Bioengineered Vaults: Self-Assembling Protein Shell—Lipophilic Core Nanoparticles for Drug Delivery

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Chemotherapy relies heavily on small therapeutic compounds, many of which are difficult to formulate and administer or suffer from biodistribution problems. Three main strategies have evolved to address these issues: direct drug modification to create a new drug with altered physicochemical properties, drug solubilization with excipients, and/or drug encapsulation within delivery systems. However, these technologies often rely upon materials that can vary greatly in size, composition, ease of preparation, dispersion, and immunogenicity. To address these issues, we have been studying the feasibility of bioengineering vaults as a next-generation drug delivery system given their collectively unique advantages of being naturally occurring, monodispersed, biocompatible, and nonimmunogenic.

ABSTRACT We report a novel approach to a new class of bioengineered, monodispersed, self-assembling vault nanoparticles consisting of a protein shell exterior with a lipophilic core interior designed for drug and probe delivery. Recombinant vaults were engineered to contain a small amphipathic R-helix derived from the nonstructural protein 5A of hepatitis C virus, thereby creating within the vault lumen a lipophilic microenvironment into which lipophilic compounds could be reversibly encapsulated. Multiple types of electron microscopy showed that attachment of this peptide resulted in larger than expected additional mass internalized within the vault lumen attributable to incorporation of host lipid membrane constituents spanning the vault waist (>35 nm). These bioengineered lipophilic vaults reversibly associate with a sample set of therapeutic compounds, including all-trans retinoic acid, amphotericin B, and bryostatin 1, incorporating hundreds to thousands of drug molecules per vault nanoparticle. Bryostatin 1 is of particular therapeutic interest because of its ability to potently induce expression of latent HIV, thus representing a preclinical lead in efforts to eradicate HIV/AIDS. Vaults loaded with bryostatin 1 released free drug, resulting in activation of HIV from provirus latency in vitro and induction of CD69 biomarker expression following intravenous injection into mice. The ability to preferentially and reversibly encapsulate lipophilic compounds into these novel bioengineered vault nanoparticles greatly advances their potential use as drug delivery systems.

KEYWORDS: vaults · nanoparticles · drug delivery systems · bryostatin 1 · HIV latency

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Vaults, the largest known ribonucleoprotein complex in eukaryotic organisms, derive their name from their multiple-arched morphology reminiscent of vaulted ceilings in gothic cathedrals.\(^8\)–\(^10\) Native vaults appear as hollow, thin-walled sub-100 nm ellipsoidal structures with well-defined monodispersity, uniform NP size of 67 nm \(\times\) 40 nm \(\times\) 40 nm, and mass of ~13 MDa.\(^11\)–\(^12\) Native vaults consist of multiple copies of three different proteins: the major vault protein (MVP, 100 kDa), vault poly(ADP-ribose) polymerase (VPARP, 193 kDa), and telomerase-associated protein 1 (TEP1, 290 kDa) along with several copies of one or more small untranslated vault-associated RNA (vRNAs).\(^8\)–\(^9\) MVP constitutes \(\sim\)75% of the native vault particle mass, and exogenous expression of MVP alone in insect cells is both necessary and sufficient for the formation of hollow vault-like particles that are morphologically identical to native vaults, but lack VPARP, TEP1, and vRNA.\(^13\) Native and recombinant vaults consist of 78 copies of MVP (39 per half vault), which begin by protruding internally into the vault lumen at the NP waistline and then proceed to run lengthwise toward either vault cap, where they become externally solvent exposed.\(^11\)–\(^14\) Novel recombinant MVP-only vaults have been created that not only retain native vault architecture but also incorporate a variety of functionalities internally and/or externally.\(^7\) Additionally, the use of an MVP interaction domain (INT, 17 kDa) derived from the C-terminus of VPARP has allowed for novel vault formation. This INT domain associates non-covalently with an internally facing MVP binding site and can be used to internally package other proteins of interest into the vault lumen via recombinant covalent attachment to INT.\(^16\)–\(^17\)

Initial bioengineering of the vault for sequestering small lipophilic compounds made use of a smaller NP known as a nanodisk (ND).\(^18\)–\(^21\) NDs (10–20 nm disordered lipoprotein complexes) were incorporated into the vault lumen, where they encapsulated all-trans retinoic acid (ATRA) for treatment against hepatocellular carcinoma cells.\(^22\) However, this approach is hindered by lipopolysaccharide-associated cytotoxicity and cumbersome synthesis.\(^23\) As such, we set out to refine and simplify our approach for vault-mediated drug delivery. Our search for a suitable candidate yielded an interesting peptide derived from hepatitis C virus (HCV). The nonstructural protein 5A (NS5A) is a 56 kDa viral phosphoprotein involved in HCV replication.\(^24\)–\(^27\) NS5A associates with the cytoplasmic leaflet of host-cell endoplasmic reticulum membranes along with other HCV proteins involved in viral replication. NS5A interaction with host membranes has been mapped to the first 31 amino acids of the protein.\(^28\)–\(^29\)

NMR analysis of this region characterized it as an amphipathic \(\alpha\)-helix (residues 5–25) of \(< 4\) nm in length that functions as a monotopic (in-plane) membrane anchor domain via lipophilic interactions between strictly conserved \(\alpha\)-helix tryptophan residues and the acyl chains of the neighboring host phospholipids (Figure 1A). Furthermore, the polar face of this amphipathic \(\alpha\)-helix also contains strictly conserved charged residues arranged in an asymmetrical distribution believed to have a putative functional role via interactions with other components of the viral replication complex.\(^30\)–\(^31\) Interestingly, these properties also allow for their self-association in water when membrane surfaces are absent. As such, NS5A1-31 was selected for recombinant attachment to the N-terminus of MVP with the assessment that the peptide would self-associate to form an internalized protein-based lipophilic ring stabilized by complementation of ionic and van der Waals forces of adjacent copies of the amphipathic \(\alpha\)-helix within the vault lumen (Figure 1B). Herein we detail the covalent attachment of the NS5A1-31 amphipathic \(\alpha\)-helix to MVP, which results in the formation of a new class of nanoparticles consisting of a self-assembling protein shell with an internalized lipophilic core that is capable of reversibly sequestering small lipophilic compounds of interest for vault-mediated drug delivery (Figure 1C).

### RESULTS AND DISCUSSION

#### Amphipathic \(\alpha\)-Helix Vault as a Self-Assembling Protein Shell—Lipophilic Core Complex

Development of the vault as a potentially viable drug delivery system is motivated by the particles’ inherent advantages: small size, self-assembly, uniform composition, monodispersity, nonimmunogenicity, and relative ease of bioengineering. However, while encapsulation of recombinant proteins within vaults has allowed for exploitation of novel functionalities, the ability to successfully incorporate and retain small molecules of therapeutic value has been relatively unexplored. Covalent attachment of a small amphipathic \(\alpha\)-helix from the hepatitis C viral protein NS5A was proposed to yield vaults containing a localized ring of lipophilic density lining the internal circumference of the NP as adjacent monomeric copies of NS5A1-31 self-interact due to their intrinsic properties. Covalent attachment of NS5A1-31 to the N-terminus of MVP did not inhibit MVP expression nor interfere with vault self-assembly. During purification, the majority of these amphipathic \(\alpha\)-helix-containing (AH) vaults were collected within the 40–45% layer of a discontinuous sucrose gradient as well-defined, intact monodisperse NPs, consistent with previous vault studies. When examined by uranyl acetate negatively stained transmission electron microscopy (TEM), AH vaults are morphologically distinct from both native and recombinant MVP-only vaults due to the presence of a significantly large region within the vault lumen that uncharacteristically lacks normal uranyl acetate staining (Figure 2A). While the absence of dark, uranyl acetate stained areas under TEM is reflective of...
structural features of the vault (i.e., the protein shell itself), the high intensity of this nonstaining region within most of the AH vaults is inconsistent with the amount of additional mass conferred onto the NP via covalent attachment of the 31 amino acid peptide NS5A1-31 to the N-terminus of MVP despite all 78 copies of the peptide being localized internally into the vault lumen at the NP waist. As such, we suspected that this additional internalized mass within AH vaults might represent additional cellular components that coassociated within the AH vault lumen via interactions with the NS5A1-31 amphipathic α-helices. As the intensity of this non-uranyl acetate staining region within AH vaults appeared to vary from vault to vault, we further hypothesized that it is dynamic in nature and could therefore be altered. When purified AH vaults were treated with 5% Tween 20 detergent followed by repurification, this nonstaining region of additional mass within AH vaults experienced a significant loss in intensity, suggesting that a sizable portion of the novel mass sequestered within the AH vault lumen is detergent soluble and therefore not part of the AH vault itself (Figure 2B).

Since TEM imaging of vaults does not faithfully represent their true 3D structure, we turned to cryo-electron tomography (cryoET) to further characterize...
this additional mass within AH vaults in their frozen hydrated state in three dimensions. CryoET tilt cross-section slices revealed that a majority of AH vaults contained internalized mass that completely traverses the vault lumen (white arrowheads), a significant distance of >35 nm with an apparent thickness of ∼3–5 nm (Figure 2C). Additionally, a minority of AH vaults showed incomplete luminal coverage (white arrows). CryoET tomography tilt slices and segmentation of the 3D reconstructions of various well-defined AH vaults further confirmed that this novel additional mass sequestered within the lumen retains a dynamic morphology (Figure 3A–E). Additionally, AH vaults lacking such internalized mass (before or after detergent treatment) remain morphologically identical to a wide variety of recombinant vaults containing short peptide sequences of various length/composition/functionality at the N-terminus of the MVP, implying that the unique identity of NS5A1-31 itself must be responsible for mediating the encapsulation of this unknown additional internalized mass within these AH vaults (Figure 3F–H).

The ability of this novel internalized mass to span the entire vault waist must be therefore attributable to the presence of additional cellular components, which we hypothesized to be host membrane lipid constituents (phospholipids, glycolipids, sterols, fatty acids, etc.) given NS5A1-31’s natural in vivo function as a monotopic lipid membrane anchor. The lack of contaminating proteins, DNA, or RNA within purified AH vaults, as well as dynamic morphological nature, also helps support the interpretation that this internally associated mass is constituted by various lipophilic material interacting with the 78 copies of NS5A1-31.
that line the inner circumference of the vault waistline. To test this hypothesis, AH vaults were subsequently incubated with the lipophilic dye 1,1\textsuperscript{0}-dioctadecyl-3,3,3\textsuperscript{0},3\textsuperscript{0}-tetramethylindodicarbocyanine perchlorate (DiD), a long-chain dialkylcarbocyanine analogue that increases fluorescent intensity in the presence of a lipophilic environment.\textsuperscript{32} DiD showed a distinct qualitative increase in fluorescence upon co-incubation with AH vaults compared to free dye or dye incubated with control vaults, thereby corroborating an improved lipophilic nature associated with AH vaults. Such an internalized lipophilic core of 3/C0\textsuperscript{5} nm in thickness and >35 nm in diameter would represent $\sim$4800 nm\textsuperscript{3} of lipophilic volume within the NP available for absorption of lipophilic compounds of interest.

**Drug-Binding Properties of AH Vaults.** To test the ability of purified AH vaults to bind therapeutic compounds, we examined their ability to selectively retain either doxorubicin (DOX), amphotericin B (AMB), or ATRA, three small therapeutic molecules of varying lipophilicity and aqueous solubilities. The AH vaults and drugs were co-incubated, and the resulting complexes separated over an overnight discontinuous sucrose gradient. While AH vaults sediment into the denser layers of the gradient due to their large molecular mass, non-vault-associated materials remain in the lighter layers of the gradient and are subsequently lost during the final ultracentrifugation at 100000 g as the NPs are pelleted. In the case of DOX, a fluorescent cytotoxic anthracycline antibiotic, neither control nor AH vaults showed any detectable retention of compound in the collected vault fraction, which is in general agreement with DOX's high water solubility of >50 mg/mL despite a LogP of 1.27.\textsuperscript{33}

Conversely, AMB, an antifungal amphipathic polynene antibiotic with poor water solubility at physiological pH of less than 0.75 mg/mL despite a LogP of 0.8, was selectively retained by AH vaults during separation with 45 molecules of AMB per single AH vault, while control vaults showed no detectable AMB association (Figure 4A).\textsuperscript{34} Similarly, ATRA, a highly toxic vitamin A-derived retinoid that regulates gene transcription with near absolute water insolubility and correlated LogP of 6.30, co-associated with AH vaults at 182 molecules of the drug per single AH vault (Figure 4B).\textsuperscript{22} However, ATRA appears to have some nonspecific association with the NP itself, as seen by its low level of association with the control vaults. The higher loading efficacy of ATRA ($\sim$75%) over AMB ($\sim$50%) might be reflective of the innate physicochemical properties between these compounds and consistent with the greater lipophilicity of ATRA. Since the potential volume of such a lipophilic microenvironment internalized within AH vaults could be as large as $\sim$4800 nm\textsuperscript{3}, it is plausible for thousands to tens of thousands of hydrophobic molecules to be encapsulated within a single AH vault. As such, our initial experimental values represent only a small fraction of their theoretical maximum capacity.

To better address maximal loading of AH vaults, we conducted smaller scale drug-loading experiments with higher titrated ratios of drug to vault. Instead of the previous 1 \mu g to 100 \mu g ratio used above, we incubated 100 \mu g of control or AH vaults with 10 or 50 \mu g of AMB (Table 1). Following incubation, vaults and their associated drug cargo were recovered from excess, unbound material by passage through a microscale gel filtration spin column. Control vaults showed low levels of drug retention of 264 and 431 molecules of AMB per single control vault for the two respective loading conditions. In contrast, AH vaults showed considerably higher AMB retention following post column separation with 1213 and 2071 molecules of AMB per single AH vault for the two conditions. It seems likely that the AMB seen in the recovered control
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sition in line with AMB's general stability. Meanwhile, the negligible loss of AMB in the AH vault samples likely results from the drug molecules being sequestered within the NP's lipophilic core, which provides greater overall stability and protection. The ability of AH vaults to encapsulate >2000 drug molecules while potentially offering a more hospitable microenvironment is an important milestone in their development as a novel excipient reservoir for solubilizing lipophilic therapeutic compounds for drug delivery.

AH Vaults for Treating HIV Latency Using Bryostatin 1. As an initial clinical application for the AH vaults, we focused on the delivery of bryostatin 1, a promising therapeutic lead for the treatment of Alzheimer's disease, cancer, and HIV/AIDS eradication. Bryostatins are a family of macrolide lactone compounds with potent neurobio-

chemical, anticancer, and immuno-modulating prop-

erties both in vitro and in vivo, which operate putatively through modulation of protein kinase C (PKC). While practical access to the bryostatins from natural sources (e.g., Bugula neritina), engineered biosynthesis, and total synthesis are still works in progress, more accessible, designed, and highly potent analogues are now becoming available. However, little attention has been directed at the formulation and delivery of these agents, issues that are of huge significance to preclinical studies on both the natural and designed analogues.

Our specific interest in bryostatin and their ana-

logues (bryologues) is driven in part from their potential use in the eradication of HIV/AIDS. In the span of a generation, AIDS/HIV has undergone a remarkable transformation from a lethal infection to a largely manageable chronic disease. However, over 33 million people remain infected with the virus. Highly active antiretroviral therapy (HAART) currently reduces viral activity to undetectable levels, thereby slowing disease progression. However, HAART does not address the issue of provirus latency within cellular reservoirs, which can resupply active virus. Latently infected CD4+ T cells can persist for decades in HAART-treated individuals and therefore represent a key barrier to disease eradication. One potential method for elimin-

ating the latent reservoir of HIV is to induce the dormant provirus to express viral proteins, which would make the host cell susceptible to immune effector mechanisms, viral cytopathic effects, and addi-

tional therapies directed toward viral proteins such as anti-HIV envelope immunotoxins. Delivery of latency activators using nanoparticles could afford a variety of benefits, including the combinatorial and targeted introduction of these agents specifically to the cell type of choice, leading to less toxicity associated with activation of nontarget cell types. To explore this possibility, we previously introduced bryostatin 1 into lipid nanoparticles and demonstrated that these particles activate latent HIV and can also be targeted toward CD4+ T cells using monoclonal antibodies. Here we have incorporated bryostatin 1 into AH vault particles and evaluated their properties in vitro and in vivo.

Bryostatin 1 (LogP of 4.25—5.40, predicted) incorporation into AH vaults was assessed by high-

performance liquid chromatography (HPLC) coupled with multiple reaction monitoring (MRM) tandem mass spectrometry (MS/MS) in lieu of spectrophotometric analysis. MRM-LC-MS/MS allowed for sensitive detection (>0.009 ng/μL) of the sodiated bryostatin 1 ion at m/z 927.4, consistent with previous reports. AH vaults were co-incubated for 30 min at 4 °C with bryostatin 1 and subsequently collected from solution using ultra-

centrifugation at 100000g. Aliquots of the starting material, spin supernatant, and the resuspended vaults were analyzed by MRM-LC-MS/MS, and the bryo-

statin 1 concentration was measured using a standard curve generated using known concentrations of

TABLE 1. AH Vault Drug-Loading Capacity Using Amphotericin B vs Control Vaults

| Control Vaults | AMB:vault starting ratio (M) | AMB:vault ending ratio (M) | AMB:vault ending ratio (post 7 days) (M) | % change |
|---------------|-----------------------------|---------------------------|----------------------------------------|----------|
| 10 μg:100 μg  | 32.6 ng:1 μg (264:1)        | 26.8 ng:1 μg (217:1)      | 18%                                    |          |
| 50 μg:100 μg  | 53.0 ng:1 μg (431:1)        | 28.0 ng:1 μg (228:1)      | 47%                                    |          |
| AH vault      | 149.2 ng:1 μg (1213:1)      | 133.6 ng:1 μg (1085:1)    | 11%                                    |          |
| 50 μg:100 μg  | 254.7 ng:1 μg (2071:1)      | 240.8 ng:1 μg (1958:1)    | 6%                                     |          |

while 4—5-fold increase in AMB associated with the AH vaults is apparently due to sequestration of the drug in their large lipophilic microenvironment. The calculated values from the 10 and 50 μg AMB:100 μg AH vault samples do not violate the starting ratio of 815:1. Rather the observed 1213 and 2071 AMB per single AH vault stems from inefficient vault recovery over the gel filtration spin column, which leads to an apparent overcalculation of the loading efficiency.

Following a 1-week storage at 4 °C, the drug-bound vaults were re-examined. Control vault samples experi-

enced a mild to severe drop in AMB content (18% loss for the 10 μg sample and 47% for the 50 μg sample, respectively). Conversely, AH vaults experienced only a minor drop in associated drug with 90% or greater of the original AMB being retained (11% loss for the 10 μg sample and 6% for the 50 μg sample, respectively). This loss of AMB in the control vault samples may be indicative of poor protection from the aqueous envi-

ronment, thereby allowing faster molecular decompo-

sition in line with AMB's general stability. Meanwhile, the negligible loss of AMB in the AH vault samples likely results from the drug molecules being sequestered within the NP's lipophilic core, which provides greater overall stability and protection. The ability of AH vaults to encapsulate >2000 drug molecules while potentially offering a more hospitable microenvironment is an important milestone in their development as a novel excipient reservoir for solubilizing lipophilic therapeutic compounds for drug delivery.

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Figure 5. (A) In vitro latent HIV activation using bryostatin 1-loaded AH vaults on J-Lat 10.6 cells for 48 h. In these assays 50 nM bryostatin 1 compound (without vaults) was used as a positive control and induced GFP expression in 30.6% (±0.6%) of cells. Error bars indicate ±1 SD (N = 3). NS not significant, *p < 0.0001 empty vault vs vault + bryostatin 1 (2-sided t test). (B) In vitro stimulation of CD69 (HIV provirus latency activation biomarker) activation using bryostatin 1-loaded AH vaults on primary human PBMCs for 24 h. Positive control stimulations with 50 nM bryostatin 1 compound (without vaults) induced CD69 expression in 69.4% (±31.3%) of cells. Error bars indicate ±1 SD (N = 4 different cell donors). NS not significant, *p < 0.01 empty vault vs vault + bryostatin 1 (2-sided t test). (C) In vivo CD69 stimulation from harvested C57/bl6 mouse splenocytes at 24 h postinjection of either media control, bryostatin 1, bryostatin 1-loaded AH vaults, or empty AH vaults. Error bars indicate ±1 SD (3–5 mice per group).

TABLE 2. Summarization of Bioengineered AH Vaults’ Ability to Selectively Associate with a Small Sample Set of Therapeutic Compounds with Different Physicochemical Properties via Noncovalent, Reversible Association within a Novel Internalized Lipophilic Core Microenvironment Generated by the Covalent Attachment of a Unique Amphipathic α-Helix Peptide (NS5A1-31) to the MVP

| therapeutic compound | solubility<sub>90</sub> (mg/mL) | LogP<sub>(octanol/water)</sub> | loading condition | drug:vault starting ratio (M) | drug:vault ending ratio (M) | detection method |
|----------------------|-------------------------------|--------------------------|-------------------|-------------------------------|-------------------------------|------------------|
| doxorubicin (580 g/mol) | 50 | 1.27 (PubChem) | 0.5 h at 4 °C, 20 h centrifugation | 10 μg:1000 μg | none detected | UV/vis spectra, DOD<sub>480nm</sub> = 11 500 M<sup>-1</sup> cm<sup>-1</sup> |
| amphotericin B (924 g/mol) | 0.75 | 0.8 (DrugBank) | 0.5 h at 4 °C, 20 h centrifugation | 10 μg:1000 μg | 5.6 ng:1 μg | UV/vis spectra, ATR<sub>406nm</sub> = 150 000 M<sup>-1</sup> cm<sup>-1</sup> |
| all-trans retinoic acid (300 g/mol) | insoluble | 6.30 (DrugBank) | 0.5 h at 4 °C, 20 h centrifugation | 50 μg:100 μg | 254.7 ng:1 μg | UV/vis spectra, ATR<sub>406nm</sub> = 150 000 M<sup>-1</sup> cm<sup>-1</sup> |
| bryostatin 1 (905 g/mol) | insoluble | 4.25–5.40 (predicted, CHEMBL and ChemSpyder) | 0.5 h at 4 °C, 1 h centrifugation | 1 μg:100 μg | 13.4 ng:1 μg | mass spec (MRM-LC-MS/MS) |

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bryostatin 1. Approximately 10.6 ± 1.4 ng of bryostatin 1 was measured in the incubated, precentrifuged sample and is in accordance with the expected value of 10 ng/μL (1 μg of bryostatin 1 per 100 μg of AH vault in 100 μL of PBS). Meanwhile, the 13.4 ± 2.3 ng of bryostatin 1 measured in the resuspended vault pellet is within experimental error and indicates that approximately 100% of the starting bryostatin 1 was retained through association with the AH vaults with ~83 molecules of the drug per single AH vault. However, because of the low availability and high cost associated with bryostatin 1, only a few initial studies were conducted using control vaults. Compared to empty AH vaults, AH vaults containing bryostatin 1 showed clear dose-dependent activation of latent HIV provirus in the J-Lat 10.6 cell line, a well-characterized model for the main T-lymphocyte cell reservoir, with activity starting at 1 ng/μL of AH vaults (Figure 5A). Alternatively, stimulation of T cells with PKC-activating compounds, such as bryostatin 1, induces cell surface expression of CD69, which occurs at similar concentrations to those required to activate HIV from latency.43,44 As such, CD69 expression can be used as a biomarker for evaluating whether bryostatin 1 delivered via association with AH vaults remains bioactive in the desired T cell type. When tested for activity in this way, bryostatin 1-loaded AH vaults activated CD69 expression in primary human peripheral blood mononuclear cells (PBMCs) obtained from four different donors in a dose-dependent manner with stimulation occurring at concentrations as low as 0.1 ng/μL AH vaults as analyzed by flow cytometry.
To evaluate whether these drug-loaded vaults are also bioactive in vivo, they were injected into C57/b16 mice at 1 µg of bryostatin 1 per 100 µg of AH vaults per mouse. At 24 h postinjection, over 90% of CD4+ T cells present within harvested splenocytes had been induced to express CD69, demonstrating that the bryostatin 1-loaded AH vaults can successfully deliver compounds in vivo (Figure 5C). Notably, these untargeted vaults also induced >70% of non-CD4+ T cells (primarily CD8+ T cells) and ~40% of non-T cells to express CD69. This activation of a broad spectrum of cell types illustrates the potential benefits of targeting the vaults more selectively to the cell type of interest (in this case, to CD4+ T cells). Methods for targeting vaults are available and are currently being explored.45 Further improvements to the HIV provirus latency activating AH vaults could also be achieved by using more potent analogues of bryostatin or prostratin.36,43,46 Second-generation AH vaults could therefore include both targeting moieties and enhanced latency-activating compounds.

CONCLUSIONS

These experiments demonstrate the novel ability of AH vaults to encapsulate various therapeutic compounds, up to >2000 molecules per single AH vault, through reversible association with a highly lipophilic interior that results from the covalent attachment of an amphipathic α-helical peptide to the MVP (summarized in Table 2). Furthermore, these drug-loaded AH vaults can deliver their therapeutic cargo, such as bryostatin 1, in vitro using both cultured and primary cell lines, as well as in vivo. The formation of these self-assembling protein shell–lipophilic core NPs benefits not only from the key properties of vaults such as monodispersity, cell specific targeting, and biocompatibility, but also from being a vault-drug formulation based approach that can completely internalize their cargo, thereby offering an additional layer of protection. As such, these novel AH vaults represent an interesting “proof of concept” approach to functionalizing the vault for potential use in drug delivery.

MATERIALS AND METHODS

AH Vault Cloning, Expression, and Purification. NSSA1-31 was PCR amplified from a construct containing HCV NSSA1-31 fused to GFP generously provided by Darius Moradpour M.D. at the Centre Hospitalier Universitaire Vaudois, University of Lausanne, Switzerland. The following primers were used to generate a ~100 bp PCR fragment with flanking Nco I restriction sites (underlined). Forward: 5′ GAATTCCTAGTGCGCCGTTCTGCAGC 3′. Reverse: 5′ CCTCTGCCATCCATGTGGCCCATGAG 3′. The resulting PCR fragment contained the amino acid residue sequence MAGSVEVDWIVCEVLSDFPKTLKAKLMPTM, which incorporates a start Met, as well as two codon sequence changes (Ser1Ala and Gly3Thr, underlined) expected to behave as silent mutations with no anticipated consequence on the structural folding of the amphipathic helix domain contained from residues 5–25. Residue Leu31 is replaced with the start Met of MVP (underlined, bold). Subsequent Nco I digestion was used to insert NSSA1-31 immediately upstream, in-frame to rat MVP cDNA previously cloned into pFastBac-1 vector using standard molecular biology techniques. This construct was subsequently used to establish a baculovirus used in AH Vault Transmission Electron Microscopy, Cryo-electron Tomography, and Retention of either doxorubicin (Sigma), amphotericin B (Sigma), or all-trans retinoic acid (Sigma) was conducted as follows. First, the series were precisely aligned using the eTom program from the Imod package. Rough alignment was performed by cross-correlation of images in the tilt series, followed by fine alignment using 10 nm gold beads as fiducial markers. A boundary model was created to adjust the tilt axis before a final, precisely aligned tilt stack was produced for each series. The aligned tilt series was then used to make a 3D reconstruction using GPU-based SIRT (simultaneous iterative reconstruction technique) reconstruction implemented in the Inspec3D program from FEI. The 3D reconstructions were saved as a stack of x–y sections stacked along the z direction. This 3D tomographic reconstruction was further processed by median filtering and pixel binning to enhance structural contrast. Individual vault particles were then segmented from the 3D density maps and displayed by volume renderings of the vault particles with packaged material with Amira (Visage Inc.). For densities corresponding to the vault particle, the known 39-fold symmetry was imposed to improve the signal-to-noise ratio for volume rendering.

AH Vault Drug Packaging and Spectrophotometric Analysis. AH vault lipophilicity was initially assayed using 5 µL of a 10 µg/µL 1,1′-diocadecyl-3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD(644 ex/665 em), Invitrogen Molecular Probes), dimethyl sulfoxide (DMSO) stock added to 1000 µg of purified AH vault, control vault, bovine serum albumin (Sigma), or alone in 1 × PBS buffer for 30 min at 4 °C with rotation and protection from light. Samples were overlaid onto 1 mL of 1 × PBS buffer in TLA100.1 rotor tubes (Beckman Coulter) and ultracentrifuged at 100000g for 1 h at 4 °C. Pellets were resuspended in 100 µL of 1 × PBS buffer, and fluorescence was measured at 665 nm.

Retention of either doxorubicin (Sigma), amphotericin B (Sigma), or all-trans retinoic acid (Sigma) was conducted as follows: 1000 µg of purified AH or CP vaults was incubated with 10 µg of each respective compound from appropriate stocks into 1 mL of 1 × PBS buffer for 30 min at 4 °C with rotation and protection from light. Samples were overlaid onto a semidiscocontinuous sucrose gradient and centrifuged at 25000 rpm (77000g) using a Beckman SW41 Ti swinging bucket rotor for 16 h at 4 °C on a Beckman-Coulter ultracentrifuge. Gradient fractions were collected and ultracentrifuged for 2 h at a magnification that gives a pixel size of 0.45 nm per pixel at the specimen level. The tomography tilt series were processed with a suite of programs to generate a segmented 3D reconstruction as follows. First, the series were precisely aligned using the eTom program from the Imod package. Rough alignment was performed by cross-correlation of images in the tilt series, followed by fine alignment using 10 nm gold beads as fiducial markers. A boundary model was created to adjust the tilt axis before a final, precisely aligned tilt stack was produced for each series. The aligned tilt series was then used to make a 3D reconstruction using GPU-based SIRT (simultaneous iterative reconstruction technique) reconstruction implemented in the Inspec3D program from FEI. The 3D reconstructions were saved as a stack of x–y sections stacked along the z direction. This 3D tomographic reconstruction was further processed by median filtering and pixel binning to enhance structural contrast. Individual vault particles were then segmented from the 3D density maps and displayed by volume renderings of the vault particles with packaged material with Amira (Visage Inc.). For densities corresponding to the vault particle, the known 39-fold symmetry was imposed to improve the signal-to-noise ratio for volume rendering.

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40,000 rpm (100000g) to pellet vaults. Collected pellets were resuspended in 1× PBS buffer and assayed for purity by either SDS-PAGE with Coomassie blue staining or by Western blotting and quantitated by bicinchoninic acid (BCA). Collected fraction samples were standardized to vaults (1 μg/vial) and further diluted 1:10 for UV/vis absorbance spectroscopy in a 100 μl microquartz cuvette from 300 to 450 or 300 to 600 nm with the long wavelength of vault-only control samples to baseline. Compound concentration was calculated using known extinction coefficients (ε: 12500 cm⁻¹ mol⁻¹ L⁻¹ for 48 h and then harvested by washing with fresh media and resuspending pellets in 2% paraformaldehyde. The percentage of GFP⁺ cells was quantified by flow cytometry using an LSRFortessa machine (BD Biosciences).

AH Vault Packaged Bryostatin 1 in Vivo Animal CD69 Stimulation and Analysis. Animal experiments were approved by the UCLA animal research committee and conformed to all local and national guidelines and regulations. C57/B6 male mice were obtained from the UCLA Department of Radiation Oncology. A 100 μg amount of AH vaults at 10 ng of bryostatin-1 per μg of vault was resuspended in 150 μL of RPMI media and then administered intravenously via retro-orbital injection. At 24 h postinjection mice were sacrificed and the spleens isolated. Spleens were disaggregated by forcing tissue through a steel mesh and then filtered through a 40 μm filter to produce a single-cell suspension. These cells were then stained for flow cytometry to analyze surface expression of relevant markers.

Flow Cytometry Analysis of Cell Surface Markers. Cells were stained by first suspending them in 50 μL of a 1:1 mix of 1× PBS and AB serum (Sigma). For human PBMC analysis the cells were stained with anti-human CD69-phycocerythrin (Beckman Coulter). For mouse spleenocyte analysis the cells were stained with a combination of anti-mouse CD3-fluorescein isothiocyanate (eBioscience), CD69-phycocerythrin (eBioscience), and CD4-peridinin chlorophyll protein (eBioscience). Following antibody body addition, the mixture was incubated at 4°C for 15 min. The cells were then washed, resuspended in 1× PBS containing 2% paraformaldehyde, and stored at 4°C. Fixed cells were analyzed using an LSRFortessa (BD Biosciences). The resultant list mode files were processed using FlowJo software (version 7.6).

Conflict of Interest: The authors declare the following competing financial interest(s): V.A.K. and L.H.R. have financial interest in a startup company, Vault Nano Inc., that has licensed intellectual property used in this study from the Regents of the University of California.

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