Development of the Vault Particle as a Platform Technology

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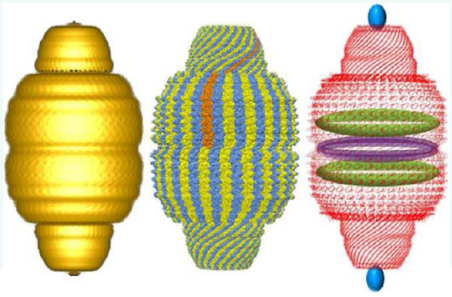
ABSTRACT Vaults are naturally occurring nanoparticles found widely in eukaryotes. The particles can be produced in large quantities and are assembled *in situ* from multiple copies of the single structural protein following expression. Using molecular engineering, recombinant vaults can be functionally modified and targeted, and their contents can be controlled by packaging. Here, we review the development of engineered vaults as a platform for a wide variety of therapeutic applications and we examine future directions for this unique nanoparticle system.



KEYWORDS: vault · nanoparticle · drug delivery · vaccine · adjuvant · immunotherapy · major vault protein (MVP) · INT (MVP interaction domain)

The discovery of the vault particle in 1986 was a serendipitous event, as these distinct ovoid shaped structures were first observed as contaminants of clathrin-coated vesicles which had been purified from rat liver and viewed under the transmission electron microscope (TEM).[1] Believing that they might be related to coated vesicles, the particles were purified to homogeneity and analyzed in more detail.[2] These initial studies revealed that the particles were unique and led to the discovery of an RNA component, distinguishing them as the largest known ribonucleoprotein particle (RNP) of the eukaryotic cell. The name vault was chosen to describe the particles' distinct multiple arched morphology, reminiscent of the vaulted ceilings of Gothic cathedrals (Figure 1).

Biochemical analysis of the particle revealed a relatively simple composition of three protein species and an RNA component. One of the proteins was considerably more abundant, making up approximately 75% of the mass of the particle. This ~100 kDalton (kDa) protein was designated the major vault protein or MVP. Two additional proteins with molecular masses of 290 and 193 kDa respectively were also detected.[2] The 290 kDa protein was later identified as TEP1, also known as the telomerase-associated protein 1,[3] and the 193 kDa protein, known as VPARP or PARP4, was determined to be related to the enzyme poly-(ADP-ribose) polymerase (PARP).[4] The RNA component (vRNA) was shown to be a small untranslated RNA[5] that associates with the vault through its binding to TEP1.[6]

Properties and proposed functions for these vault components and the native vault particle have been reviewed[7−12] and will only be briefly commented on here. A number of studies have implicated vaults in a broad range of cellular functions including nuclear-cytoplasmic transport, mRNA localization, drug resistance, cell signaling, nuclear pore assembly, and innate immunity.[12] The three vault proteins (MVP, VPARP, and TEP1) have each been knocked out individually and in combination (VPARP and TEP1) in mice.[6,13−15] All of the knockout mice are viable and no major phenotypic alterations have been observed. *Dictyostelium* encode three different MVPs, two of which have been cloned and knocked out singly and in combination.[16,17] The only phenotype seen in the *Dictyostelium* double knockout was growth retardation under nutritional stress.[17] If vaults are involved in one or more essential cellular functions, it seems likely that redundant systems exist that can ameliorate their loss.

Vault particles purified from rat liver have a mass of ~13 MDa.[18] Cryo-electron microscopy (cryo-EM) determined the particle dimensions to be 41 nm × 41 nm × 72.5 nm,

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making them the largest cytoplasmic ribonucleoprotein particle ever described.[19] Vaults have also been purified from other eukaryotes including mice, slime mold, torpedo electric eel, rabbit, cow, and sea urchin, and their unique multiple-arched morphology is highly conserved. Purified particles are virtually indistinguishable by TEM.[16,17,20−22] This conservation of structure is a result of the high conservation of the unique MVP coding sequence. Homologues of MVP have been identified in numerous species from protozoa to metazoans and even in chanoflagellates, the sister group to metazoa. Yet to date, no MVP homologues have been identified in yeast, worms, insects, or plants. The genomes of several unicellular eukaryotes including *Dictyostelium, Trypanosomes, Leishmania*, and *Acanthamoeba* encode multiple MVP homologues. The distinct vault structure is determined solely by the assembly of approximately 78 copies of MVP into the outer shell of the particle. The elegant arrangement of the MVP chains has been mapped by both low and high resolution crystal structures which correlate well with models predicted from low resolution cryo-EM image reconstructions[23,24] (Figure 2).

Vault Engineering. The idea that vaults could be engineered grew out of analysis of MVP expression in insect cells using the baculovirus system.[25] Initially MVP with an N-terminal 31 amino acid tag was expressed. The tag included a 6-histidine sequence as a means of purifying expressed MVP chains, however when the soluble fraction was passed over a NTA-Ni resin, none of the MVP protein bound to the column. This suggested that the histidine residues in the tag were not accessible to bind the column, yet all of the MVP was in the soluble fraction. When this fraction was centrifuged at 100 000*g* and the supernatant (S100) and pellet (P100) were analyzed by immunoblotting, all of the MVP was found in the P100 fraction suggesting particle assembly. Using a modified vault purification protocol, the assembly of MVP into vaults was demonstrated and the particles were characterized by TEM.[25] As insects do not have endogenous vaults (the Drosophila genome was lacking a gene for MVP and attempts to purify vaults from uninfected Sf9 cells were negative), it was concluded that these particles were formed entirely from the expressed MVPs. Analysis of the purified insect cell vaults revealed that the particles were assembled solely from the MVP and no high molecular mass TEP1- or VPARP-like protein bands were observed.[25] Remarkably, recombinant vaults are virtually indistinguishable from natural vaults when examined by TEM (Figure 1).

The notion that the entire vault structure could be formed by assembly of multiple copies of just one protein was surprising. Previous symmetry and stoichiometry calculations and models from cryo-EM reconstruction studies, led to the hypothesis that MVP constituted the barrel portion of the particle while TEP1, VPARP, and vRNA made up the caps.[18,19] The MVP baculovirus expression results invalidated these models and instead revealed that all of the information needed for assembly of the entire regular vault structure (as seen by TEM and cryoEM) was encoded entirely by MVP.[25] Cryo-EM difference mapping of rat liver vaults treated with ribonuclease suggested a location for the vRNA inside the particle[26] and vaults purified from TEP1 and VPARP knockout mice were likewise analyzed leading to models for the locations of these vault components inside.[19]

In addition to individual empty vault particles, some of the MVP expressed in insect cells assembled into larger particles whose structures appeared to be made

VOCABULARY: Adjuvant - a pharmacological or immunological agent often included in vaccines to enhance the recipient's immune response to a supplied antigen, but do not in themselves confer immunity; **chemokine** - a family of small proteins secreted by cells. Their name is derived from their ability to induce directed chemotaxis in nearby responsive cells. Some chemokines, such as CCL21, are considered pro-inflammatory and can be induced during an immune response to recruit cells of the immune system to a site of infection; **Chlamydia trachomatis** - an obligate intracellular human pathogen that is one of three bacterial species in the genus *Chlamydia. C. trachomatis*, is a prominent cause of sexually transmitted infection, with approximately 92 million cases occurring annually, and is an instigator of female reproductive dysfunction; **dendritic cells** - immune cells whose main function is to process antigens and present them on their surface to be recognized by other cells of the immune system, such as T-cells; **endocytosis** - a process by which cells take up molecules (such as proteins) by engulfing them. It is used by all cells of the body because most substances important to them are large molecules that cannot pass through the cell membrane; **immune cells** - white blood cells, or leukocytes are cells of the immune system involved in defending the body against both infectious disease and foreign materials. Five different and diverse types of leukocytes exist and all are derived from a multipotent cell in the bone marrow known as a hematopoietic stem cell. Leukocytes are found throughout the body, including the blood and lymphatic system; **T cells** - or T lymphocytes belong to a group of white blood cells known as lymphocytes. They play a central role in cell-mediated immunity. They can be distinguished from other lymphocytes, such as B cells and natural killer cells, by the presence of a T cell receptor on their surface. They are called T cells because they mature in the thymus; **vaccine** - a biological preparation that improves immunity to a particular agent that can cause a disease. A vaccine is often made from a weakened or killed form of a microbe, a toxin or a surface protein from a disease causing agent. The vaccine stimulates the body's immune system to recognize the agent as foreign, destroy it, and "remember" it;

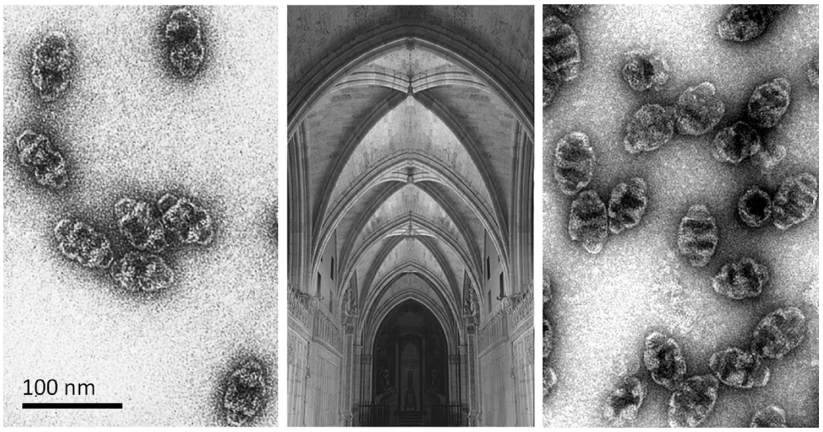

Figure 1. Natural and recombinant vault particles. Vault particles purified from rat liver were deposited on a carbon-coated grid and stained with uranyl acetate prior to viewing by TEM (left panel). Recombinant vaults (cp-MVP, see Table 1) purified from baculovirus infected Sf9 insect cells were likewise examined at the same magnification (right panel). Center panel illustrates the vaulted ceiling of a gothic cathedral.

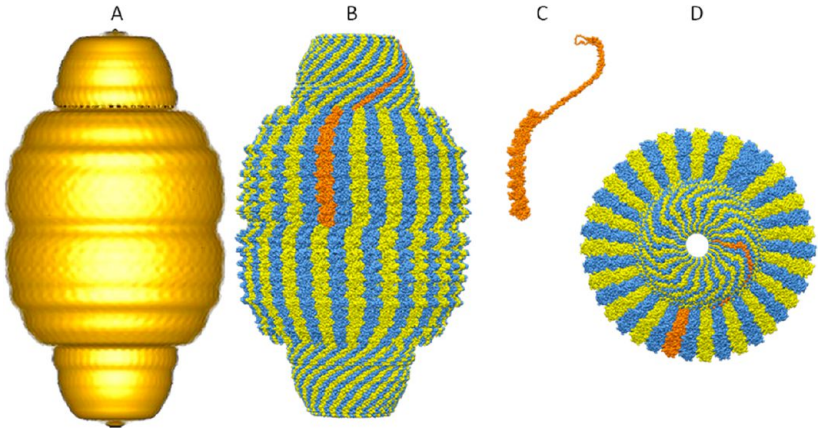

Figure 2. Vault Structure. Vault particles were analyzed by cryoEM and X-ray crystallography. The cryoEM reconstruction (A) is recombinant CP-MVP vaults, with imposed D48 symmetry at 16 Å resolution. Contributed by Dr. Phoebe Stewart (Case Western Reserve University) based on Figure 5 in Mikyas *et al*.[19] The space-filling models were produced with the UCSF Chimera software package using the 3.5 Å resolution native rat liver vault structure determined by Tanaka *et al*.[24] A side view (B) and end view (D) are shown with alternating chains colored blue and yellow and 5a single MVP chain colored orange. The orange chain from the vault side view is also shown alone (C).

up of multiple half-vaults. These structures, which were referred to as "vaultimers", could be grouped into larger half-vault aggregates (three, four, five, six half-vaults *etc.*).[25] The prevalence of vaultimers was later correlated with the addition of N-terminal tags on the expressed MVP, as vaults assembled from some other tags and untagged MVP were mostly devoid of these structures (Figure 1).[19] The structural arrangement of a single MVP chain into the assembled vault was modeled using cryo-EM difference mapping, where N-terminal tags were found to be located at the vault waist on the inside of the particle with longer tags having greater internal density.[19] Likewise, vaults assembled from MVP containing a C-terminal tag displayed extra density at the top and bottom of the vault, suggesting that the N-terminus began at the inside of the waist of the particle and extended all the way to the particle cap where the C-terminus was exposed at the surface (Figure 3).[19,27] Numerous extensions have been added to MVP cDNA and expressed using the baculovirus protein expression system to form modified vault particles. These various N- and/or C-terminal MVP recombinant particles have been named using an X-MVP-Y nomenclature where X describes the N-terminal tag and Y describes the C-terminal tag. The various tagged vaults are summarized in Table 1.

The model for the MVP alignment in the vault was essentially proven when an ~9 Å X-ray crystal structure of recombinant vaults purified from insect cells was carried out.[23] A further refinement to 3.5 Å resolution using crystallized rat liver vaults verified the low resolution structure prediction with a few notable differences.[24] First the chain route at the cap showed a more direct path and a double iris prediction was not substantiated in the higher resolution model. More importantly, the structures of these two very different particles (one recombinantly formed solely from MVP and the other purified from tissue and composed of all

vault components) were essentially identical. The 3.5 Å structure predicted 78 copies of MVP per vault which contradicted earlier scanning transmission electron microscopy (STEM) and stoichiometry analysis[18] which predicted 96 copies of MVP per particle, fitting the 8-fold symmetry seen in freeze etch images of open vault "flowers". More recent STEM and single particle end view cryo-reconstructions of recombinant vaults are more consistent with the 78 copy number (unpublished); however, the possibility still remains that vaults may have a variable number of MVPs (78 ± *n*).

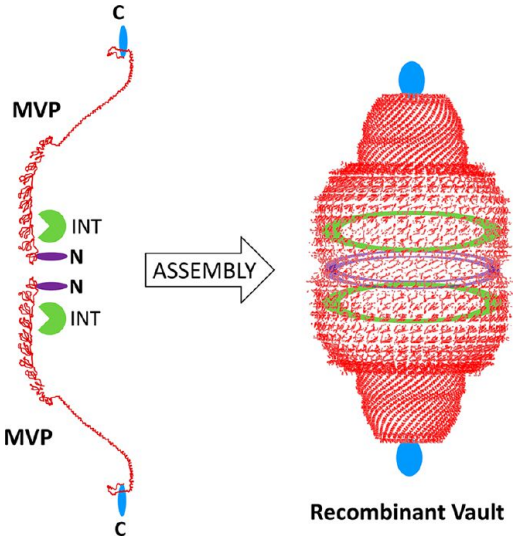

Figure 3. Schematic diagram of vault engineering. Two single MVP chains are illustrated on the left in red with the locations indicated where additional amino acids can be added at the N-terminus (purple) and C-terminus (blue). The INT peptide is illustrated in green aligned with its binding site on MVP. Assembly of the various MVP chains into a vault is illustrated on the right. The purple MVP N-terminal peptide extensions are located on the interior surface of the engineered vault particles at the waist (shown as two purple discs). The blue MVP C-terminal peptide extensions are located on the exterior surface of the particles at the end of the caps. When vaults are packaged with exogenous proteins fused to the INT domain, these proteins form two rings of density inside the particle (shown as two green discs).

Vault Packaging. With the goal of packaging foreign proteins into empty recombinant vaults to impart new properties, attention was focused on a previous study that utilized MVP as the bait in a yeast two-hybrid analysis and identified a C-terminal domain of VPARP as an MVP interacting protein.[4] The shortest VPARP C-terminal domain that was shown to interact with MVP was a 162 amino acid C-terminal fragment (VPARP residues 1563−1724) designated the MVP interaction domain (INT). INT could be reduced to 147 amino acids (VPARP residues 1563−1709) without any apparent loss of MVP binding.[28] As the INT domain was responsible for binding VPARP to MVP, it was hypothesized to act as a zip code directing the protein to the inside of the vault particle. This targeting ability was confirmed when INT was fused to firefly luciferase and coexpressed with MVP in Sf9 insect cells.[29] Luciferase-INT was found to copurify with recombinant vaults, and cryo-EM difference mapping revealed that the fusion protein was packaged inside the particles into two rings of density, one above and one below the particle waist[29] (Figure 3). The approximate location for the INT binding site has been independently mapped by NMR to MVP residues 113−221, the third and fourth repeat domains of MVP.[30] The NMR analysis fits nicely with the location determined by cryo-EM difference mapping. Enzymatic activity analysis of the packaged luciferase-INT indicated that the inside of the vault was not freely accessible to ATP. This was the first indication that the shell of the vault provides some protection to packaged contents.[29]

A number of different proteins have been fused to INT and all can be packaged into recombinant vaults (Table 2). The packaging process was further examined and found to not require cotranslation with MVP.[31]

TABLE 1. Recombinant Vaults Displaying Additional Motifs

vault type	N-terminal modification[a]	C-terminal modification[a,b]	reference
MVP			19
CP-MVP	cysteine-rich peptide		19
GFP-MVP	green fluorescence protein		29
CP-MVP-Z	cysteine-rich peptide	Z domain binds IgG	27
His-T7-MVP	His-T7 epitope tag		19,25
VSVG-MVP	VSVG epitope tag		19,25
pVI-MVP	adenovirus membrane lytic peptide (aa 34−53)		42
pVI-MVP-Z	adenovirus membrane lytic peptide (aa 34−53)	Z domain binds IgG	42
MVP-TAT		TAT tag a cell-penetrating peptide	74
MVP-VSVG		VSVG epitope tag	27
VSVG-MVP/MVP-EGF	VSVG epitope tag	epidermal growth factor	27
CP-MVP-RGD	cysteine-rich peptide	RGD for integrin binding	105
CP-MVP-RGD-STP	cysteine-rich peptide	RGD-Strep tag	105

[a] The following peptide tags were added to the N-terminus of MVP: His-T7 (MGSSHHHHHHSSGLVPRGSH MASMTGGQQPW); CP (MAGCGCPCGCGA); VSVG (MGYTDIEMNRLGKP); and pVI (AFSWGSLWSGIKNFGSTVKN). [b] The following peptide tags were added to the C-terminus of MVP: Z domain (FMNQQQRRFYEALHD PNLNEEQRNAKIKSIRDD); HIV-Tat 48 (GRKKRRQRRRAHQ); and RGD-Strep (RGDAWRHPEFGG).

TABLE 2. INT Fusion Proteins Packaged Inside Vaults

INT-fusion protein	fusion protein function	reference
INT	binds to MVP—packaging	4,28,31
GFP-INT	green fluorescence	29
mCherry-INT	red fluorescence	27
pVI-INT	adenovirus membrane lytic domain (aa 34—114)	40
CCL21-INT	chemokine	61
CCL21-mCherry-INT	chemokine, red fluorescence	61
MOMP-INT	membrane protein antigen	48
Luciferase-INT	enzymatic, light emission	29
OVA-INT	model antigen	58
ΔApo-AI-INT	forms a nanodisk with lipid	82

In fact purified INT fusion proteins were found to be packaged inside purified recombinant vaults by simply mixing the two components and incubating the mixture on ice for 30 min. This packaging was thought to occur *via* vault "breathing", a process previously characterized for virus particles.[32,33] As purified vaults are occasionally observed as half vault structures, a transient half-vault/whole-vault dynamic could also explain INT protein packaging.[31] The INT targeting domain has also been modified to allow it to sequester compounds within vaults that are not encoded in DNA. Recombinant INT containing an additional 31 amino acids at the N-terminus (included a 6-His tag, a thrombin cleavage site, and a T7 tag) was used to shuttle bound species into vaults.[28] This was accomplished utilizing Ni-NTA-nanogold, a material with affinity to the 6-His tag at the N-terminus of INT. A specific interaction of the gold clusters with 6-His-tagged recombinant INT was demonstrated, as well as the ability of INT to shuttle gold probes inside the vaults and bind to the vault interior.

Vault Dynamics. Fluorescence resonance energy transfer (FRET) was observed from polyethylene glycol (PEG) fused hybrid cells that expressed either CFP or YFP labeled vaults, indicating that vaults could exchange major vault protein (MVP) subunits *in vivo*.[34] Investigation into the mechanism of this exchange *in vitro* using epitope-tagged recombinant vaults suggested that they were capable of rapidly separating at the particle waist and reassembling back into whole vaults, supporting a half vault exchange mechanism.[34] It remains to be demonstrated whether this exchange is responsible for the *in vivo* FRET observations or if half vault exchange is strictly an *in vitro* phenomenon.

Fluorescence spectroscopy, multiangle laser light scattering, and the quartz crystal microbalance were used to probe recombinant vault conformational changes in response to variations in solution pH.[35] Vaults were found to disassemble into halves at pH 3.4; however, this conformational change was irreversible.[35] A variety of spectroscopic techniques (*i.e.*, circular dichroism, fluorescence spectroscopy, and light scattering) along with EM were used to characterize the structural stability of vaults over a wide range of pH (3—8) and temperature (10—90 °C). Ten different conformational states of the vaults were identified over the pH and temperature range studied with the most stable region at pH 6—8 below 40 °C and least stable at pH 4—6 above 60 °C. A unique intermediate molten globule-like state was also identified at pH 6 and ~55 °C. EM imaging showed the opening of intact vaults into flowerlike structures when transitioning from neutral to acidic pH.[36]

While vaults dissociate into halves at pH less than 4.0, covalent cross-linking of available amine groups was found to prevent this low pH dissociation. When a cleavable, amine-reactive bifunctional coupling reagent was used, cross-linked vaults stayed whole at low pH; however, they dissociated into halves at low pH when the cross-links were cleaved.[37] In contrast, covalent cross-linking of cysteine sulfhydryl groups, made available at the vault waist *via* an N-terminal CP tag (Table 1), did not bestow pH stability on treated vaults, suggesting that sulfhydryl-reactive cross-linkers do not couple opposite vault halves, rather they introduce cross-links within each individual vault half.[37]

Interestingly a semiconducting polymer [poly-(2-methoxy-5-propyloxy sulfonate phenylene vinylene), MPS-PPV] was demonstrated to be encapsulated inside recombinant vaults presumably driven by charge—charge interactions.[38] While the electrostatic environment inside a vault is not completely understood, the ease of inclusion of MPS-PPV (an anionic polyelectrolyte) suggests the presence of a significant number of positively charged amino acids at the inner surface of vaults in contrast to the exterior. The results indicate that a polymeric polyanionic drug should also be encapsulated inside the vaults in a manner similar to that used for MPS-PPV. If such a drug could then be depolymerized (for example using a pH change or external irradiation), this would create a slow release system.[38]

Vault Targeting to Cells. Vaults packaged with an INT-tagged fluorescent protein (GFP or mCherry) have been added to HeLa cell cultures and their uptake monitored using confocal microscopy.[27,29] This uptake, which probably occurred by endocytosis, was not specific, nor efficient, suggesting that targeting strategies would need to be developed if vaults were going to be useful as a general delivery vehicle. By adding C-terminal peptide extensions to MVP, recombinant vaults were formed with these peptides accessible on the exterior surface of the particles at the caps.[27] Two different tags were engineered onto the C-terminus of MVP to facilitate targeting to epithelial cancer cells (A431) *via* the epidermal growth factor receptor (EGFR): a 33 amino acid Fc-binding peptide (called the Z domain[39]) and the 55 amino acid epidermal growth factor (EGF). The modified vaults were found to bind specifically to A431 cancer cells either directly (EGF modified vaults) or as mediated by a monoclonal antibody (anti-EGFR) bound to recombinant vaults

containing the Z domain. EGFR was chosen as it is upregulated in numerous cancer cell types. Thus both specific (peptide-directed) and general (antibody-mediated) methods could be used to target recombinant vault particles to cells.[27]

Vault Targeting to the Cytoplasm. Since endocytosed material is often transferred to the lysosome for degradation, vault-mediated delivery of therapeutic agents to cells may require escape of the particle from the endocytic compartment. Untargeted recombinant vault particles likely enter cells *via* macropinocytosis or phagocytosis but lack demonstrable membrane penetrating activity.[40] Many viruses have solved this problem by producing proteins that can open the endocytic membrane. Adenovirus enters cells *via* endocytosis and the reduced pH of the endocytic compartment facilitates partial disassembly of the viral capsid concomitant with the release of adenovirus protein VI (pVI).[41] The N-terminal region of pVI contains a putative amphipathic α-helical domain (amino acid residues 34−54) that exhibits potent membrane lytic activity as measured by the disruption of artificial lipid membranes (liposomes).[41] To explore the feasibility of improving vault penetration into target cells, the membrane lytic domain of pVI was incorporated into the interior of recombinant vault particles *via* fusion to the INT targeting domain.[40] The membrane lytic activity of the pVI domain was retained upon incorporation into vault particles. Moreover, internalization of vault/pVI-INT particles into murine macrophages promoted codelivery of a soluble ribotoxin or a plasmid encoding GFP.[40] These findings indicated that vault particles could be modified to enhance cell uptake of selected biomolecules. One problem with this approach was that relatively large numbers of vault/pVI-INT particles ($>10^6$/cell) were needed to mediate toxin and gene delivery. In fact relatively high amounts of adenovirus particles (*i.e.*, 20 000 per cell) are also needed to efficiently deliver ribotoxin.[41] The higher numbers of particles needed for vault/pVI-INT delivery likely arise from the fact that, unlike adenovirus, these nanoparticles lack a cell targeting ligand and a cell surface vault receptor has yet to be characterized. To remedy this situation, vaults engineered with MVP C-terminal EGF (see above) were packaged with pVI-INT and specifically targeted to the EGF receptor. Using this targeting approach, cells could be transfected with ∼50 000 vaults per cell.[42] An even more efficient membrane lytic vault was produced by fusing the 20 amino acid membrane lytic domain of pVI directly to the N-terminus of MVP.[41] By combining this construct with the Z domain, a single vault structure (referred to as the pVI-MVP-Z vault) was obtained that, when complexed with anti-EGFR antibodies, could deliver biomaterials by a highly efficient process requiring as few as 500 vaults per cell. This vault nanoparticle targeted specific cell surface receptors, yet it retained its ability to package exogenous protein payloads. These particles were quite effective at facilitating the delivery of plasmids to the cytoplasm of cells as measured by plasmid expression.[42] Furthermore these particles were considerably less toxic than a commercial transfection reagent in the A431 cell line that was used in this study.[42]

When the endocytosis of antibody bound pVI-MVP-Z vaults was directly monitored by packaging mCherry-INT into the particles, fluorescence microscopy indicated that these vaults escaped endosomes in areas surrounding the outer rim of cells in less than 5 min after vault addition. This result was consistent with previous studies which indicated that vaults containing pVI caused a rapid and progressive disruption of liposomes within ∼2 min.[40] The very rapid interaction between pVI and the endosomal membranes likely occurred shortly after formation of the endosomal compartment. By 30 min, vaults were already released from presumed endosomes and located independently within the cytoplasm, while vault particles without pVI were still trapped in endosomes and early lysosomes. Significant disruptions of the endosomal membrane must have occurred in order for the fluorescent mCherry-INT protein to enter the cytoplasm. However, cells did not appear damaged by the endosome disruption induced by the pVI-MVP-Z vault, as they regained a normal morphology and could proliferate, indicating that the endosome disruption did not appear to be cytotoxic.[42] Thus vaults have been successfully engineered to specifically deliver cargo to the cytoplasm of cells with high efficiency.

Therapeutic Applications: Vaccines. All human cells thus far analyzed have been shown to contain vaults with quantities varying from a few thousand per cell to in excess of 100 000 per cell.[43] As a naturally occurring nanocapsule, the vault particle may be useful as a therapeutic delivery system. The particle has many of the properties thought to be advantageous for such a system. First, the particle's size (<100 nm) is small enough to prevent it from being trapped in the kidney and/or liver if administered intravenously, a fate that is often seen with structures above 200 nm, and particles less than 200 nm can freely access the draining lymph nodes when injected intradermally.[44] Second, the particle has a large interior space, large enough to encapsulate hundreds of proteins. Third, the protein shell of the vault offers some protection from external proteases; and fourth, vaults appear to be biodegradable. Finally, vaults are nonimmunogenic (see below). Engineering of the C-terminus of MVP allows the particle to be targeted to cell surface receptors for delivery of selected cargo. Two additional properties of the recombinant vault particle that may make it an ideal structure to engineer as a therapeutic delivery system are its monodispersity and regularity. Unlike chemically synthesized nanoparticles, vaults produced

TABLE 3. Properties of Vaults Compared to "Ideal" Delivery Vehicles

PROPERTY	"IDEAL"	VAULTS
Size /Payload	<100 nM , homogenous, regular , large payload	✓ 70 nM , homogenous, regular , ✓ 50 x 10^6 Å^3 internal volume
Stability	Serum, tissue / protect payload	✓ Highly stable/protects contents Serum, tissue stability (TBD) *
Safety	Biocompatible Non-immunogenic Non-toxic	✓ Natural protein/biocompatible ✓ Non-immunogenic Toxicity (TBD)
Purity	No adventitious agents Pyrogen free	TBD
Production	Scalable/batches reproducible	✓ Scalable/batches reproducible
Dispersity	Monodisperse	✓ Monodisperse
Surface	Non-fouling (low surface opsonization)	✓ Likely to be non-fouling**
Delivery	Targetable, retainable	✓ Targetable, retained
Release	Controlled release	✓ Slow release kinetics of packaged proteins
Multifunctionality	Proteins/drugs/DNA/RNA etc..	✓ Proteins/drugs /polymers

* TBD, to be determined. ** As a natural protein capsule found in all higher eukaryotes, surface opsonizaton has not been observed nor is it expected.

by cellular expression appear to be entirely regular given that they can be crystallized and their structure characterized by X-ray diffraction. Vaults also remain monodisperse even when kept at a high concentration. A summary of the various properties that are desired in a nanocarrier to those of the vault is shown in Table 3. Vaults have most of the "ideal" properties and thus far no properties that predict problems with the platform have been seen; however, toxicity and purity assessments await more extensive animal and human testing. Liposomes, the most highly applied nanoparticle platform to date, also satisfy most of the desired properties listed in Table 3, however, liposome stability and targeting can be problematic and the controlled release of encapsulated drugs has not been easy to regulate. INT fusion proteins packaged into vaults have been shown to be slowly released. Release kinetics are dictated by the affinity of INT with its binding site on MVP. It should be possible to "tune" protein release by amino acid alterations in the INT sequence and/or the MVP INT binding site. Virus capsids are similar to vaults as they are naturally occurring protein capsules; however, issues of toxicity and immunogenicity seriously impact the use of viruses as nanocarriers especially in applications where multiple administrations are required.

Vaults are highly stable structures *in vitro*, and a number of studies indicated that the particles are nonimmunogenic. First, purified rat vaults were unable to induce antibodies in rabbits unless they were hemocyanin cross-linked.[2] Second, a large panel of human autoimmune antibodies has been screened and no evidence was found for autoantibodies against any vault proteins (unpublished). Finally, immunogenicity testing of recombinant vaults in rats using a rather aggressive subcutaneous injection routine, showed no immunoreactivity against the recombinant vaults (unpublished). As detailed above, vaults can be engineered and expressed using the baculovirus expression system, and heterologous proteins can be encapsulated using the INT packaging domain strategy. The internal cavity of the recombinant vault is large enough to accommodate multiple immunogenic proteins and an initial application developed for the particle was to use vaults as mucosal adjuvants.

Since vaults are the size of small microbes and are expected to freely access the draining lymph nodes,[44] a vault particle containing an immunogenic protein would be expected to be readily phagocytosed by dendritic cells. Further, as recombinant vaults containing proteins can be produced and purified in large quantities, the particle could serve as a viable vaccine delivery scaffold if the vaults proved facile for generating mucosal immunity. To produce an optimal mucosal immune response, mucosal associated lymphoid tissue needs to be stimulated. For example, airborne allergens enter nasal surfaces where they stimulate the induction of immune responses within nasal-associated lymphoid tissue (NALT).[45] Because mucosal immune surfaces are interconnected, immune stimulation at one mucosal surface produces an immune response at distant mucosal surfaces.[46] Immune cells and antibodies can even appear at vaginal surfaces following stimulation of the distant nasal mucosa.[47] A study using *Chlamydia trachomatis* infection was carried out to test the utility of vaults as a mucosal vaccine delivery platform.[48] This infection relies on cell-mediated mucosal immune responses for elimination

and is a significant burden on health care. *C. trachomatis* is a prominent cause of sexually transmitted infection, with approximately 92 million cases occurring annually[49] and is an instigator of female reproductive dysfunction.[50] To eradicate infection, T helper immune cells (Th1) must be present within vaginal tissues.[51] However, a vaccine has not yet been produced which induces sizable Th1 immune responses in mucosal tissues.

Delivery of particles to dendritic cells (DC) through the Fc immunoglobulin receptor (FcR) has been proposed as an effective vaccination strategy for boosting cell-mediated immune responses against *C. muridarum* infection and other pathogens.[52,53] Further, Th1 responses and immunity against chlamydial genital infection were shown to require the presence of FcR on DC.[53] Vaults engineered to display the Fc-binding Z peptide (see above and Table 1) were shown to bind to and be taken up by DC, presumably through bound mucosal immunoglobulins or through direct binding to the Fc receptor.[48] Vaults were engineered to package the major outer membrane protein (MOMP) of *Chlamydia muridarum,* a highly immunogenic protein that has been used as an antigen to lessen development of infertility after Chlamydia infection.[54,55] A MOMP-INT fusion protein was produced and packaged into recombinant cp-MVP-Z vaults (Tables 1 and 2). These MOMP-vaults were efficiently taken up by DC in culture where they induced DC maturation and the secretion of cytokines and chemokines necessary for producing immune responses. The mechanism of MOMP-vault action on DC was shown to be through TLR-independent activation of inflammasomes. Further, vaults did not cause DC to secrete factors associated with tissue inflammation and were therefore referred to as "smart adjuvants".[48] Using a *C. muridarum* genital infection model,[56] mice vaccinated with MOMP-vaults were found to have a reduced bacterial burden following chlamydial genital infection as compared to a promising liposome preparation[57] which contained the same antigen (MOMP). Figure 4 illustrates the protective effect of intranasal vaccination with the MOMP-vault and live *C. muridarum,* compared to a control vault (GL-vault) which imparts no protection. This study indicated that vaults can serve as a novel adjuvant for inducing protective immunity against microbial infection at mucosal surfaces while limiting excessive inflammation.[48]

To further probe the mechanism of vault-induced immunity, a follow-up study was performed to characterize the types of immune responses elicited by engineered vault nanocapsules compared to another type of nanocarrier, liposomes, using a well-characterized model antigen, ovalbumin (OVA).[58] Ovalbumin is a highly immunogenic antigen and has often been used as a proof of principle for numerous vaccination strategies.[59,60] Full-length ovalbumin was fused to INT and

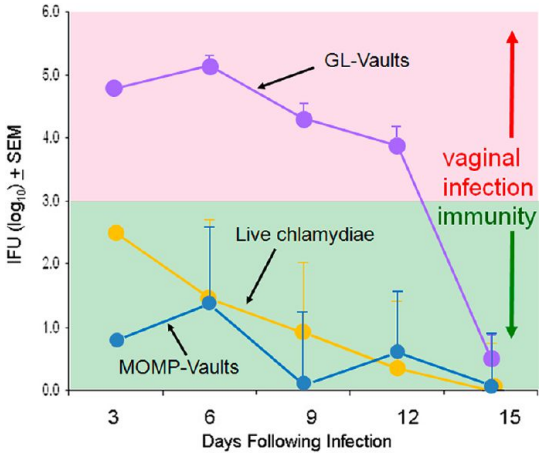

Figure 4. MOMP-vaults reduce *C. muridarum* infection. The bacterial burden following a challenge infection was determined from vaginal swabs to be statistically reduced in the MOMP-vault immunized group compared to the positive control group that had been previously immunized intranasally with live *C. muridarum* (Live chlamydiae). Like the mice immunized with live chlamydiae, the mice immunized with the MOMP-vault developed a robust immunity to a challenge infection. As a control, mice immunized with vaults containing the green fluorescence protein fused to INT (GL-Vaults) did not develop immunity until 15 days after the challenge infection, a process that occurs naturally in mice in response to chlamydia (from ref 48).

packaged into recombinant vaults (OVA-vaults). Immunization of mice with OVA-vaults was more effective at generating cellular immunity, as characterized by increased numbers of OVA responsive memory CD8+ and CD4+ T cells. Modification of the vault, by addition of the ``Z'' domain, altered the level of anti-OVA Ig subclass as shown by an increased IgG1:IgG2C ratio. Furthermore immune responses against OVA induced by OVA-vault nanoparticles differed from those induced by ovalbumin-packaged liposomes. An important feature of vault adjuvants was the robust induction of CD8+ and CD4+ memory T cells.[58] Taken together, these two studies indicated that vaults could be used as subunit vaccines which can generate both cellular and humoral immunity and they provide a rationale for using vault nanocapsules to develop vaccines against antigens for human pathogens and cancer.[48,58]

Therapeutic Applications: Tumor Immunotherapy. Since proteins packaged into vaults through INT binding interactions appear to be reversibly associated with the particles,[31] vaults seem most appropriate for applications where the slow release of proteins or peptides is desired. One particularly promising use of vaults has been to deliver an immune activating peptide intratumorally for the purpose of initiating anti-tumor immune responses in lung cancer. In this study the effect of vaults engineered to deliver CCL21 on the growth of tumors was evaluated *in vivo*.[61] CCL21 is a lymphoid chemokine able to attract a variety of immune cells including T cells and dendritic cells (DC)

through binding to the chemokine receptor CCR7.[62−65] Intratumoral administration of recombinant CCL21 reduces tumor burden in murine lung cancer models;[66] however, the antitumor activity required high and frequent dosing because proteins administered intratumorally are not retained locally for prolonged periods. To overcome this limitation, DC have been engineered for intratumoral CCL21 delivery.[67,68] A phase I clinical trial in advanced nonsmall cell lung cancer has been initiated using intratumoral injection of DC transduced with an adenoviral vector expressing the CCL21 gene (Ad-CCL21-DC).[69] The procedure for treating patients in this trial is cumbersome, expensive, and time-consuming and therefore vaults were engineered to deliver CCL21 in an attempt to circumvent autologous DC preparation and minimize batch to batch variability.

Although empty vaults were mildly chemotactic, packaging the chemokine (CCL21-INT) into the vault particle (CCL21-vault) synergistically enhanced T cell migration, suggesting that the sustained release of CCL21 by the vault could establish a steep chemotatic gradient.[61] Mice implanted subcutaneously with Lewis Lung (3LL) tumor cells develop tumors rapidly, and these tumors are not attacked by the mouse immune system. Intratumoral injection of CCL21-vaults was found to promote the recruitment of T lymphocytes and DC into the tumor microenvironment leading to a robust antitumor response (Figure 5). The results demonstrated that the vault design is effective at inhibiting the growth of established tumors since a single injection of CCL21-vaults led to significant inhibition in tumor growth compared to controls.[61] The CCL21-vault formulation was as effective as frequent high doses of recombinant CCL21.[66] Vaults engineered to release CCL21 hold significance for wide application as an "off the shelf" reagent for treatment of a broad range of malignancies.

The intratumoral delivery of a nanoparticle therapeutic, such as described above, could be advantageous over intravenous delivery. Systemic delivery often results in nonspecific ``off-target'' tissue damage and particle clearance can reduce efficacy. With the rapid advance of precision guided technologies, new methods of delivering drugs directly to the site of the cancer (local treatment), rather than systematically, have been developed and are gaining traction. For example, recent clinical trials on patients with glioblastoma multiforme (GBM) have shown that the injection of the chemotherapeutic drug carboplatin by convection-enhanced delivery directly into the peritumoral region provoked a significant killing of GBM cells at concentrations that were not toxic to normal brain.[70,71] Patients with ductal carcinoma *in situ* breast cancer that were injected with a low dose of doxorubicin directly into their mammary ducts were found to have tumors less than half the size of those receiving the drug intravenously.[72] When doxorubicin-loaded

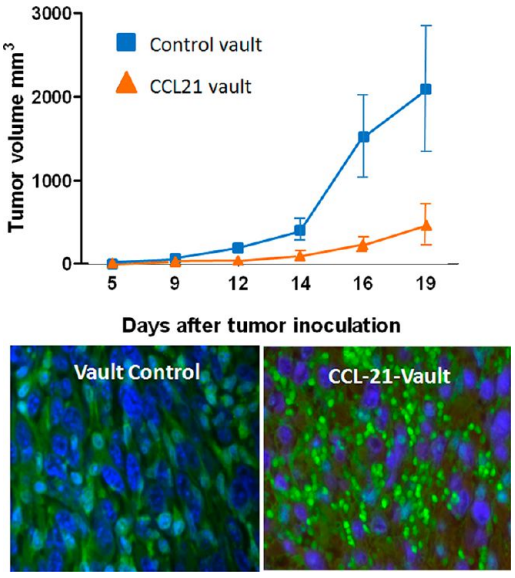

Figure 5. CCL-21-vaults inhibit the growth of Lewis Lung tumors by attracting immune cells. (Top) Mice were implanted with 3LL tumor cells and after nine days established tumors were injected intratumorally with 200 ng of control vaults (blue squares) or CCL21-vaults (red triangles). Tumor growth was assessed by measuring their diameters using calipers up to day 19. (Bottom) Transgenic mice engineered to express enhanced green fluorescent protein (GFP) under the direction of the human ubiqutin C promoter (UBC-GFP mice) were used to establish 3LL tumors lacking GFP expression. These tumors were treated with intratumoral injection of control or CCL21-vaults as above and on day 19 tumor tissue was frozen, sectioned, fixated onto slides, and counterstained with the nuclear dye DAPI (blue). Control tumors (bottom left panel) demonstrate very limited green fluorescence (infiltrating cells). In contrast, prominent tumor-infiltrating lymphocytes, small round green fluorescent cells originating in the host animal are evident in tumors from the CCL21-vault treated mice (bottom right panel) (from ref 61).

nanoparticles (NPs) were delivered by inhalation to the lungs of animals with nonsmall cell lung carcinoma, a highly significant improvement in survival compared to all control groups was observed and this mode of delivery was associated with lower side effects.[73]

In an effort to optimize the vault-based drug delivery system for direct, nonsystemic applications in cancer therapy, a cell penetrating peptide (CPP) was engineered onto the exterior surface of vaults.[74] CPPs have emerged as a valuable class of short peptide sequences that have the capacity to deliver macromolecular cargos that can be 100-fold higher in molecular weight than the peptides alone.[73,75] A 13 amino acid peptide (GRKKRRQRRRAHQ), derived from a truncated version of the HIV1 TAT protein[76,77] was bioengineered onto the C-termini of MVP, resulting in localization of this peptide at the end of the vault caps. Multiple techniques were used to demonstrate that vaults expressing surface TAT peptides (TAT-vaults) showed increased binding to a wide variety of cell types and these bound vaults were internalized at higher efficiencies than vaults lacking the CPPs.[74]

The ability of the TAT-modified vaults to bind to a wide variety of cells predicts that this moiety may be able to potentiate the antitumor effects of the CCL21-vault described above for direct tumoral injection. Furthermore, the cell-binding properties of the TAT-vault could also be an advantage for applications using intratumoral injection of vaults engineered to contain cytotoxic drugs (see below).

Therapeutic Applications: Delivery of Hydrophobic Drugs. The vaccine and chemokine delivery applications described above take advantage of the INT packaging domain that binds to the inside of the vault particle to package protein-based therapeutics. Packaging traditional drugs into vaults presents an entirely different challenge. Small hydrophobic drugs with promising potential *in vitro* are often limited in their *in vivo* use due to poor pharmacokinetic and pharmacodynamic properties. The development of nanoparticle-based platforms have enhanced the delivery of current therapeutic compounds and circumvented the adverse pharmacological properties of these conventional drugs.[78] These new drug delivery systems overcome current limitations by offering new environments for improved solubility, thereby eliminating the need for many toxic organic solvents. Common examples include the use of dendrimers, liposomes, or conjugation to polymers, such as polyethylene glycol.[79−81] The latter two have had considerable success and have been approved for clinical use despite existing pitfalls, such as size limitations and lack of tissue targeting. With the goal of creating a vault capable of encapsulating therapeutic compounds for drug delivery, a strategy to package another nanoparticle, known as a nanodisk (ND), into the vault lumen was developed.[82] Nanodisks are small discoidal lipid bilayer fragments derived from a truncated form of apolipoprotein-AI (Apo-AI, amino acids 44−200) containing a series of amphipathic helices that encircle the disk's circumference in a beltlike manner, resulting in a nanoparticle with an average diameter of approximately 10−20 nm.[83,84] As a lipid bilayer, NDs provide a rich lipophillic domain that can absorb hydrophobic compounds.

NDs have been previously shown to bind a variety of drugs and improve their effectiveness.[85−88] Although these NDs can be modified for tissue-specific targeting directly, lipid surfaces remain exposed, potentially allowing for exchange with surrounding cell membranes, which could limit their usefulness as a delivery system.[89] It was hypothesized that vaults could be used to shield drug-loaded NDs from the external medium. Packaging of NDs into the vault was achieved by fusing the INT domain to a truncated form of Apo-AI to form ΔApo-AI−INT. A modified ND was then assembled, designated as nanodisk−INT (NDI), and shown to be packaged into recombinant vaults. These particles retained drug during ND formation and packaging into the vault.[82] As a proof of principle, a drug previously shown to bind ND, all-trans retinoic acid (ATRA), was selected.[87] ATRA is a gene transcription regulator that acts by binding to receptors for retinoid acid and retinoid X.[90,91] This binding leads to changes in a variety of genes involved in cell proliferation, differentiation, and apoptosis. ATRA has been shown to be useful in the treatment of a variety of illnesses ranging from acne to cancer.[92−94] However, ATRA is not without significant drawbacks including being highly insoluble, teratogenic, and having the possibility of leading to retinoic acid syndrome; properties that could be improved if it were used in a delivery system.[95,96]

ATRA could be selectively and stably incorporated into NDI-packaged vaults and these particles would retain the biological activity of the sequestered drug.[82] Incorporation of ATRA into vaults is a promising first step toward developing the vault as a system capable of delivering small hydrophobic drugs. We expect that the general strategy of sequestering lipids into the vault particle will allow the vault to be used with a myriad of therapeutic compounds, thereby making the vault a general and versatile vehicle for drug delivery.

Future Directions. As a delivery system the vault nanoparticle has incredible potential, yet only a few applications have thus far been investigated. *In vitro* applications, such as the use of vaults as delivery regents for biological materials, are likely to be the first commercial applications for the particle. One could envision the pVI-MVP-TAT vault as a versatile reagent that could be packaged with an INT-fusion protein of choice to facilitate delivery into cells. Although vaults have yet to be engineered to package DNA, the pVI-MVP-TAT vault might still be useful as a transfection reagent, as plasmids added to cells together with these vaults would be facilitated in their entry into the cell cytoplasm by a bystander effect.[42] Alternately Z-tagged vaults could be used to transfect cells normally resistant to transfection by binding the particles to antibodies specific for cell surface receptors. Targeting to endosomes, lysosomes, or cytoplasm does not appear to be difficult.[40,42]

Therapeutic delivery using vaults in humans will require the production of GMP vaults and the extensive toxicology, absorption, distribution, metabolism, and excretion testing that is required of all new drugs. Additional chemical characterization of particle structure, composition, quality, stability, and purity will also be required as these steps are obligatory for all new nanomaterials.[97−99] High yield bulk production will need to be worked out for the vault particle, either in Sf9 cultured cell systems or new protein production platforms such as insect larva.[100] Large scale and reproducible particle purification methodologies will need to be developed, preferably without ultracentrifugation steps. The FDA is concerned with drug

manufacturing and insists on consistency in batch to batch production and potency.

Modifying the vault particle so that it can be used in a number of other applications is certainly possible and a few variations are worth mentioning here. A high priority for new therapeutics is the development of effective gene therapy vectors that can selectively and efficiently deliver a gene to target cells. Significant challenges to gene delivery include protection of labile DNA, specific tissue and cell targeting, transport across cell membranes, localization to the appropriate subcellular compartment and controlled release. A wide variety of strategies for delivery of DNA exist, including viral and nonviral vectors. Interest in nonviral vectors has grown from the promise that they may be simpler to use, more readily adaptable to large-scale production, and able to avoid an immune response (reviewed in[101,102]). In addition, a major drawback of current viral-vector technology is that gene transfer can occur nonspecifically to cells other than the desired target cells. This not only decreases the overall efficacy of the viral vectors, it increases the risk of inducing neutralizing antibodies against the transgene product.[103,104] Nonviral methods that have been developed include naked plasmid DNA injection, the gene gun, electroporation, and encapsulation into liposomes or nanoparticles (reviewed in ref 101). None of these approaches are ideal, due to one or more limitations, which include poor biocompatibility, lack of targeting specificity, inability to control release, low efficiency, and narrow flexibility. It is tempting to speculate that vault particles engineered to package nucleic acids could overcome most if not all of these limitations. A number of approaches toward nucleic acid packaging are possible including engineering of DNA or RNA binding domains into the inside of the particle either through fusion of protein domains to the N-terminus of MVP or to INT. These approaches could also be useful for engineering the particle to sequester siRNAs.

As the current technology for vault engineering is most highly developed for protein packaging, future applications should also be considered where unstable proteins may be stabilized by packaging into the vault particle. This could be useful for increasing the stability of circulating enzymes in the bloodstream or for increasing the half-life of cytoplasmic enzymes that could be engineered to associate with endogenous vault particles as a means of protecting them from intracellular digestion. Although materials packaged into vaults by virtue of the INT binding domain appear to be released from the particle, it will be important to engineer this binding interaction so that protein release can be controlled and regulated. Applications can be anticipated where the release of contents could range from minutes to days, and thus a complete understanding of the binding dynamics between INT and MVP is necessary. Just as protein packaging into the vault particle may be useful for protein stabilization, it could also be a useful means of reducing the concentration of toxic proteins in the cellular environment. Vaults could be engineered to sequester toxins released by pathogenic organisms by placing toxin binding sites on the inside of the particle possibly through fusion to MVP. Combining toxin sequestration with an enzymatic step to inactivate the toxin, possibly through an INT-targeted degradative enzyme, would result in a vault bioreactor. Such bioreactors, housed in the constricted reaction volume of the vault lumen should present an ideal geometry, as small molecule reaction byproducts could diffuse out of the permeable vault shell while bound protein catalysts would be retained where high concentrations of substrates are sequestered.

The direct engineering of the vault particle has thus far been limited to peptide additions to the N- and C-terminus of MVP. Although these approaches have led to a variety of applications, many other approaches should be possible. The highest resolution structure of the vault produced by X-ray diffraction is 3.5 Å, as higher resolutions are obtained, precise amino acid substitutions can be planned so as to alter the internal environment of the vault to facilitate binding of specific cargo. Substitutions that alter the outside of the particle could be used to facilitate targeting and alter pharmacokinetics and pharmacodynamics. tRNA suppressor mutations could allow for incorporation of unnatural amino acids to enable highly specific, high-yield click chemistry modifications to be carried out inside and outside the particle.

More dramatic modifications may also be possible such as increasing and decreasing the length and altering the shape of the vault to tailor the size of the internal cavity. These changes will likely require knowledge of vault assembly, a process which at the present time is completely uncharacterized. In fact, too little attention has been focused on vault formation which has been assumed to occur automatically in the cell cytoplasm from individual MVP monomers through a self-assembly process. However, the size, complexity, and homogeneity of the finished particle, coupled with the fact that MVP monomers have not been found to reassemble into vaults, suggest significant assembly assistance could be required by chaperones known and yet to be discovered.

In light of the variety and ease of vault engineering that can be anticipated, it is tempting to speculate a number of futuristic applications for the particle ranging from intracellular biological sensors to flexible nanomachine component parts. These flights of imagination are possible because of the elegant structure of the vault and the myriad of possibilities for engineering the particle using standard molecular biology techniques.

Conflict of Interest: The authors declare no competing financial interest.

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