PMA HCs through the thiol groups and reacted with biotin *via* the amine groups to generate PMA/PVPON_{biotin} HCs. Furthermore, RAFT polymerization made it possible to controllably synthesize PVPON of various molecular weights, resulting in linkers of different lengths for biofunctionalization of the HCs. The advantage of using PVPON as a linker is that the amount immobilized can be easily controlled by the molecular weight of PVPON and, therefore, the number of chains attached to the surface. Additionally, the immobilization of modified PVPON occurs *via* multilayer assembly, leading to PMA/PVPON_{biotin} HCs, which can be biofunctionalized. To demonstrate the general applicability of this functionalization protocol, two monoclonal antibodies, humanized A33 monoclonal antibody (huA33 mAb)³¹ and epidermal growth factor receptor monoclonal antibody (EGFR mAb),³² were biotinylated,



Scheme 1. Schematic representation of PMA/PVPON_{biotin} hydrogel capsule (HC) formation, surface biofunctionalization, and specific binding to cancer cells. Silica particles are sequentially coated with thiolated poly(methacrylic acid) and poly(*N*-vinyl pyrrolidone) *via* hydrogen bonding at pH 4 (the process is repeated four times). Thiolated and biotinylated PVPON (yellow layer with green dots) is deposited and cross-linked as the final layer, followed by core removal. Upon exposure to physiological pH, the capsules swell and the nonmodified PVPON is released, resulting in biotin-functionalized PMA/PVPON_{biotin} HCs. Subsequently, antibodies complexed with NeutrAvidin (depicted in blue and pink) are coupled to these HCs through biotin/NeutrAvidin interactions. The incorporation of antibodies on the surface of HCs leads to enhanced binding and internalization by human colorectal cancer cells (shown in purple).

complexed with NeutrAvidin, and individually coupled to PMA/PVPON_{biotin} HCs. Using human colorectal cancer cells (LIM1899), which endogenously express both A33 antigen and EGFR on the cell surface,³³ we demonstrate significantly enhanced cellular binding and internalization of these antibody (Ab)-functionalized capsules compared with control human immunoglobulin (IgG)-functionalized capsules, suggesting that these capsules can specifically interact with cells through antibody/antigen recognition. We anticipate that the versatility of the functionalization approach reported in this study will help realize a range of biofunctionalized carriers with potential for targeted therapeutic delivery.



Scheme 2. Schematic diagram showing RAFT polymerization of PVPON and end group transformation. First, poly(*N*-vinyl pyrrolidone) (PVPON) was synthesized by RAFT polymerization using *O*-ethyl *S*-(phthalimidomethyl) xanthate as a RAFT agent. The deprotection step was performed by treating the polymer with hydrazine hydrate in methanol and, subsequently, with sodium borohydride. Ellman's reagent was allowed to react with the thiol functionality for characterization and storage purposes.

RESULTS AND DISCUSSION

RAFT Polymerization of PVPON. The ability to control both the molecular weight distribution of polymers and their functional end groups makes RAFT polymerization advantageous for bioconjugation. Specifically, the presence of thiocarbonylthio functionality in RAFT-derived polymers enables bioconjugation through the relevant thiol.³⁴ PVPON was synthesized in the presence of the *O*-ethyl *S*-(phthalimidomethyl) xanthate RAFT agent using azobisisobutyronitrile (AIBN) as the initiator. The xanthate RAFT agent used in this study has been shown previously to provide good control over “less activated” monomers (LAMs; e.g., vinyl acetate, 1-vinyl-2-pyrrolidone).³⁵ In addition, this RAFT agent has the capacity to generate a protected amine end group that can be modified subsequently to give bioreactive end functionality.^{35–37} Following polymerization, the molecular weights observed from NMR and GPC were close to the theoretical molecular weights (7, 15, and 30 kDa) and the polydispersities were narrow (below 1.20, Table 1). These values are in good agreement with those reported for RAFT polymerization of 1-vinyl-2-pyrrolidone.^{35,38,39} It is desirable that polymerization is not brought to full conversion to avoid thiocarbonylthio end group loss via radical–radical polymer coupling and other radical side reactions.⁴⁰ Thus, the monomer conversion of the prepared polymers was kept below 85% (final column, Table 1).

RAFT PVPON Modification. Hydrazine has a bifunctional role in the aminolysis of RAFT-derived polymers.⁴¹ As a strong nucleophile, it not only drives the hydrozoinolysis of the phthalimidomethyl group of the RAFT PVPON polymer with the desired α -amino functionality but also gives an exposed thiol functionality by cleaving the thiocarbonylthio end group. The deprotection step was performed by treating the RAFT PVPON polymer with hydrazine hydrate in methanol. The conversion of the phthalimidomethyl group to α -amine was

TABLE 1. Molecular Weights and Polydispersities for PVPON Obtained by Polymerization of 1-Vinyl-2-pyrrolidone (50% v/v in Dioxane) in the Presence of the *O*-Ethyl *S*-(Phthalimidomethyl) Xanthate RAFT Agent and AIBN 60 °C

\bar{M}_n^{theo} , kDa ^a	\bar{M}_n^{NMR} , kDa ^b	\bar{M}_n^{GPC} , kDa ^c	\bar{M}_w/\bar{M}_n	Conv _{monomer} , %
7	7.3	7.2	1.08	60.7
15	12.2	17.2	1.18	80.7
30	27.0	32.3	1.15	80.8

^a Theoretical number-average molecular weight (\bar{M}_n). ^b Number-average molecular weight (\bar{M}_n) determined from ¹H NMR spectra. ^c Number-average molecular weight (\bar{M}_n) and polydispersity (\bar{M}_w/\bar{M}_n) determined by gel permeation chromatography.

confirmed by ¹H NMR spectroscopy (Figure S1a in Supporting Information), in which the terminal phthalimidomethyl group has a characteristic ¹H NMR chemical shift as two broad “doublets” at δ 7.71 and 7.83 ppm. The ¹H NMR spectra clearly indicate the disappearance of the phthalimide end group, reflecting the successful conversion to α -amine (Figure S1b). The amine end group could not be detected by ¹H NMR, as its chemical shift appears between δ 0.5 and 4.5 ppm as a broad signal, which would be overlapped by polymer backbone signals.

Although the use of hydrazine hydrate can transform the thiocarbonylthio end group to a thiol functionality (while deprotecting the amine), the two-step approach was used to ensure the presence of a thiol end group on the PVPON polymers (Scheme 2). To obtain polymer chains with a thiol end group, the thiocarbonylthio moiety on the RAFT PVPON chain was cleaved with sodium borohydride. The resulting thiol was immediately reacted with Ellman's reagent to form a mixed disulfide with a characteristic absorbance at 335 nm. The terminal thiocarbonylthio group on the RAFT-synthesized PVPON has a characteristic absorbance maximum at 305 nm, and the disappearance of the peak at 305 nm and the appearance of a peak at 335 nm confirm the successful reaction (Figure S2). Further evidence of the reaction between the polymer and the 2-nitro-5-thiobenzoate (NTB) moiety of Ellman's reagent was obtained by ¹H NMR spectroscopy, which revealed the appearance of additional signals at chemical shifts of 7.54 and 7.77 ppm (Figure S1c). The conjugation of the thiol to the NTB moiety protects the terminal thiol from side reactions and, more importantly, is primed for thiol-disulfide exchange.

To allow selective bioconjugation, the modified PVPON with α -amine and protected ω -thiol can be reacted with a small molecule that facilitates covalent linkage to an amine, such as *N*-hydroxysuccinimidyl (NHS) ester-activated biotin. Biotin binds with high affinity to avidin and its derivatives, such as streptavidin or NeutrAvidin.²⁷ *N*-Hydroxysuccinimidyl ester-activated biotins (NHS-biotin) are most commonly used because NHS esters react efficiently with primary amino groups

at pH 7–9 to form stable amide bonds.⁴² PVPON with an α -amino end group was reacted with biotin-*N*-hydroxysuccinimidyl ester (biotin-NHS) at pH 7.5 for at least 2 h. Biotin incorporation was confirmed using the EZ-Link Sulfo-NHS-LC-Biotinylation kit. For the given samples, a ratio of 0.9 to 1 was obtained. This result provides additional evidence for the phthalimide conversion into a primary amine and shows retention of amine reactivity.

PMA/PVPON HC Formation. To obtain hollow capsules, we used the layer-by-layer (LbL) approach, which comprises the sequential deposition of polymers onto sacrificial particles. In previous studies, we have shown that the polymer pair PVPON and PMA_{SH} can be adsorbed onto particles without compromising their colloidal stability, a feature essential for reliable capsule assembly.¹⁹ Oxidative cross-linking of PMA_{SH} and removal of the core particles generate stable PMA hydrogel capsules (HCs). In this study, RAFT-derived PVPON was modified to possess a single terminal thiol group, through which the PVPON was attached to generate PMA/PVPON HCs following the removal of the particle templates. The multilayer assembly was monitored using flow cytometry analysis of PVPON modified with an Alexa Fluor 488 (AF488) fluorescent label coupled through the α -amino group. The sequential deposition of the PMA_{SH} and AF488-PVPON resulted in a linear increase in capsule fluorescence (Figure S3). We have previously shown the release of PVPON layers from intact capsules following a pH change from acidic pH (pH 4) to physiological pH (pH 7.2) due to disruption of hydrogen bonding between the polymer pairs.⁷ However, with the current capsules, flow cytometry reveals that around 72% of the PVPON was retained within the capsules on increasing the pH to 7.2 (Figure 1a). This result was confirmed when using fluorescence microscopy (Figure 1b,c). To further verify if the polymer remains encapsulated within the capsules or attached to the PMA network, PMA capsules were assembled using fluorescently labeled, modified PVPON (activated thiol group on the one side and reacted with a Alexa Fluor 488 dye through amine group on the other side) only on the outermost layer (while in the other layers commercially available PVPON was used). In this way, the change in pH should release PVPON, and residual fluorescence will be due to covalently attached PVPON. Flow cytometry was used to quantify the remaining fluorescence on the surface of the capsules, and the amount of PVPON retained was found to vary, depending on factors such as the thiolation degree of the PMA, molecular weight of the polymer, or the cross-linker used (Figure 2).

Our previous studies on PMA HC formation suggested that the optimal thiol percentage to obtain stable and well cross-linked capsules was between 9 and 12%.^{9,11} Similar results were observed in this

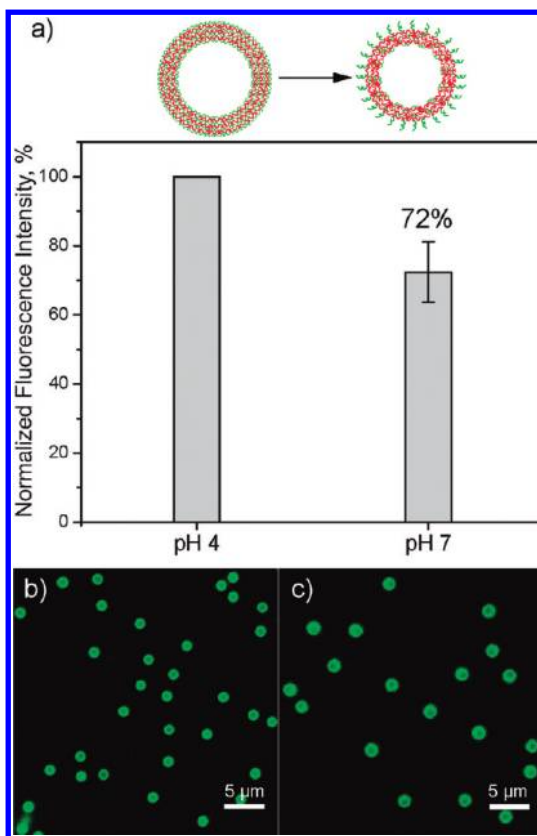


Figure 1. Change in the normalized fluorescence intensity of PMA/AF488-PVPON hydrogel capsules templated from 1 μ m diameter SiO₂ particles. (a) Fluorescence intensity change of capsules measured by flow cytometry upon changing the pH from 4 (normalized fluorescence intensity, the initial fluorescence readings of the capsules was set to 100%) to 7.2 (percentage calculated relative to the initial fluorescence). (b) Fluorescence microscopy images of PMA/AF488-PVPON hydrogel capsules taken at pH 4. (c) Fluorescence microscopy images of PMA/AF488-PVPON hydrogel capsules taken at pH 7.2, where the capsules swell and release the PVPON non-linked to the PMA network.

study for PVPON integration (Figure S4). PMA thiolation of less than 9% provides a lower number of thiols required for efficient cross-linking between the thiol groups of PVPON and PMA HCs. Thiolation of more than 20% produces a more hydrophobic PMA with a more coiled polymer structure, which we postulate leads to more intramolecular cross-linking and, consequently, less thiol groups available for intermolecular interactions,⁹ such as the formation of disulfide bonds between modified PVPON and PMA_{SH}.

It is possible to calculate the number of chains on the surface of the capsules by using the surface coverage of PVPON (1.4 mg m⁻²)¹¹ and assuming that the efficiency of the reaction between α -amine and NHS-ester approaches 100% (see Supporting Information). The highest percentage of polymer retained, and consequently the highest number of chains on the surface of the capsules, was observed with low molecular weight PVPON (7 kDa) (Figure 2a). Each individual

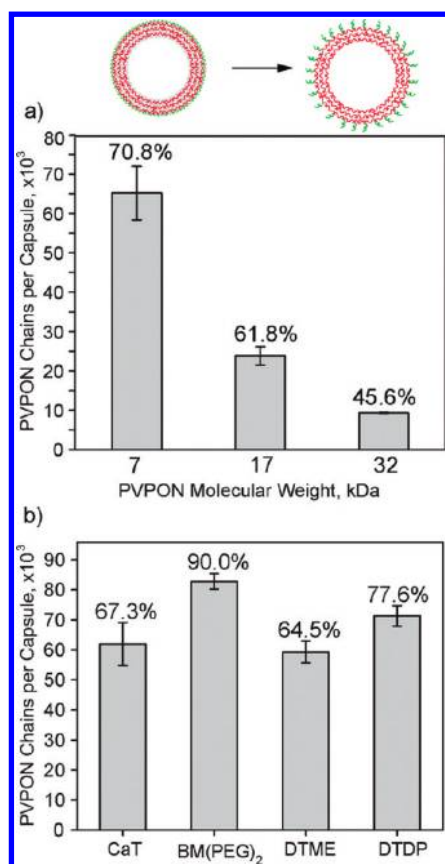


Figure 2. PVPON retention on the surface of capsules templated from 1 μm diameter SiO_2 particles as a function of various factors. (a) PVPON molecular weight (the degree of PMA thiolation was kept constant at 11%). As the molecular weight of PVPON increased, the percentage of PVPON retained together with the number of chains decreased, suggesting a dilution of the thiol content on the surface. (b) Cross-linking agent used (the molecular weight of the PVPON was 7 kDa, and the thiol content of PMA_{SH} was 12%). The most efficient cross-linker was BM(PEG)₂, while the others were found to have an intermediate effect on PVPON retention.

polymer chain possesses a single thiol end functionality. We would expect that more chains of lower molecular weight polymer can be adsorbed onto a given surface area compared to larger chains, resulting in an increased number of thiol groups available for incorporation into the PMA_{SH} network. As the molecular weight of PVPON increased, the percentage of PVPON retained and the number of chains decreased, suggesting a dilution of the thiol end group and the main chain) (Figure 2a).

The use of various cross-linkers to stabilize PMA HCs also modulates the degree of PVPON retention on the surface of the capsules (Figure 2b). In previous studies, we have described the formation of disulfide-stabilized PMA capsules using the oxidizing agent Chloramine T (CaT) or by thiol-disulfide exchange with a thiol-activated polymer.⁴³ More recently, we have extended the cross-linking approach by implementing homobifunctional cross-linking reagents containing the

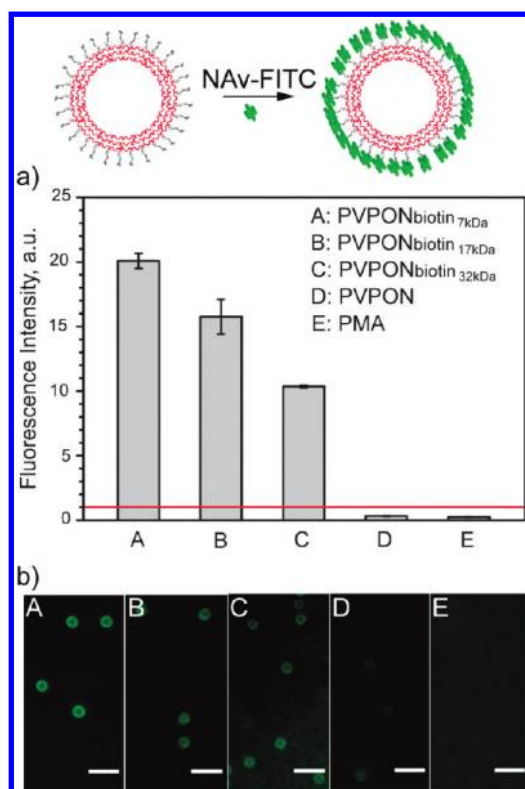


Figure 3. Specific interaction between PMA/PVPON_{biotin} HCs and NeutrAvidin. (a) Fluorescence intensity of the capsules measured by flow cytometry, which showed decreasing fluorescence with increased molecular weight of the polymer on the surface. The red line represents the background fluorescence. (b) Corresponding fluorescence microscopy images. The scale bars are 10 μm .

thiol-reactive moiety, maleimide.¹⁹ The use of the oxidizing agent (CaT) alone showed only around 70% efficiency of PVPON incorporation, suggesting incomplete conversion of thiols into disulfide linkages. Cross-linking using reagents with thiol-reactive groups (*i.e.*, maleimides) demonstrated a different behavior. In this study, we used two homobifunctional cross-linkers, namely, BM(PEG)₂ and DTME, which contain noncleavable short poly(ethylene glycol) and a redox-cleavable disulfide bridge, respectively. Figure 2b shows that the most efficient cross-linker was BM(PEG)₂. This is most likely due to the more hydrophilic nature of BM(PEG)₂ in aqueous solution compared to DTME (which is mostly insoluble in aqueous solution), allowing better penetration into the layers. Dithiodipyridine (DTDP) is another cross-linking reagent that is accessible for thiol-disulfide exchange. It has intermediate hydrophobicity, and not surprisingly, it has a corresponding intermediate effect on PVPON retention (Figure 2b). We have recently shown promising results for the cross-linking of PMA HCs with DTDP for improved cargo retention.⁹ It was determined that the rate of reaction between DTDP-activated thiol and the neighboring thiol on the PMA chain is sufficiently fast to establish a disulfide bridge between the polymer chains.

Since there is only a single thiol at the terminus of the modified PVPON, it is clear that not all of the PVPON can be retained on the surface of the capsules.

Biofunctionalization of PMA HCs. We next investigated the potential of generating two biorelevant end groups for biofunctionalization of the capsule surface by incorporating biotin into PVPON (PVPON_{biotin}). We chose to conjugate this small biomolecule (also known as vitamin H) because of the highly specific interaction and high affinity with avidin²⁷ and its derivatives, such as NeutrAvidin (NAv). The use of biotinylated antibodies and their specific interaction with avidin for tumor targeting is reported elsewhere.^{44,45} PMA HCs were assembled with PVPON_{biotin} on the surface of the capsules, followed by incubation with FITC fluorescently labeled NeutrAvidin (NAv-FITC). The effect of the molecular weight of PVPON_{biotin} on the interaction between the biotinylated capsules and the fluorescent protein was monitored by flow cytometry and fluorescence microscopy (Figure 3a). Flow cytometry analysis of the capsules showed decreasing fluorescence with increased molecular weight of the polymer on the surface. This suggests that the presence of higher molecular weight modified PVPON on the surface results in a smaller number of functional end groups for interaction with the NAv-FITC. The control capsules that lack biotin showed minimal fluorescence arising from NAv-FITC (Figure 3b (D,E)), suggesting negligible nonspecific interactions between the NAv and the surface of the capsules.

Monoclonal antibodies that can selectively target cancer cells have been exploited for effective cancer therapies.⁴⁶ Studies have shown that nanoengineered drug carriers enhance efficacy by increasing the effective concentration of the payload compared to free drug.¹³ Therefore, biofunctionalization of drug carriers with monoclonal antibodies (mAb) is expected to further improve the therapeutic index by specific targeted delivery. We previously reported on the incorporation of antibodies on the surface of PMA-coated silica particles through electrostatic interaction,²⁶ but this approach is only feasible when charged molecules are present on the surface. To overcome this limitation, herein we developed a versatile method to functionalize PMA HCs with monoclonal antibodies *via* the strong interaction between NeutrAvidin and biotin. The PMA/PVPON_{biotin} HCs were incubated with the NeutrAvidin/biotin-IgG complex (ratio 1:2). To verify the coupling with IgG, the capsules were further incubated with secondary (Fab specific) fluorescent IgG-FITC antibodies. To distinguish the capsules from the FITC-labeled protein, PMA/PVPON_{biotin} HCs were AF633 fluorescently (red) labeled using the remaining thiol groups on the capsules and AF633 maleimide dye. As shown in Figure 4a, the AF633-labeled PMA/PVPON_{biotin} HCs exhibited strong green fluorescence arising from IgG-FITC. In contrast, the AF633-labeled PMA/PVPON

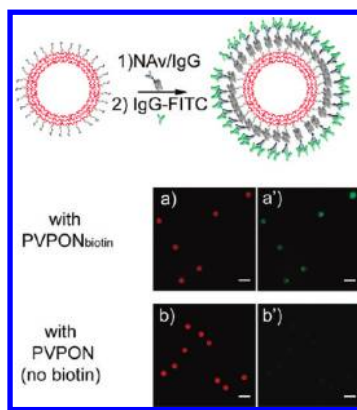


Figure 4. Incorporation of primary IgG onto the surface of the PMA/PVPON_{biotin} capsules followed by verification with secondary FITC fluorescently labeled IgG. (a) AF633 dye-labeled PMA HCs (red) with attached 7 kDa biotin-PVPON, (b) AF633 dye-labeled PMA HCs with attached 7 kDa PVPON without biotin. Green fluorescence originated from secondary IgG-FITC (a',b'). The AF633-labeled PMA/PVPON_{biotin} HCs exhibited strong green fluorescence arising from IgG-FITC. In contrast, the AF633-labeled PMA/PVPON HCs (without biotin) showed negligible levels of green fluorescence. The scale bars are 10 μ m.

HCs (without biotin) showed negligible levels of green fluorescence. From the known number of PVPON chains on the surface, and assuming that each PVPON chain incorporates one complex, the number of attached antibodies is calculated to be between $(2-20) \times 10^4$ Ab per capsule, depending on the linker length (Table S1). To demonstrate the targeting effects using the antibody-functionalized PMA/PVPON_{biotin} HCs, two monoclonal antibodies (mAb), humanized A33 mAb (huA33 mAb) and the anti-epidermal growth factor receptor mAb (EGFR mAb), were used individually to functionalize the AF633-labeled PMA/PVPON_{biotin} HCs. A human colorectal cancer cell line, LIM1899,⁴⁷ was chosen for incubation with the Ab-functionalized capsules, as these cells endogenously express both A33 and EGF receptors on the cell surface.³³ First, to evaluate the cell surface binding, Ab-functionalized capsules were incubated with LIM1899 cells at 4 °C for 1 h with a capsule-to-cell ratio of 100:1. Incubation at this temperature (4 °C) prevents cellular internalization by endocytosis. Following this treatment, the unbound capsules were removed and the cells were washed with PBS twice and then analyzed by flow cytometry. In the case of huA33-functionalized capsules, three different lengths of PVPON_{biotin} were tested as a linker to functionalize the capsules, as chain length can be crucial for efficient interaction between the antigen and antibody. After the incubation, over 90% of cells were associated with capsules for all three types of huA33 mAb-functionalized PMA/PVPON_{biotin} HCs, and in contrast, significantly less cellular association was demonstrated for the corresponding IgG-functionalized PMA/PVPON_{biotin} HCs (Figure 5a). It is also worth noting that the highest mean fluorescence intensity of

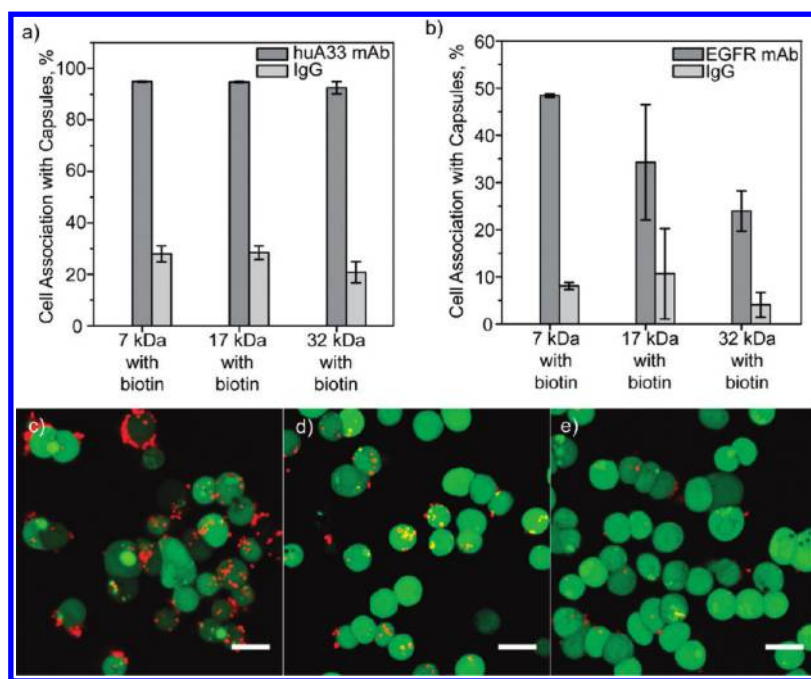


Figure 5. Selective binding of targeted PMA/PVPON_{biotin} HCs to cancer cells. (a) Flow cytometry analysis of huA33 mAb-functionalized capsule association with LIM1899 colorectal cancer cells. (b) Flow cytometry analysis of EGFR mAb-functionalized capsule association with LIM1899 cells. (c–e) Representative CLSM images of cellular uptake of Ab-functionalized capsules: CellTracker green CMFDA stained LIM1899 cells incubated with (c) huA33 mAb-, (d) EGFR mAb-, and (e) IgG-functionalized PMA/PVPON_{biotin} HCs (red labeled with AF633). The EGFR mAb-functionalized capsules were inside the cells as evidenced by colocalization of the red and green fluorescent signals (yellow). The scale bars are 20 μ m.

the cells was shown when the 17 kDa PVPON_{biotin} was used, suggesting that the length of the linker plays an important role in the maximal binding between A33 antigen and huA33 mAb on the surface of the capsules (Figure S5). To demonstrate the general applicability of this approach, anti-EGFR mAb was used to functionalize the PMA/PVPON_{biotin} HCs. First, the capsules were functionalized with the antibody with linkers of different length. The cells were then incubated with the functionalized capsules at 4 °C for 1 h with a capsule-to-cell ratio of 100:1, followed by analysis using flow cytometry. Consistent with huA33 mAb-functionalized capsules, all three types of EGFR mAb-functionalized capsules showed enhanced binding on the cell surface compared to IgG-functionalized capsules (Figure 5b). Similarly, the percentage of cells associated with EGFR mAb-functionalized capsules was dependent on the length of the linker used since the cellular binding with the capsules decreased as the length of the linker increased (Figure 5b). Furthermore, it is worth noting that the highest percentage of cells associated with huA33 mAb-functionalized capsules was about 90%, whereas the highest percentage of cells associated with EGFR mAb-functionalized capsules was about 50%, suggesting that the targeting effect is also strongly dependent on the specific antibody.

Following the specific binding of the antibody-functionalized capsules with the cell membrane, we sought to investigate the cellular uptake of these functionalized capsules. On the basis of the binding results,

we chose to use 7 kDa PVPON_{biotin} to couple with antibodies, as it was most effective in targeting cells. First, the LIM1899 cells were stained with CellTracker green CMFDA to outline the entire cell. The AF633-labeled Ab-functionalized PMA/PVPON_{biotin} HCs (huA33 mAb-, EGFR mAb-, and IgG-functionalized capsules) were then incubated with the cells at 37 °C for 1 h to allow for internalization to occur. After treatment, the cells were washed with PBS to remove excess capsules and analyzed by confocal microscopy. Both huA33 mAb-functionalized and EGFR mAb-functionalized capsules showed stronger cellular association (including cell surface binding and cell uptake) compared with IgG-functionalized capsules (Figure 5c–e), in good agreement with cellular binding results obtained by flow cytometry. It was also notable that whereas huA33 mAb-functionalized capsules were strongly associated with the cell membrane, the EGFR mAb-functionalized capsules were inside the cells, as evidenced by colocalization of the red and green fluorescent signals. This suggests that the kinetics of internalization are different when the capsules are functionalized with two different antibodies. It has been reported that huA33 antigen may be associated with cell adhesion complexes, such as tight junctions, and huA33 mAb exhibits prolonged membrane retention.⁴⁸ In contrast, a study has shown that, upon binding of ligand to the EGFR on the surface of the cells, the complex aggregates into clusters and is rapidly internalized.⁴⁹ Consistent with these findings, PMA/PVPON_{biotin} HCs with

huA33 mAb stay predominantly on the cell surface, and only small portions are further internalized. In comparison, EGFR mAb-functionalized capsules are internalized by the cells to a larger extent.

Taken together, our results have shown that the functionalization of capsules with monoclonal antibodies leads to specific interactions of capsules with cells *via* antibody–antigen recognition. The interactions between Ab-functionalized capsules and cells are a complex phenomenon that depends on several variables, including the expression level of antigen on the cell surface, the coverage of antibodies on the capsule surface, the accessibility of antigens and antibodies, as well as cell-intrinsic properties. Future studies will be aimed at obtaining detailed knowledge of each variable to achieve optimized targeting effects for specific antibodies.

CONCLUSION

This paper demonstrates a generic and facile method for functionalization of disulfide-stabilized hydrogel

capsules (HCs) with macromolecules using a heterotelechelic linker, poly(*N*-vinyl pyrrolidone) containing thiol and amine end groups. Our data reveal that the number of chains attached to the surface can be easily controlled by the molecular weight of the linker, consequently controlling the amount of protein immobilized on the capsules. To demonstrate the general applicability of this functionalization protocol, two monoclonal antibodies were individually coupled to PMA/PVPON_{biotin} HCs. The antibody-functionalized capsules demonstrate significantly enhanced cellular targeting effect compared to control human immunoglobulin (IgG)-functionalized capsules. Moreover, our data demonstrate that the different antibodies on the capsules lead to different internalization efficiencies, suggesting that the antibody–antigen interaction actively mediates the cellular uptake of capsules. Given the versatility of this functionalization approach, these disulfide-bonded capsules have the potential for a wide range of diagnostic and therapeutic applications.

MATERIALS AND METHODS

Materials. 1-Vinyl-2-pyrrolidone was purchased from Aldrich and was purified prior to use by filtering through a column of basic alumina. 2,2'-Azobis(isobutyronitrile) (AIBN), dithiothreitol (DTT), 5,5'-dithiobis-2-nitrobenzoic acid (Ellman's reagent) (ER), 2-(*N*-morpholine)ethane sulfonic acid (MES), 3-(*N*-morpholino)propanesulfonic acid (MOPS), *N*-chloro-*p*-toluenesulfonamide sodium salt (chloramine T), poly(*N*-vinylpyrrolidone) (PVPON, MW = 10 000 g mol⁻¹), dimethyl sulfoxide (DMSO), dioxane, sodium borohydride (NaBH₄), sodium phosphate buffer saline (PBS), and sodium acetate were purchased from Sigma-Aldrich and were used as received. Hydrochloric acid (HCl), sodium hydroxide (NaOH), and acetone were obtained from Merck. Hydrazine hydrate and diethyl ether were purchased from BDH. Pyridine dithioethylamine hydrochloride (PDA-HCl) was obtained from Shanghai SpeedChemical Co Ltd. Alexa Fluor 488 C5 maleimide (AF488) and Alexa Fluor 633 C5 maleimide (AF633) reactive dyes were purchased from Invitrogen. Poly(methacrylic acid) (PMA, MW = 15 000 g mol⁻¹) was purchased from Polysciences Inc. and was used as received. Silica particles (1.04 μm diameter) were purchased from MicroParticles GmbH, Germany. High purity water with a resistivity greater than 18 MΩ·cm was obtained from an in-line Millipore RiOs/Origin system (Milli-Q water).

The humanized A33 monoclonal antibody (huA33 mAb) and Mu528 IgG2a anti-EGFR antibody were produced in the Biological Production Facility Melbourne—Austin Branch of the Ludwig Institute for Cancer Research, Australia, and stored in phosphate buffered saline (PBS, pH 7.2) containing 15 mM sodium azide. Anti-human IgG (Fab specific)-FITC antibody produced in goat was obtained from Sigma-Aldrich as a solution in 0.01 M PBS, pH 7.2, containing 15 mM sodium azide.

PVPON Synthesis and Modification. The phthalimidomethyl xanthate RAFT agent and the corresponding RAFT-synthesized PVPON were obtained as previously described.³⁵ The polymers were analyzed using ¹H NMR and GPC, the results of which are shown in Table 1.

The terminal phthalimide group of the RAFT-synthesized PVPON was converted into a terminal primary amine group by hydrozinolysis. Then, 250 μL of hydrazine hydrate (BDH) was added to a 100 g L⁻¹ solution of RAFT-derived PVPON in methanol, and the resulting mixture was stirred at 60 °C overnight. The excess hydrazine hydrate was neutralized by careful

addition of 37% HCl to adjust it to neutral pH. The _{NH2}PVPON was isolated by precipitation into acetone, filtered, and dried under vacuum overnight.

To ensure full transformation of the thiocarbonylthio end group to a thiol, the RAFT-derived polymer or PVPON after hydrozinolysis was dissolved in 1 M NaBH₄ (Sigma-Aldrich) solution in water, and the reaction mixture was incubated at room temperature (25 °C) for 2 h. The excess sodium borohydride was neutralized by careful addition of 37% HCl and was adjusted to pH 7.5 by careful addition of 1 M HCl and NaOH solutions while buffered by the addition of phosphate buffer (0.1 M). To this mixture was added an excess of 0.5 M Ellman's reagent (ER, Sigma-Aldrich) in 0.1 M phosphate buffer at pH 7.5, and the reaction was allowed to proceed for 1 h. The resulting polymer (_{NH2}PVPON_{ER}) was isolated using a NAP-25 column (GE Healthcare) and recovered by freeze-drying. The introduction of the 2-nitro-5-thiobenzoate (NTB) moiety is for the purposes of prolonged storage of the polymer and its characterization.

Fluorescent labeling of _{NH2}PVPON_{ER} was carried out by incubating 10 mg of polymer in phosphate buffer saline (0.1 M Na₂HPO₄, 0.15 NaCl, pH 7.2) with 10 μg of 1 g L⁻¹ Alexa Fluor 488 carboxylic acid succinimidyl ester dye (AF488NHS, Invitrogen) in DMSO. The reaction between the primary amine end group and *N*-succinimidyl ester on the fluorescent dye was allowed to proceed for 2 h, after which the polymer was purified using a NAP-5 column (GE Healthcare) and recovered by freeze-drying.

Biotinylation of the polymer was carried out by incubating 10 mg of the _{NH2}PVPON_{ER} in 1 mL of phosphate buffer saline (0.1 M Na₂HPO₄, 0.15 NaCl, pH 7.2) with a 20-fold molar excess of 0.1 mM Sulfo-NHS-LC-Biotin (Pierce). The reaction between the primary amine on the polymer end group and NHS on the biotin was allowed to proceed for 2 h, after which the polymer was purified *via* column chromatography using a NAP-5 column (GE Healthcare) and recovered by freeze-drying. Biotin incorporation in the polymer was confirmed using a EZ-Link Sulfo-NHS-LC-Biotinylation kit (Pierce). Biotinylated polymer was added to a mixture of 2-(4-hydroxyphenylazo)benzoic acid (HABA, Aldrich) and avidin (Pierce), and the unknown amount of biotin in a solution was estimated by measuring the absorbance of the HABA-avidin solution, before and after addition, at 500 nm using an Agilent 8453 diode-array UV–vis spectrophotometer. The change in absorbance relates to the amount of biotin in the sample and is calculated as moles of biotin per mole of polymer.

Multilayer Assembly on Particles. The assembly of PMA HC was performed using a 5 wt % particle suspension (MicroParticles, GmbH) in a similar way to that reported previously.¹¹ For multilayer assembly, PMA with a thiolation degree of 12%⁹ and PVPON (commercially available (Sigma-Aldrich) or RAFT-synthesized PVPON (RAFT-PVPON)) were used.

Biotinylation of Antibodies. Biotinylation of antibodies was carried out according to the procedure provided with the EZ-Link Sulfo-NHS-Biotinylation kit (Pierce). This procedure was applied to every type of antibody used in this work. In particular, the aliquot of immunoglobulin (IgG, Sigma-Aldrich) antibody in PBS was mixed with 5 × molar excess of 10 mM Sulfo-NHS-Biotin (Pierce) in water to obtain no more than two biotin groups per antibody molecule. The mixture was kept under constant mixing at 4 °C overnight. The next day the biotinylated antibodies were recovered using Zeba Desalt spin columns (Thermo Scientific) according to the manufacturer's instructions. Estimation of biotin incorporation into the antibody was determined according to the procedure provided with the EZ-Link Sulfo-NHS-Biotinylation kit in the same way as described above for the polymer.

Functionalization of the Capsule Surface. SiO₂ particles were assembled with multilayers as described above, except for the last layer, for which biotinylated PVPON was deposited. The rest of the hollow capsule formation process was followed as reported previously.¹¹ Following capsule formation, capsules redispersed in 50 μ L of MES buffer (50 mM, pH 6) were fluorescently labeled by mixing them with 5 μ L of 1 g L⁻¹ Alexa Fluor 633 maleimide in DMSO followed by overnight storage. Fluorescently labeled capsules were isolated from the free dye by rinsing three times with MES buffer (50 mM, pH 6). The capsules were then functionalized using the biotin-NeutrAvidin (NAv, Pierce) interaction. In particular, 20 μ L of the sample was diluted 10 times with PBS and incubated with a large excess of premixed NeutrAvidin/antibody complex to prevent capsule aggregation for 1 h at 4 °C. The complex was prepared by rapidly mixing 2 parts of antibody (0.1 g L⁻¹) with 1 part of NAv (0.1 g L⁻¹). Excess NAv/antibody was removed through centrifugation/redispersion (4 cycles of 3500g, 5 min). Taking into account the known number of PVPON chains on the surface, and assuming that each PVPON chain incorporates one complex, the number of antibodies can be calculated for each sample (Table S1). However, the final number of antibodies will exceed that calculated since all samples were mixed with an excess of biotinylated antibodies to prevent capsule aggregation. To estimate quantitatively the number of antibody molecules immobilized on the surface of the capsules was not possible due to a large excess of antibody used.

Cell Culture. The human colorectal cancer-derived cell line LIM1899⁴⁷ was maintained in RPMI media (Gibco) containing 10% fetal bovine serum (FBS) and ADDS (10.8 μ g mL⁻¹ α -thioglycerol, 0.025 U mL insulin, 1 μ g mL⁻¹ hydrocortisone) at 37 °C in a 5% CO₂ humidified atmosphere and subcultured prior to confluence using trypsin/EDTA.

Capsule Binding to Cells. Cells were suspended in PBS after trypsinization. An aliquot of the cell suspension (2×10^5 cells) was incubated with IgG-, huA33 mAb-, or EGFR mAb-functionalized AF633-labeled capsules at a ratio of 100 capsules to 1 cell at 4 °C for 1 h. After incubation, cells were washed with PBS twice. The cell pellet was resuspended in 1 mL of PBS and analyzed by flow cytometry. The percentage of cells associated with capsules was determined by the acquisition of the signal from AF633. Analysis was performed using FlowJo v8.1 (TreeStar) software.

Capsule Imaging. Differential interference contrast (DIC) images were acquired using an inverted Olympus IX71 microscope equipped with a DIC slider (U-DICT, Olympus) with a 60 × oil immersion objective lens.

Capsule Cellular Uptake. LIM1899 cells were plated at 8×10^4 cells/well into the wells of an 8-well Lab-Tek I chambered coverglass slide (Thermo Fisher Scientific) and allowed to adhere overnight. The cells were then labeled with CellTracker green CMFDA (10 μ M) according to the manufacturer's protocol. Subsequently, the cells were incubated with Ab-functionalized PMA/PVPON_{biotin} HCs (labeled with AF633) for 1 h (37 °C, 5% CO₂), followed by three washes with PBS. Optical sections

were acquired with a Leica laser-scanning confocal microscope (TCS SP2, Leica, Germany).

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Supporting Information Available: ¹H NMR spectra of the modification of the end groups of RAFT-derived PVPON; UV-vis spectra of the PVPON with a xanthate end group; multilayer buildup; effect of PMA thiolation on PVPON retention on the surface of HCs; fluorescence associated with huA33 mAb- and IgG-functionalized PMA/PVPON_{biotin} HCs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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