ABSTRACT: The hepatitis B virus-like particle (HBV VLP) is an attractive protein nanoparticle platform due to the availability of 240 modification sites for engineering purposes. Although direct protein insertion into the surface loop has been demonstrated, this decoration strategy is restricted by the size of the inserted protein moieties. Meanwhile, larger proteins can be decorated using chemical conjugations; yet these approaches perturb the integrity of more delicate proteins and can unfavorably orient the proteins, impairing active surface display. Herein, we aim to create a robust and highly modular method to produce smart HBV-based nanodevices by using the SpyCatcher/SpyTag system, which allows a wide range of peptides and proteins to be conjugated directly and simply onto the modified HBV capsids in a controlled and biocompatible manner. Our technology allows the modular surface modification of HBV VLPs with multiple components, which provides signal amplification, increased targeting avidity, and high therapeutic payload incorporation. We have achieved a yield of over 200 mg/L for these engineered HBV VLPs and demonstrated the flexibility of this platform in both biosensing and drug delivery applications. The ability to decorate over 200 nanoluminases per VLP improved detection signal by over 1500-fold, such that low nanomolar levels of thrombin could be detected by the naked eye. Meanwhile, a dimeric prodrug-activating enzyme was loaded without cross-linking particles by coexpressing the modiﬁcation sites for engineering purposes. Although direct protein insertion into the surface loop has been demonstrated, this decoration strategy is restricted by the size of the inserted protein moieties. Meanwhile, larger proteins can be decorated using chemical conjugations; yet these approaches perturb the integrity of more delicate proteins and can unfavorably orient the proteins, impairing active surface display. Herein, we aim to create a robust and highly modular method to produce smart HBV-based nanodevices by using the SpyCatcher/SpyTag system, which allows a wide range of peptides and proteins to be conjugated directly and simply onto the modified HBV capsids in a controlled and biocompatible manner. Our technology allows the modular surface modification of HBV VLPs with multiple components, which provides signal amplification, increased targeting avidity, and high therapeutic payload incorporation. We have achieved a yield of over 200 mg/L for these engineered HBV VLPs and demonstrated the flexibility of this platform in both biosensing and drug delivery applications. The ability to decorate over 200 nanoluminases per VLP improved detection signal by over 1500-fold, such that low nanomolar levels of thrombin could be detected by the naked eye. Meanwhile, a dimeric prodrug-activating enzyme was loaded without cross-linking particles by coexpressing orthogonally labeled monomers. This along with a epidermal growth factor receptor-binding peptide enabled tunable uptake of HBV VLPs into inflammatory breast cancer cells, leading to efficient suicide enzyme delivery and cell killing.

KEYWORDS: protein nanoparticles, bioconjugation, modular, biosensing, drug delivery

Nanotechnology has taken great strides in creating a new generation of sensing and therapeutic tools. The small size of nanomaterials allows them to interact with targets on a molecular level and provides unique optical readouts for highly sensitive bioassays. Additionally, sizes are optimal for both promoting passive accumulation in tumor tissues through the enhanced permeability and retention effect and for targeting cell uptake through receptor-mediated endocytosis. Protein nanoparticles are a particularly attractive material for biosensing and delivery applications, as they are made of biocompatible protein building blocks that self-assemble into uniform structures that can withstand harsh chemical and physical conditions. Their small size in conjunction with their multunit makeup provides a large surface area to volume ratio on which functional components may be attached to the individual building blocks and thereby presented at a high local density. This multivalent capability is highly desirable for biosensing and delivery applications, as it provides valuable properties, such as signal amplification for reporter domains, avidity of targeting moieties for enhanced cell binding, and high payloads of therapeutic outputs. A "plug and play" protein nanoparticle that can be customized for a variety of applications would be highly valuable; however, an efficient and biocompatible method for assembling multiple functions onto a single protein nanoparticle is crucial for optimal performance.

Many chemical and biological techniques have been utilized in modifying protein nanoparticles with functional components. Traditional chemistries using reactive amino acid side groups, however, are nonspecific and do not provide control over the orientation in which a functional protein is displayed on the nanoparticle. Unnatural amino acids (UAAs) have been incorporated into protein nanoparticle monomers to provide bioorthogonal reactive groups for site-specific conjugation; however, protein expression yields are limited by UAA incorporation efficiency, and additionally, protein nanoparticle surface charge affects conjugation efficiency.

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Furthermore, solution conditions for some of these chemical conjugation strategies may be unfavorable for more fragile protein components. Biological methods overcome these concerns as they take place under benign, physiological conditions. Genetic fusions to termini or flexible loops in self-assembling monomers can be used for the incorporation of foreign domains into protein nanoparticles; however, not all protein nanoparticles can tolerate the insertion of large native protein structures. For those that can tolerate the addition of small peptides, enzymatically mediated, site-specific protein conjugation strategies, such as sortases and SpyCatcher/SpyTag, can be utilized. Recently the SpyCatcher/SpyTag system has gained particular attention for its fast kinetics in a robust range of solution conditions. By simply mixing the 15 kDa SpyCatcher protein with the 13-amino-acid SpyTag, the two protein components spontaneously form an isopeptide bond between the active lysine and aspartic acid residues. Unlike sortases, the SpyCatcher reaction is irreversible, and thus a higher extent of conversion can be achieved. SpyCatcher/SpyTag has been exploited in a variety of protein nanoparticle platforms ranging in size and number of possible conjugation sites from 12 nm ferritin cages displaying 24 copies of SpyCatcher to 56 nm AP205 bacteriophages displaying 420 SpyTags. These have been applied to modular vaccines, biocatalytic nanocompartments, and nonchromatographic IgG purification. More recently, customizable nanoparticle sensors and drug delivery vehicles are beginning to be explored. Fusing the SpyCatcher to the nanoparticle scaffold is of particular interest in order to minimize the perturbation to complex decorations. In pursuit of this modular toolset, the Spy&Go affinity purification system was developed for the simple separation of SpyTagged moieties. Current nanoparticle platforms, however, are limited to insertions at exposed termini or only small peptide insertions into interior loops. Termini located at axes of symmetry or assembly interfaces restrict the insertion size to the SpyTag; further, the accessibility to the conjugation sites becomes hindered, lowering the decoration capacity. While the 180 N-termini for the 29 nm AP205 bacteriophage are well distributed across the capsid surface, the reported yields of AP205-SpyCatcher have been low (1.1 mg/L). A SpyCatcher nanoparticle platform that can display a large number of accessible SpyCatcher proteins and be produced in high yields, therefore, is desirable.

The hepatitis B viral-like particle (HBV VLP) is particularly attractive for the development of vaccine and drug delivery platforms, as the spikey exterior provides highly accessible locations for surface modifications using many of the chemical and biological strategies described above. The HBV VLP is assembled from 240 copies of the core-forming protein HBc, which dimerizes, then polymerizes into a 34 nm icosahedral capsid, providing a large number of modification sites for multifunctionalization. The four-helix bundles of the HBc dimer make up the surface spikes. These have been modified through insertion of foreign domains into the flexible c/e1 loop, residues 74–84, exposed at the tips, where even small native proteins have been inserted into the loop without impacting capsid formation. The range of proteins that can be inserted, however, is limited by the geometry of the foreign domain. This has been alleviated through the SplitCore version of the HBV, where the N (residues 1–79) and C (residues 80–183) core fragments are expressed as a split protein so that new C’ and N’ termini are generated at the loop area and are available for genetic fusion. This split complementation format, however, lowers protein nanoparticle yields.

Herein, we present a modular nanocarrier platform using the HBV VLP, where SpyCatcher is inserted into the c/e1 loop. These particles are able to be produced in E. coli shake flasks in high yields. Various functional components fused to a SpyTag can be conjugated onto the surface by simply mixing with our HBV-SpyCatcher VLPs (Figure 1). We show that the HBV-SpyCatcher can be decorated in a versatile fashion with close to 240 functional moieties, making this approach ideal for...
transforming HBV-SpyCatcher into a modular platform capable of facile customization with different targeting and detection components. To demonstrate this flexibility, we recruited different protein components to create highly tunable VLP devices for either high-sensitivity immuno-detection or receptor-mediated drug delivery into inflammatory breast cancer cells. The ability to tune the decoration density allows highly modular control of signal amplification, cell uptake efficiency, and cancer cell killing using delivered yeast cytosine deaminase (yCD) for prodrug activation.

RESULTS AND DISCUSSION

The HBV core protein, HBc, is amenable to genetic fusions within the exposed, c/e1 epitope loop, as long as the guest protein has compatible geometry where the N and C termini are close in proximity.38 Given this design consideration, we argue that the 9 kDa minimized SpyCatcher protein39 should fit within the c/e1 loop without disrupting VLP assembly. To test this possibility, SpyCatcher flanked by flexible linkers36 was inserted between residues 78 and 81 of HBc149, the truncated core protein lacking the C-terminal RNA-binding protamine domain. The resulting HBV-SpyCatcher fusion protein was expressed in E. coli, and the soluble cell lysate was examined by SDS-PAGE (Figure 2A, lane 1). A band corresponding to the size of the HBV-SpyCatcher fusion protein was detected, making up on average 25% of the total soluble cell lysate. The soluble fraction of the lysate was further examined through sucrose gradient sedimentation, where intact HBV particles have been observed to sediment into fractions 7–10.40 The HBV-SpyCatcher band was observed to fall tightly within fractions 8–11 (Figure S1A), suggesting that the fusion protein likely retained the ability to assemble into VLPs. VLP formation was further verified by pooling fractions 8–11 and imaging by transmission electron microscopy (TEM) (Figure 2B). Particles with an average size of 33.5 ± 0.7 nm diameter (p = 0.01) (Figure 2C) were observed, confirming the formation of intact HBV-SpyCatcher hybrid VLPs.

To demonstrate that the SpyCatcher displayed on the VLP surface remains active, HBV-SpyCatcher soluble cell lysate was mixed with soluble cell lysate containing SpyTag-GFP (Figure 2A, lane 2) at a three to one volume ratio, and a sample was taken directly after mixing (Figure 2A, lane 3). The appearance of a larger, conjugated band (58.4 kDa), after allowing the reaction to complete, confirmed that the SpyCatcher was functional (Figure 2A, lane 4). The reaction product was further examined through sucrose gradient sedimentation. Both HBV-GFP and unreacted HBV-SpyCatcher co-sedimented in fractions 9–12 (Figure S1B). Green fluorescence also was visible within the lower portion of the HBV-GFP sucrose gradient, whereas without the HBV, the GFP remained at the top (Figure 2D). Intact VLPs were again detected by TEM from the pooled fractions 9–12 (Figure 2B). Consistent with the surface decoration of GFP, the average diameter of 36.3 ± 0.7 nm (p = 0.01) was larger than that of the wild-type VLP (p < 0.01) (Figure 2C), and the surface morphology appeared more irregular than the undecorated HBV-SpyCatcher VLPs.

For subsequent studies, HBV-SpyCatcher VLPs were first partially purified through ammonium sulfate precipitation at 20% saturation. A sucrose gradient of the ammonium sulfate precipitated HBV-SpyCatcher VLPs can be found in Figure S2A. The average estimated yield of VLPs after precipitation was 220 mg of protein per liter of culture. This yield is almost 10-fold greater than previously reported SpyCatcher-mi3 cAGE.41 It is possible that the flexibility of presenting the SpyCatcher in a loop rather than the termini allows VLPs to assemble in high yields.

Given the 240 available modification sites on the HBV-SpyCatcher VLP, it is an ideal platform for biosensing as many output components as can be localized to the surface (Figure 1) to provide signal amplification that is far greater than what has been reported for similar nanoparticle biosensor platforms, such as apoferritin (24 sites),7 SpyCatcher polymer (30 sites),41 and E2 (60 sites).3 To create a modular strategy to incorporate any antibody of interest as the sensing domain, a small, antibody-binding Z-domain was used to recruit the desired IgG by binding to the Fc region with nanomolar affinity.42 To display the Z-domain on the HBV-SpyCatcher VLPs without any steric hindrance from a dense layer of reporter outputs, the Z-domain was fused to the N-terminus of a 40-repeat elastin-like polypeptide (ELP), and a SpyTag was fused to the C-terminus. This design not only allows Z-ELP-SpyTag to serve as an input sensor domain but also adds the flexibility to further polish the VLPs. Thermally responsive ELP biopolymers undergo a reversible phase transition, providing
simple purification through cycles of thermal precipitation and resolubilization.\textsuperscript{43} E2 protein nanocages sortase-ligated with ELP have been purified using this method.\textsuperscript{19,44,45} For the reporter domain, a small (22.2 kDa), monomeric nanoluciferase (NanoLuc) from \textit{Oplophorus gracilirostris} was utilized to provide high sensitivity for an ELISA-like detection scheme.\textsuperscript{5}

To evaluate the conjugation capacity of the 240 available SpyCatcher sites, HBV-SpyCatcher VLPs were first conjugated with 5% of the sites to Z-ELP and 5% of the sites to SpyTag-GFP. This yielded about 12 Z-ELP and 12 SpyTag-GFP per nanoparticle, as estimated via densitometry. A sucrose gradient of these VLPs after an ELP polishing step demonstrated intact nanoparticles as shown in Figure S2B. We next decorated with an increasing number of NanoLuc, which yielded VLP probes with a range of 42 to 211 NanoLuc per VLP (Figure 3A). Through SDS-PAGE, it was observed that at the maximum NanoLuc decoration the unmodified HBV band was 98% consumed, demonstrating 235 SpyCatcher sites on the surface were accessible. This result further highlights the advantage of the SpyCatcher/SpyTag chemistry compared to other strategies, such as affinity interactions\textsuperscript{6,7} and even covalent sortase A chemistry,\textsuperscript{5} where decoration with output domains failed to approach the theoretical maximum attachment sites. Compared to other SpyCatcher/SpyTag platforms with high numbers of conjugation sites, the HBV-SpyCatcher outperforms in carrying capacity for similarly sized decorations. For example, P22-WB-SpyTag with 360 possible sites could only accommodate 150 copies of 22 kDa affibodies,\textsuperscript{24} whereas 2xSpyTag-AP205 with 360 possible sites reached 193 copies of a 33 kDa antigen.\textsuperscript{26}

By normalizing the VLP concentrations using the internal GFP standard, the NanoLuc activity of the VLPs was determined through measuring luminescence output. Interestingly, NanoLuc conjugated to the VLPs showed a higher activity than free enzymes at the same concentration (Figure 3B). On average, there was a 2.7-fold activity enhancement. Studies of scaffolded enzymes have shown that immobilization to a scaffold can lead to an increase in activity, which can be attributed to the microenvironment created by the scaffold.\textsuperscript{46−48} The enhancement in activity observed is beneficial, as it can provide an even higher degree of signal amplification than simply increasing the number of NanoLuc.

The NanoLuc sensors were evaluated in an ELISA format for detecting the cancer biomarker thrombin. Human thrombin was coated on 96-well plates and blocked. Figure 3. Increasing copies of NanoLuc on HBV VLPs provide enhanced luminescence for a thrombin ELISA assay. (A) HBV-VLPs were reacted with increasing copies of SpyTag-NanoLuc to create luminescent VLPs. (B) Luminescence of NanoLuc conjugated to HBV-VLPs compared to free NanoLuc at the same concentration was measured, and the error bars represent the standard deviation from three replicates. (C) HBV-VLP probes with increasing copies of NanoLuc were used in an ELISA detection of 5 nM human thrombin. Fold enhancement compared to the 1 input to 1 output control protein (Z-domain-ELP-NanoLuc) is presented with the standard deviation from three replicates. (D) Visual detection of luminescence from the 5 nM human thrombin ELISA assay in both the dark and low light. (E) The detection limit of the 211 NanoLuc HBV is presented with the standard deviation from three replicates. (F) Visual detection limit of the 211 NanoLuc HBV.
Antithrombin antibody was first added followed by 50 nM of the NanoLuc VLPs. Signal amplification was observed compared to a control protein in which the Z-domain was fused directly to a NanoLuc with an ELP linker in the middle (Figure 3C). Compared to the control, the HBV sensor showed up to 1521-fold amplification in signal at 120 copies of NanoLuc per HBV. Beyond 120 copies of NanoLuc the relative luminescence did not increase further due to such high quantities of enzyme bound to wells. The high level of amplification for the HBV-SpyCatcher VLPs enables the detection of 5 nM thrombin by the naked eye using as few as 42 NanoLuc per VLP (Figure 3D). The detection limit for the 211 NanoLuc HBV was 750 pM thrombin (Figure 3E). This exceeded the 2.5 nM detection limit of the Z-ELP-NanoLuc control, increasing the sensitivity by 3.3-fold (Figure S3). Visible detection for the 211 NanoLuc HBV could still be observed in the dark with as little as 1 nM thrombin (Figure 3F). The ability to visibly detect antigens at low nanomolar concentrations is highly useful as a low-cost technology for environmental sensing in many developing countries. Customizing the amount of detection agent to visually detect desired antigens at relevant concentration ranges provides flexibility in adapting this sensor for simple readouts for a variety of targets.

The signal amplification for the HBV-SpyCatcher is much greater than reported for NanoLuc-based E2 and SpyCatcher polymer sensors, and it extends beyond sheer copy number and the 2.7-fold activity enhancement. A key difference is the avidity of the antibody-binding domain compared to the control. The E2 cages displayed five Z-domains with a structured cohesin domain linker compared to a singular Z-domain as a control. In comparison, we are displaying 12 structured cohesin domain linker compared to a singular Z-control. The E2 cages displayed avidity of the antibody-binding domain compared to the polymer sensors, and it extends beyond sheer copy number greater than reported for NanoLuc-based E2 and SpyCatcher polymer sensors. The E2 cages displayed avidity of the antibody-binding domain compared to the polymer sensors, and it extends beyond sheer copy number greater than reported for NanoLuc-based E2 and SpyCatcher polymer sensors. Additionally, the polydispersity of randomly assembled particles creates competition between smaller polymers with fewer copies of NanoLuc and the larger, higher-copy polymers, lowering overall signal compared to uniformly assembled nanocages and VLPs.

The modularity of the HBV-SpyCatcher design also allows easy redecoration of the VLPs as a drug delivery vehicle by incorporating a receptor-targeting moiety, along with a reporter and a cytotoxic output (Figure 1). To demonstrate the facile functionalization of the HBV with binding moieties that could direct it toward any disease target of interest, we chose the phage-selected GE11 peptide, which binds the epidermal growth factor receptor (EGF-R) with an affinity of 22 nM. EGF-R is up-regulated in a number of cancers, and recently GE11 was shown to selectively target EGF-R-positive inflammatory breast cancer (IBC) cells. Furthermore, the uptake efficiency of GE11-labeled protein was shown to increase with clustering multiple copies of the GE11. Given the multivalency of the HBV platform, we hypothesized we could tune the uptake of HBV particles into IBC cells by modulating the conjugation density. To track VLP uptake, we included a GFP reporter. Yeast cytosine deaminase, a cancer suicide enzyme that converts produg 5-FC to chemotherapy 5-FU, was chosen as an output domain.

GE11 peptides were conjugated onto HBV VLPs using a hydrophilic ELP [AV60] backbone chosen for its high transition temperature (55.2 °C) in order to avoid VLP aggregation at higher conjugation densities. Increasing amounts of GE11-ELP (0%, 5%, 10%, and 15% of HBV sites) were conjugated to HBV-GFP VLPs along with control ELPS so that the total amount of conjugated ELP (15% of HBV sites) remained the same for all samples (Figure S4A). IBC cells were treated with equal amounts of VLPs, and cell uptake was measured with flow cytometry. A representative histogram of the sample populations (Figure 4A) shows that while there appears to be some uptake in VLPs without GE11 modification, a significant increase in fluorescence is seen at 5% decoration, demonstrating the enhancement attributed to receptor-mediated endocytosis. Further modification with GE11 provided a proportional increase in the mean fluorescent intensity (Figure 4B). Fluorescent images also were taken to visualize VLP uptake in IBC cells (Figure 4C and Figure S5). Punctate, green spots surrounding DAPI-stained nuclei were observed with increasing green intensity for VLPs with higher GE11 decoration. The ability to tune the amount of uptake by controlling the targeting avidity provides another layer of customization in therapeutic dosing and target specificity, as too high of an avidity can also increase background uptake in low-receptor-expressing noncancer cell types.
Figure 5. Delivery of HBV-yCD to IBC cells for prodrug activation. (A) Representative flow cytometry histograms of IBC cells treated with HBV-VLPs decorated with and without yCD. (B) Mean fluorescence intensities taken from flow cytometry. The error bars represent the standard deviation from three replicates. (C) Fluorescent microscope images of IBC cells treated with HBV-GE11 and HBV-GE11-yCD with images taken with DAPI and GFP filters overlaid. The scale bar is 100 μm. (D) MTT cell viability assay of cells treated with prodrug and active drug controls along with HBV-yCD alone and HBV and 5-FC controls and HBV-yCD with prodrug. The error bars represent the standard deviation from six replicates.

Directed enzyme prodrug therapy has been widely explored in a variety of platforms for targeted activation of chemotherapies in diseased tissues.52,53 yCD is of particular interest in that it converts a nontoxic prodrug molecule into an FDA-approved chemotherapy drug, 5-FU.54 The advantage in using this model enzyme is that prodrug conversion does not depend on endosomal escape, as the prodrug and activated drug are membrane permeable. Given that yCD is a dimer, direct conjugation of SpyTag-yCD dimers with HBV-SpyCatcher VLPs would result in VLP cross-linking. To avoid cross-linking, a strategy was devised by coexpressing SpyTag-yCD with yCD-His6 so that a population of singularly SpyTagged-yCD dimers would assemble (Figure S6A). Dimers containing a His6 tag were recovered through Ni-NTA affinity chromatography. Purified yCD was then reacted with HBV-VLPs at 5% of the total SpyCatcher sites, and the VLPs were ELP purified to remove the non-SpyTagged yCD population (Figure S6B). This simple strategy expands the types of proteins that can be incorporated into the HBV-VLP from monomers to dimers without any cross-linking issues.

HBV VLPs decorated with 15% GE11, 10% GFP, and either 0 or 5% yCD were applied to IBC cells (Figure S4B). Uptake was first measured with flow cytometry to determine if the addition of the yCD had any impact on cell uptake (Figure 5A). Mean fluorescence intensity values of the populations show no significant difference in IBC uptake with the yCD incorporated (Figure 5B), and the fluorescent images of the IBC cells containing HBV-yCD VLPs look similar to the images of IBC cells that had internalized HBV VLPs without yCD (Figure 5C and Figure S7). After confirming delivery, cells were treated with 5-FC. MTT cell viability assays demonstrated prodrug activation in HBV-yCD-delivered, 5-FC-treated cell samples, which exhibited cytotoxicity levels similar to the cells treated with just 5-FU (Figure 5D). Addition of 5-FC alone showed only a small degree of cytotoxicity as compared to the untreated control. HBV samples with prodrug but no yCD produced similar results to the prodrug-only control, and HBV-yCD samples without prodrug were similar to the untreated samples, showing that the HBV carrier is noncytotoxic. These results demonstrate how customized HBV carriers can be designed to deliver active suicide enzymes to cancer cells for treatment. The tailorable nature of the platform would allow for the adaptation of this technology for targeting different receptors, and the number of available conjugation sites provides tunable drug dosing for maximum efficacy.

CONCLUSIONS

In summary, we have developed a modular protein nanoparticle platform that can be easily produced in E. coli in high yields and can self-assemble 23S functional protein moieties through simple mixing. We demonstrated the biologically favorable conditions for protein assembly not only retained functionality but in the case of NanoLuc also enhanced the resulting activity by 2.7-fold due to microenvironment changes through enzyme clustering on the VLP. The significantly higher level of luminescence enabled the naked-eye detection of antigens at low nanomolar concentrations. This visual detection could be useful in the development of environmental sensors for use in the field or point of care diagnostics where visual readouts, without the use of bulky and expensive detection equipment, are needed. More importantly, this property could potentially be exploited with other enzymes for nanobiocatalyst applications.

In addition to biosensing, the modularity of our strategy allows easy customization toward cell targeting and drug delivery. Tunable drug delivery vehicles were created where the amount of cell uptake could be controlled by varying the targeting ligand density. The modular platform not only has potential for incorporating different targeting ligands but also could be used for incorporating multiple targeting ligands at controlled ratios for enhanced specificity in cell engagement.31,55 We further demonstrated that we could attach active cargo for directed enzyme prodrug therapy. Since only 12 copies of yCD are necessary to achieve the equivalent performance to free 5-FU, there is ample room for the incorporation of other types of functional moieties including endosomal escaping peptides and stealth agents. HBV is a particularly promising vehicle for drug delivery, as protein cargos also can be fused to the C-terminus to enable interior loading.36 Previously, an siRNA-binding protein was incorpated to encapsulate 240 copies of siRNA57 and more recently, a doxorubicin-binding peptide was presented in the interior for high drug loading.38 These strategies in combination with the presented exterior decoration platform could be used to craft more advanced carriers for drug delivery applications.
However, given the interest of HBV VLPs as vaccine carriers, this platform could serve as a more modular and efficient assembly strategy for those applications as well.

**METHODS**

Materials. All genetic manipulation was performed in *E. coli* strain NEBst (New England Biolabs, Ipswitch, MA, USA) ([Cam 2, 2017] α-galactosidase) and a pET24a vector containing NcoI and SacI at the upstream and primers NcoI-NcoreFOR and SacI-Ccore149-H6_REV. The Z-domain was inserted using Gibson Assembly. The Z-ELP plasmid was digested with NcoI and NdeI, and ELP[AV-60] was inserted between NdeI and NcoI sites cloned upstream NdeI where the Z-domain was inserted. The final construct was cloned between sites NdeI and XhoI of the pET24a vector, using an annealed and phosphorylated primers NdeI-A(AAAAK)\_A-BamHI\_FOR and BamHI-A(AAAAK)\_A-NdeI\_REV. GFP was amplified from primers BamHI-GFP\_FOR and Xhol-GFP\_REV and ligated between BamHI and Xhol so that the His6-Tag in the pET24a vector was in frame. SpyTag-NanoLuc was cloned from the SpyTag-ELP [KVF8-40] plasmid by amplifying the NanoLuc with primers Ndel-NanoLuc\_FOR and Xhol-NanoLuc\_REV, digesting the product and SpyTag-ELP [KVF8-40] plasmid with Ndel and Xhol, and ligating the two together.

SpyTag-yCD was cloned into a pCDS-1b vector that previously contained a SpyTag between Ncol and Ndel, a (G4S)3 linker between Ndel and EcoRI, and another gene between EcoRI and Xhol. The yCD was amplified using primers EcoRI-yCD\_FOR and XhoI-FLAG-yCD\_REV. Both the product and pCDS-1b vector were digested with EcoRI and Xhol and ligated together. The yCD-His6 construct was made by amplifying yCD with primers Ndel-yCD\_FOR and XhoI-yCD\_REV, digesting the product and vector pET24a with Ndel and Xhol and ligating the two together.

**Protein Expression.** HBV-SpyCatcher, SpyTag-ELP-GFP, SpyTag-NanoLuc, and SpyTag-ELP constructs were grown in Terrific Broth (TB) supplemented with 50 μg/mL kanamycin. SpyTag-GFP was grown in TB supplemented with 100 μg/mL ampicillin. The SpyTag-yCD and yCD-His6 constructs were coexpressed in TB with 50 μg/mL kanamycin and 50 μg/mL streptomycin. HBV-SpyCatcher was expressed in 125 mL shake flasks with 30 mL of media. SpyTag-ELPs, SpyTag-NanoLuc, and SpyTag-yCD/yCD-His6 were expressed in 60 mL shake flasks with 25 mL of media. ELPs were expressed in 1 L Erlenmeyer flasks with 100 mL of media. All cultures were grown at 37 °C and 250 rpm to an OD600 of 0.7–1.0, after which, HBV-SpyCatcher was expressed overnight at 20 °C and 250 rpm using leaky T7 expression. ELP constructs were expressed overnight at 37 °C and 250 rpm using leaky T7 expression. SpyTag-NanoLuc was expressed overnight at 25 °C and 250 rpm using 200 μM IPTG, and SpyTag-ELP constructs were expressed overnight at 20 °C and 250 rpm using 200 μM IPTG. SpyTag-yCD/yCD-His6 was expressed overnight at 20 °C and 250 rpm using 500 μM IPTG. Cells were harvested through centrifugation at 3000g and 4 °C. The cells were resuspended in a TN150 buffer (50 mM Tris, 150 mM NaCl at pH 8.0) to optical densities (OD600) between 20 and 50. Cells were re-suspended in a Fisher Scientific sonicator (Pittsburgh, PA, USA). The lysate was clarified via centrifugation at 17 969g at 4 °C for 20 min.

**Protein Purification.** ELP constructs were purified through inverse transition cycling (ITC). Ammonium sulfate stock solution (3M) was added to protein lysate to a final concentration of 0.5 M to aggregate ELPs. The protein was centrifuged down at 16 000g at 4 °C for 20 min. The pellet was incubated on ice with cold TN150 buffer for 20 min and resuspended via pipetting. The protein was spun again at 16 000g and 4 °C for 20 min to remove insoluble contaminants. This process was repeated a second time to further purify the ELP protein. Concentrations were measured using A280 with theoretical extinction coefficients.

HBV-SpyCatcher was purified via ammonium sulfate precipitation at 20% saturation on ice. After 30 min of incubation on ice, the protein was collected through centrifugation at 17 969g at 4 °C for 20 min. The pellet was resuspended in TN150 buffer by dialysis against TN150 buffer overnight at 4 °C. Afterward, a further spin at 17 969g and 4 °C for 30 min was performed to remove aggregates. The concentration and yield of HBV-SpyCatcher protein was estimated by a combination of Bradford Assay (BioRad) and densitometry. The Bradford assay was performed according to the manufacturer’s instructions using a BSA standard curve. Densitometry was performed on coomasie-stained SDS-PAGE gels using the Image Lab S.1 software package (BioRad), where the “Lanes and Bands” tool was used to quantify the percentage of each band in the sample. The HBV-SpyCatcher band percentage was multiplied by the concentration measured by the Bradford assay to determine HBV concentration. Yield was calculated by multiplying the concentration by the total volume of purified sample and dividing by the original culture volume.
Sucrose gradient sedimentation was performed using a 10% w/v to 60% w/v sucrose step gradient. Equal volumes of sucrose solutions (10%, 20%, 30%, 40%, 50%, and 60%) and protein were loaded into polystyrene tubes (Beckman-Coulter, Indianapolis, IN, USA). These were ultracentrifuged in a SW60-Ti rotor (Beckman-Coulter) at 60,000 rpm at 20 °C for 40 min. Fractions were taken from the top of the gradient and were analyzed via SDS-PAGE. VLP-containing fractions were pooled for dialysis against TN150 and were further examined by TEM.

SpyTag-GFP-His6 and SpyTag-NanoLuc were purified via Ni-NTA affinity chromatography (Thermo Scientific, Rockford, IL, USA). Lysate was bound to the column in TN150 buffer with 10 mM imidazole. The column was washed with TN150 buffer with 30 mM imidazole and eluted with TN150 and 250 mM imidazole. The protein was then dialyzed into TN150. The yCD combo was initially purified via Ni-NTA affinity chromatography to remove any SpyTag-yCD homodimer. This was done similarly to SpyTag-GFP-His6. The protein was dialyzed into TN150, then reacted to HBV-SpyCatcher. The complex was purified with ITC to remove yCD-His6 homodimer. Protein concentrations were determined using the Bradford assay and densitometry.

SpyTag Reactions and Device Assembly. SpyTag reactions were performed either overnight at 4 °C or at room temperature for 5 h. HBV and SpyTagged protein were mixed together at the desired molar ratios and allowed to react in TN150 at pH 8. To fully decorate the HBV, the SpyTagged protein was added in 5-fold excess to ensure maximal consumption of the HBV receptor. After assembly, HBV nanoparticles were purified by ITC. Densitometry was performed to evaluate the average number of decorations per particle. The band percentages were converted to molar percentages by dividing each by its respective molecular weight and dividing that number by the sum of the total corrected band percentages. Molar percentages were multiplied by 240 to get an average number of decorations per VLP.

Transmission Electron Microscopy. HBV samples were applied to carbon-coated grids (Electron Microscopy Sciences, Hatfield, PA, USA) and stained using 2% uranyl acetate. Images were taken with a Zeiss Libra 120 transmission electron microscope (Oberkochen, Germany). Particle sizes were measured across 25 separate images using ImageJ (NIH, Bethesda, MD, USA), and statistical significance was determined with a two-sample t test (p < 0.01).

ELISA Detection. Human thrombin (Sigma-Aldrich, St. Louis, MO, USA) was coated onto white, 96-well, high-bind plates (Thermo Scientific) by incubating 50 μL of thrombin in 100 mM sodium carbonate buffer pH 9.6 for 2 h at room temperature. The wells were washed with TBST (50 mM Tris, 150 mM NaCl, pH 7.5 with 0.05% Tween20) and then blocked overnight at 4 °C with 1% BSA (Sigma-Aldrich) in TBS (50 mM Tris, 150 mM NaCl, pH 7.5). The wells were washed four times with TBST. A mouse anti-thrombin antibody (Santa Cruz Biotechnology, Dallas, TX, USA) was bound at a concentration of 15 nM for 1 h at room temperature. The wells were washed four times with TBST, and the HBV nanoparticles were applied at a concentration of 50 nM in TN150 with 0.05% Tween 20 and 1% BSA and allowed to incubate for 1 h. The plate was washed eight times with TBST, and 80 μL of the Nano-Glo luciferase assay reagent (Promega, Madison, WI, USA) was added to the wells. Luminescence was measured using a Synergy H4 microplate reader (BioTek, Winooski, VT, USA).

Mammalian Cell Culture. SUM149 inflammatory breast cancer cells were utilized as an EGF-R-positive cell line. Cells were cultured at 37 °C and 5% CO2 in Ham’s F12 medium supplemented with 5% FBS, 1% (v/v) penicillin/streptomycin, 1% (v/v) mycoplasma antibiotic supplement, 1% (v/v) glutamine, 5 μg/mL insulin, 2.5 μg/mL transferrin, 200 ng/mL selenium, and 1 μg/mL hydrocortisone according to previously established methods. For delivery experiments, HBV nanoparticles were resuspended after ELP purification into PBS and stored at 4 °C until use. SUM149 cells were seeded into an eight-well Nunc Lab-Tek chamber slide (Thermo Scientific) coated with a collagen film (1.5 mg/mL collagen I bovine protein in 0.02 M acetic acid in DI water). Cells were grown overnight until approximately 70% confluency. Nanoparticles were applied to cells at a concentration of 25 nM in Opti-MEM reduced serum medium. Cells were allowed to incubate with nanoparticles for 3 h. Cells were washed three times with PBS to remove nanoparticles that were not internalized. The cells were then fixed with 10% neutral buffered formalin (Sigma-Aldrich) and stained with 300 nM DAPI (Thermo Scientific) according to the manufacturer’s protocol. The fixed cells were imaged with a Leica DFC350 FX microscope (Leica Microsystems, Wetzlar, Germany).

Flow Cytometry. SUM149 cells were seeded in a tissue culture treated 12-well plate (Corning Inc., Corning, NY, USA) at a density of 3 × 104 cells per well and allowed to incubate overnight at 37 °C. The following day, 25 nM of nanoparticles in Opti-MEM medium were applied to the cells and incubated for 3 h (Figure 4B) or 4 h (Figure 3B) before washing three times with PBS. Cells were trypsinized and centrifuged at 800 rpm for 4 min before spent media was removed and live cells were resuspended in cold PBS (Figure 4B) or fixed with 10% neutral buffered formalin (Figure 3B). The GFP fluorescence intensity of 1.5 × 105 cells was measured with a NovoCyte flow cytometer (ACEA Biosciences, Inc., San Diego, CA, USA). The mean fluorescence intensity ± the standard deviation of three replicates was reported.

MTT Viability Assay. In a tissue culture treated 96-well plate (Corning Inc.), SUM149 cells were seeded at 1 × 104 cells per well and incubated overnight. Opti-MEM medium with 25 nM nanoparticles was added to cells and incubated for 3 h. Following incubation, cells were washed three times with PBS to remove extracellular protein and treated with 500 μg/mL of either 5-FC or 5-FU in Ham’s F-12 medium. Cells were then allowed to incubate for 48 h at 37 °C. After 48 h, 10 μL of 12 mM MTT (Thermo Fisher) and 100 μL of medium were added to each well and allowed to incubate for 4 h. After labeling the cells with MTT, 85 μL of media was removed from the wells and 50 μL of DMSO was added. Cells were then incubated at 37 °C for 15 min, and the absorbance was measured at 540 nm on a Synergy H14 microplate reader. To determine the cell viability, the background fluorescence of MTT/media alone was subtracted and the samples were normalized to the absorbance of untreated cells. The mean viability ± the standard deviation of six replicates was reported.

ASSOCIATED CONTENT

Supporting Information

Additional figures; a table of oligos used for molecular cloning; a list of amino acid sequences of all protein constructs used; and the DNA sequence of the HBV-SpyCatcher construct from promoter to terminator (PDF)

AUTHOR INFORMATION

Corresponding Author
Wilfred Chen — Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, Delaware 19716, United States; orcid.org/0000-0002-6386-6958; Email: wilfred@udel.edu

Authors
Emily J. Hartzell — Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, Delaware 19716, United States
Rachel M. Lieser — Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, Delaware 19716, United States; orcid.org/0000-0002-7597-0672
Millicent O. Sullivan — Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, Delaware 19716, United States
Complete contact information is available at: https://pubs.acs.org/10.1021/acsnano.9b08756

Notes
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