VAULTS: NATURES GARBAGE COLLECTORS

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Abstract

Since the discovery of vaults, several different applications have been investigated. Vaults are the biggest known eukaryotic ribonucleoproteins. Through engineering vaults, empty structures can be produced, opening up a wide range of applications for those particles. From therapeutic applications like vaccines or delivery vehicles to enzyme stabilization for enzymatic bioremediation, recombinant vaults can be used in diverse areas. This review will highlight the different applications of engineered vaults for a wide range of therapeutic applications. It highlights the promising results of using vaults for the immobilization of enzymes, able to degrade wastewater contaminants and dramatically reduce the reproductive toxicity of the side products. Challenges and future directions will be discussed to highlight future direction and potential unexplored fields for the application of vaults.

Introduction

When vaults were first discovered in 1986, nobody expected the vast number of different applications for which these structures could be used. First discovered as contaminants of clathrin-coated vesicles, these particles were purified and further investigated using transmission electron microscopy (TEM) and biochemical analysis.[1] This investigation revealed an RNA component, making them the largest known ribonucleoprotein particle of eukaryotic cells.[1,2] After the first structural analysis of the vault was conducted, they received the name they are known for today due to their similarities to the *vaulted* ceilings of Gothic cathedrals.[2] Further analysis revealed the components of the vault: the major vault protein (MVP), the telomerase-associated protein 1 (TEP1), and an enzyme related to the poly-(ADP-ribose) polymerase (VPARP).[2–4] Besides these proteins, a small untranslated RNA (vRNA), which strongly associates with TEP1, was also discovered.[5,6]

Using a baculovirus expression system, it was demonstrated that MVP alone is responsible for forming a vault, contradicting the then accepted model that all of the parts mentioned above are necessary for the vault structure.[7] These studies suggested that only MVP is required to form the typical structure of vaults, with around 78 copies per vault.[8,9] With these findings, the possibility of packaging different proteins within an empty vault was explored.

In order to achieve packaging, it was explored how to encapsulate proteins inside vaults. VPARP is strongly associated with the MVP.[4] It was determined that a 162 amino acid domain of VPARP is responsible for this interaction.[10] This discovery inspired scientists to further explore this domain as a possible route to encapsulate proteins into vaults. The MVP interaction domain, abbreviated "INT", was first fused to firefly luciferase.[11] After co-expression and cryo-EM difference mapping, it was revealed that the enzyme was successfully incorporated within vaults and was still fully functional.[11] Furthermore, it was discovered that co-expression of the MVP and the INT fused protein was unnecessary to incorporate proteins into vaults, as simple incubation of vaults together with the protein was enough to do so.[11] Since this discovery, several other proteins have been incorporated to the inside of vaults by fusion to INT.

Even though their exact function is unknown, numerous possible applications of these unique particles have been explored. Vaults are found in all human cells.[12] As a naturally occurring nanocapsule, they are an exciting delivery vehicle for different therapeutic materials. Vault size below 100 nm prevents the particle from being trapped in the kidney or liver. The large interior space makes the particle ideal for packaging hundreds of proteins inside, and the outer protein shell is able to protect the inside from external proteases and harmful conditions.[13] Furthermore, by engineering the carboxy terminus of the vaults, they can be directly targeted to the cell of interest.[14] This property makes them an ideal candidate for different therapeutic applications, such as vaccines. This concept was successfully applied to prevent *Chlamydia trachomatis* infection in mice. Vaults were able to induce protective immunity and were able to prevent excessive inflammation.[15]

Vaults are not limited to medical applications. They can also be used in the field of environmental remediation to enhance the stability of isolated enzymes in order to improve biodegradation.[16] The paper, plastic and food packaging industry produces many contaminants that are released into the environment.[17] Some of these products can negatively influence reproductive health.[18,19] Therefore, scientists have been exploring closely-related analogues.[20,21] However, these also show similar concerns in

regard to their reproductive health toxicity.[20,21] Therefore, using enzymes for bioremediation has been thoroughly explored. At first, fungal whole cells were used, but due to their susceptibility to environmental conditions and the possibility of altering the microbial microflora, the focus was switched to isolated enzymes.[22] To lower the required concentration and costs of applying enzymes for biodegradation, those enzymes were then fixed onto a surface.[23] However, due to the covalent bonds used to immobilize enzymes on the surface, those solid supports often lead to diffusion resistance, lowering the activity of the enzyme.[24] Therefore, fusing the INT domain to the enzyme of interest and subsequently packaging it into vaults as a potential alternative was explored.[25] A study by Wang et al. demonstrated that when manganese peroxidase (MnP) was packaged into vaults, not only the efficiency of the enzyme increased but also the reproductive toxicity of the side products of Bisphenol A (BPA), a prevalent wastewater contaminant, and its alternatives decreased dramatically.[25] These results show another promising potential for the application of vaults.

This review explores the great potential of these structures. Different applications will be discussed, ranging from vaccines and drug delivery to enzymatic bioremediation. Furthermore, remaining challenges and future perspectives on the usage of vaults, especially in biodegradation, will be discussed. We hope this review will highlight the potential of vaults and prompt applications for vaults in new areas.

Vaults - How it all began

Vaults were first identified using flatbed gel electrophoresis.[1] While separating a subpopulation of clathrin-coated vesicles, Kedersha et al. found two novel structures that have not been previously described.[1] One composed of a single peptide (Mr 142.00) and the other an ovoid species, which was dominated by a polypeptide (Mr 104.000).[1] Upon further modification of the method, it was possible to separate the coated vesicles and ovoid particles.[2,26] After negative staining of the new particles and analysis via TEM, they received the name they are known for. First described as novel cytoplasmic ribonucleoprotein particles, their name changed to vaults, inspired by their resemblance to cathedral vaults (see Figure 1).[1,2] Each half of this structure can be opened in a flowerlike structure, with 8 petals surrounding a central ring.[4,27,28] Three-dimensional cryo-EM reconstruction gave further insight into the structure of vaults. Two protruding caps on both ends of the vaults and a thin-walled midsection form the barrel-shaped vault.[28]

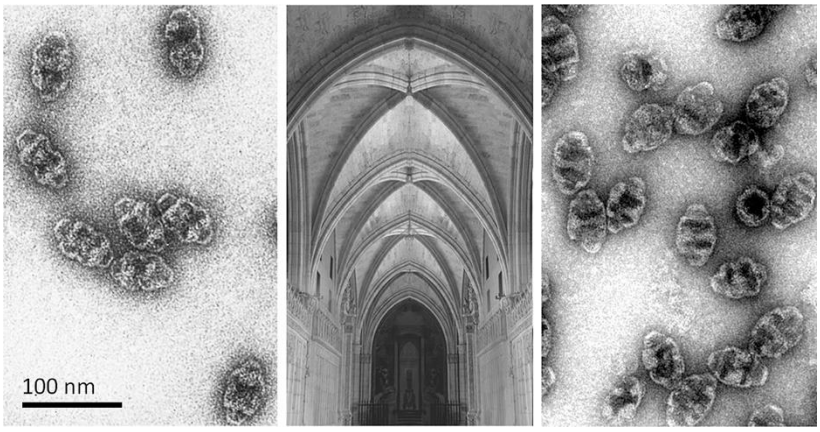

FIGURE 1: Left panel shows natural occurring vaults, and the right panel shows recombinant vaults. Both exhibit the typical barrel shape resembling cathedral vaults (middle panel) (from ref [9])

With a size of 35x65 nm, these newly found particles are larger than ribosomes.[2] SDS PAGE gave further information about this new structure.[2] Vaults contain several minor and five major species, predominated by a polypeptide (Mr 104.000), which accounts for around 70% of the mass.[2,4] Their structure is highly uniform, and their morphology is somewhat similar to that of a clathrin-coated vesicle.[2] However, an SDS PAGE revealed that vaults do not contain clathrin.[2] Furthermore, immunological studies revealed that vaults have no relation to coated vesicles, as no cross-reactivity between the polypeptides was observed.[2]

Vaults also seem to be more stable than coated vesicles, as they were able to survive treatment with 2M urea and several proteases like trypsin or *S.aureus V-8* protease.[2] When using proteinase K, a non-specific proteinase, most of the vault species was degraded except for one species with Mr 37.000.[2] Further investigation showed that this species might not be a protein at all, as it was destroyed by RNase A.[2] This newly found RNA consists of around 140 bases and has an unusually low molar ratio of adenosine of around 12%.[2]

This new RNA sparked the interest of researchers and led to further investigations.[27] At this point, it was known that vaults consist of 3 protein species and one small RNA, the vault-associated RNA (vRNA).[27] Cryo-EM reconstruction and difference mapping of TEP1 knockout mice placed the position of the vRNA in the cap of the vault.[28] Further Cryo-EM analysis of RNase treated vaults confirmed this position.[6,28] Vaults were found

throughout various eukaryotes like amphibians, avians and the slime mold.[27] They are mainly located in the cytoplasm, with a number of 10,000 to 100,000 vaults per cell.[4] The length of the vRNAs can vary between 86 – 141 bases, depending on which eukaryote it was isolated from, with mammalian vRNAs sharing around 80% identity.[4] Even though the length of the vRNA may differ between species, their secondary structures are clearly related, suggesting that the vRNA may be a component essential for the function of the vault. However, the exact function is currently not known.[5] vRNA does not seem to play an essential role in the structure of the vault, as the degradation of the RNA does not lead to an alteration of the vault structure.[4] But what about the other vault proteins? Vaults contain 3 proteins: the major vault protein (MVP), poly(ADP-ribosyl)polymerase (VPARP) and Tep1, with masses of 104, 193 and 240 KD, respectively.[4]

Kickhoefer et al. found that VPARP, with its poly(ADP-ribose) polymerase activity, gives vaults an enzymatic activity.[4,6] In response to DNA damage, PARP has the ability to catalyse the formation of ADP-ribose-polymers, vPARP does not have this property.[4] The precise role of vPARP is not known, however, it could enhance the interaction of vaults with other proteins or may influence the change of the vault's conformation.[4] An interesting finding from Kickhoefer et al. was that MVP is a potential substrate for vPARP, besides its ability to ADP-ribosylate itself.[4] Furthermore, VPARP contains a BRCA1 COOH terminus domain called BRCT, which is also found in the normal PARP. Those domains may enhance protein interactions and are often found at the NH2 or COOH terminus.[4] PARP also has a function in DNA repair. It gets activated upon DNA damage and binds to a single or double-strand breaks, where it then attaches ADP-ribose moieties to itself or other nuclear proteins.[29,30] Some scientists believe that multidrug resistance in cancer could be related to DNA damage, as many chemotherapeutics are DNA-damaging agents. Finding this unique domain made scientists hypothesise that vaults could have an influence on multidrug resistance in response to DNA damage, especially as some multidrug-resistant cell lines showed an upregulation of vaults.[12,31] However, the slight excitement was quickly gone when it was shown that upon DNA degradation with UV light, the distribution of vaults and its activity did not change. The exact activation of VPARP and also PARP, therefore, remain a mystery to this day.[4]

The 240 KD vault protein is identical to the mammalian telomerase associated protein 1 (TEP1), which could influence the vault's structure, function or assembly.[4] With a yeast RNA-protein interaction assay, Kickhoefer et al. have shown that vRNA specifically interacts with TEP1.[6] In *mTep1*-deficient mice, the vault structure is unaltered. However, less density of vRNA is found in the caps of the vaults, and their half-life also decreased several fold.[6] vRNAs of *mTep-/-* vaults do not stably associate with vaults and reached a concentration below a detectable level.[6] This shows that TEP1 is not necessary for vault stability, however, this protein plays an essential role in the stability of the vRNA as well as its association with vaults. Although it is not clear how TEP1 is able to stabilize vRNA, most likely, the stabilization occurs through direct binding or protecting it from degradation by packaging it in the vaults.[6]

Last but not least, the 100 KD protein, also known as MVP, which makes up 70% of the vaults mass.[4] To explore the exact function of the MVP, a baculovirus expression system was used. MVP was engineered with an N-terminal 31 amino acid tag.[7,9] Part of this tag was a 6-histidine sequence in order to be able to purify the expressed MVP on an affinity column.[7,9] However, to the researchers surprise, none of the MVP bound to the NTA-Ni resin in the column, meaning that the histidine from the tag was not

accessible and, therefore, unable to bind to the column.[7,9] Through centrifugation, the MVP was found in the pellet, and upon further investigation, the formation of particles was revealed.[7,9] The formation of vault particles was further confirmed with a modified vault purification protocol and TEM imaging. Due to the fact that insects do not contain vaults, those purified vaults resulted from the expressed MVP.[7,9] This also confirmed that MVP is the main factor in forming vaults, as there was no visible band of TEP1 or VPARP, and the expressed MVP vaults were structurally indistinguishable from natural vaults.[7,9] This finding completely transformed the model of vaults, as it was hypothesized beforehand that TEP1, VPARP and vRNA are responsible for forming the caps and MVP the typical barrel shape.[9,32,33] However, this investigation revealed that all the information needed to form a structural vault is encoded in the MVP.[7,9] This revelation led to a further investigation into how MVPs are arranged in vaults. For that, Cryo-EM difference mapping was used.[9,33] This revealed the location of N-terminal tags to be at the vault's waist inside of the particle.[9,33] With the increasing length of the tag, the density at the waist increased as well. When the tag was added to the C-terminus, increased density was found at the top and bottom of the vault. [9,33] This demonstrated the arrangement of the single MVP chains within vaults, with the C-terminus at the top and the N-terminus at the waist (see Figure 2).[9,33] Furthermore, it was previously predicted that 96 copies of the MVP are found within one vault.[9,32] However, this was disproven when a 3.5 A° X-ray crystal structure was carried out, predicting 78 instead of 96 copies.[8,9,32]

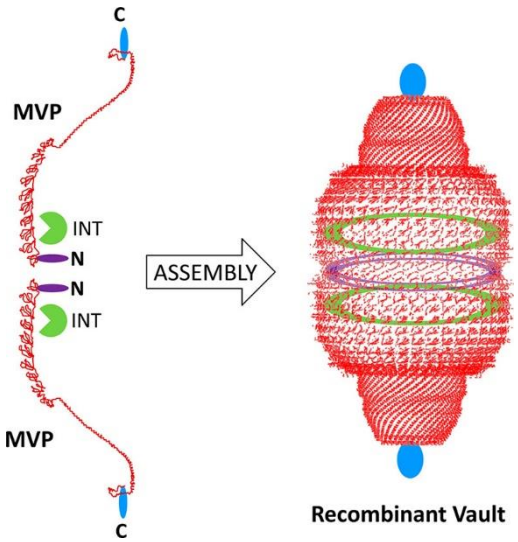

FIGURE 2: Schematic of vault engineering. The left side shows two single MVP chains. Purple indicates where the additional amino acids can be added at the N-terminus, which are located at the waist of the vault particle when assembled (purple belt). If proteins are packaged into vaults via the INT domain, their location is in two bands around the waist (green belts) (from ref [9])

But what is the function of vaults? This is a question which scientists have been arguing about since the vault was discovered. Later reconstruction of the vault showed a hollow particle, suggesting that vaults may have a function as a carrier.[4,28] Surprisingly, even though vaults have been mainly located in the cytoplasm, some have been found in the nuclear fraction.[4,34] Therefore, Kickhoefer et al. have proposed that vaults may function as a nuclear pore complex plug, especially due to their similar mass, structure and size.[4,34] Through antibodies specific to the MVP, it was shown that vaults might interact with the nuclear pore complex and play a role in transporting RNAs and proteins between the nucleus and the cytoplasm.[2,5] Furthermore, their size is

similar to the central transporter.[28] This suggests that vaults could play a role in transporting proteins between the cytoplasm and the nucleus.[28] However, if vaults would play a role as a central transporter, they should always be localized with the NPC.[28] This is not the case, and vaults have also not been found in yeast, which contains NPCs.[28] So, are they part of nuclear transport? Maybe vaults are not part of the nucleocytoplasmic transport but may act as a transporter for a subset of molecules.[28] Abbondanza et al. found further evidence for vaults as carriers. They found increased levels of vaults when they studied the estrogen receptor.[4] Furthermore, they found that vaults coimmunoprecipitated with the human estrogen receptor and the level of MVP increases when cells are treated with estradiol.[28] Therefore, vaults may also play a role in transporting steroid hormones between the cytoplasm and the nucleus.[28] Unfortunately, these studies have not been reproduced or followed up by other labs. An increased level of vaults has also been found by scientists in multidrug-resistant cancer cell lines. A possible explanation for that phenomenon could be the binding of vaults to the drug or preventing the delivery of the drug to its site of action.[4] In lung cancer cell lines, it was observed that instead of elevated known drug efflux pumps like P-glycoprotein (Pgp) or MDR-related protein (MRP), they showed an elevated level of a protein first described as lung resistance-related protein (LRP), which later was revealed to be human MVP.[28] Another possible role of vaults could be connected with motility. Scientists have found the vaults at the ends of actin filaments.[28,34] This was further confirmed by studied the rat brain, as high amount of vaults were found at two motile cell types.[28,34] The actual function of vaults still remains a mystery. However, presence in many tissue types, that vaults have an integral cellular function, possibly in cellular transport of macromolecules.[28]

The potential of vaults

Even though the exact function of vaults is still unknown, the hollow structure of recombinant vaults with an internal volume of 5 x 10^7 $Å^3$ makes them an ideal candidate for packaging proteins.[11] The initial idea of engineering vaults as a delivery vehicle came from the analysis of the expression of MVP, revealing that MVP alone is responsible for forming the typical barrel structure of vaults.[7,9]

In order to package proteins into vaults, proteins have been tagged with a polypeptide domain called INT (MVP interaction domain).[11] This domain was first identified with a yeast two-hybrid analysis and is located at the C-terminus of VPARP.[4,9] It is able to direct the tagged protein into the vaults and is responsible for the interaction between VPARP and the MVP.[11]

The ability to direct proteins into the inside of vaults was confirmed by fusing firefly luciferase to INT and subsequently co-expressing the fusion protein with MVP in Sf9 insect cells.[9,11] Copurification and cryo-EM difference mapping revealed that the luciferase was successfully packaged inside the vault, located at two rings above and below the waist of the vault.[9,11] Several proteins have been packaged into vaults since then; some examples can be found in Table 1. After this discovery, the packaging process was improved, and it was found that mixing the INT-fused protein and vaults together for 30 mins is enough to package the protein of interest inside the vaults.[9,35,36] The exact mechanism of packaging is unknown; however, the packaging occurs either through 'breathing', normally observed in viruses or through a half-vault/whole-vault dynamic, allowing the INT-fused protein to enter the vault.[9,35,36]

TABLE 1: INT Fusion Proteins (adapted from ref [9])

INT-fusion protein	Function of protein	reference
GFP-INT	Green fluorescence	11
mCherry-INT	Red fluorescence	14
Luciferase-INT	Light emission	11
CCL21-INT	Chemokine	37
pVI-INT	Adenovirus membrane lytic domain	38
MnP-INT	Degradation of contaminants	20
OVA-INT	Immunogenic antigen	39
PmpG-INT	Chlamydial antigen	40
MOMP-INT	Immunogenic protein	15

After successfully incorporating different proteins into vaults, possible applications of vaults were explored, one of them using vaults as adjuvants for vaccines. Due to their size (<100 nm), large interior space and their protein shell able to give protection from external proteases, vaults are ideal candidates as a delivery vehicle for vaccines.[9,13] Furthermore, vaults do not exhibit immunogenicity. This has been tested by exposing rabbits to purified rat vaults, which did not induce any immune response. Furthermore, when human autoimmune antibodies were screened, no autoantibodies were found against vault proteins.[9] Also, as all human cells contain between a few thousand and 100 000 vaults per cell, they are naturally occurring nanocapsules, making them an interesting adjuvant for vaccines.[9,12] By engineering the C-terminus of the MVP, vaults can be directed to cell surface receptors and therefore allow targeted delivery.[9] One interesting application was developed by Champion et al., where vaults

were used as a mucosal vaccine delivery platform against a *Chlamydia trachomatis* infection.[9,15] In order to induce an immune and Th1 response against *C. muridarum*, it has been shown that the Fc immunoglobulin receptor (FcR) on dendritic cells (DC) is necessary. [41,42] With this information, vaults were engineered to contain the Z peptide, a peptide able to bind to the FcR (cp-MVP-Z vaults).[15] These engineered vaults were then packaged with the major outer membrane protein (MOMP) of *C. muridarum*. This protein is highly immunogenic and has been used before to reduce infertility after an infection.[43,44] MOMP was fused with the INT domain before being packaged into the cp-MVP-Z vaults.[15] As demonstrated in Figure 3, mice that were vaccinated with the vaults containing MOMP had a lower bacterial burden compared to liposomes containing MOMPs and additionally reduced excessive inflammation.[45] This study showed that vaults were able to induce protective immunity against a *C. muridarum* infection.[45]

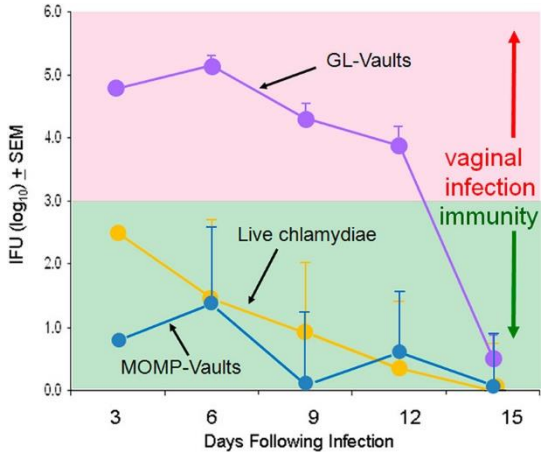

FIGURE 3: Vaults packaged with MOMPs are able to reduce *C. muridarum* infection. The bacterial burden is statistically reduced in mice that were previously immunized with MOMP-vaults compared to mice that were intranasally immunized with live *C. muridarum*. Mice immunized with vaults containing green lantern (GL-vaults) showed no immunity. (from ref [9])

As previously mentioned, recombinant vaults have an empty interior, able to package a significant amount of proteins. Therefore, the next step of exploring a potential therapeutic application was straightforward - vaults as delivery vehicles for drugs, especially hydrophobic drugs. Hydrophobic drugs often have very promising *in vitro* results but fail due to pharmacokinetic and pharmacodynamic properties.[9,46] Therefore, vaults would be a promising delivery vehicle to improve those properties. Many other delivery vehicles, including liposomes or dendrimers, have been explored as a potential solution to delivery problems. However, even though successful, some pitfalls like size limitations and tissue targeting remain.[9,47,48] Buehler et al. developed a promising system where so called nanodisks, small discoidal lipid bilayer fragments, were incorporated into the interior of vaults.[9,49] An advantage of nanodisks is their lipophilic interior, which can absorb hydrophobic compounds.[9,50–53] Although several different hydrophobic drugs have been bound to nanodisks and their effectiveness was improved, the exposed lipid surface of the disks remains a problem when it comes to delivery. This surface makes them susceptible to interact with other cell types and increases the risk of off-target effects.[9,54]

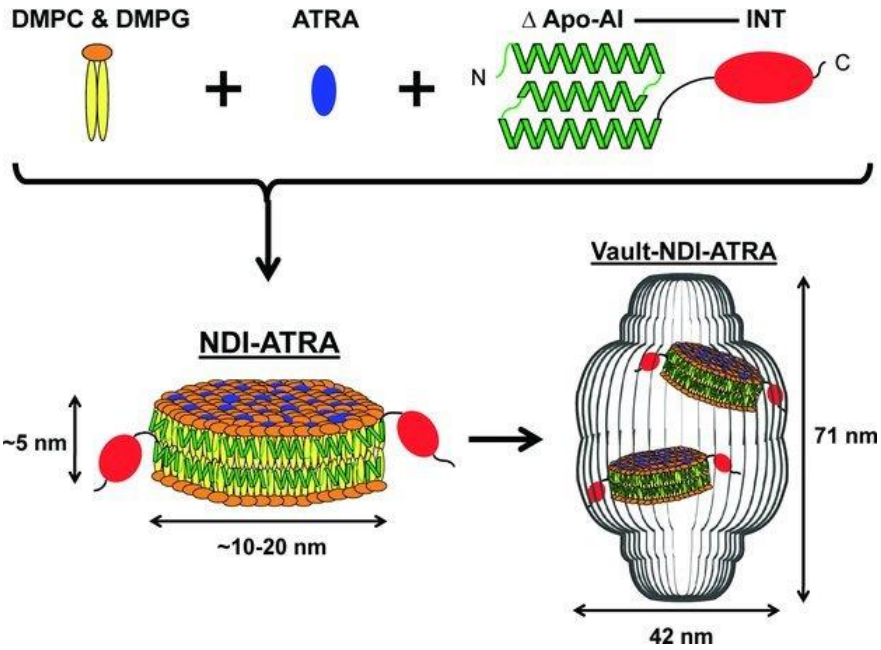

FIGURE 4: Schematic of ATRA nanodisk incorporation into vaults. ATRA absorbance occurs during the formation of the nanodisk (NDI). Through fusing the INT domain to the NDI, this construct is incorporated into the vault particle

So, where do vaults come in? Vaults could be used as a shield to protect nanodisks from the environment.[49] In order to achieve this, the INT domain was fused to a truncated form of Apo-AI, which was then used to assemble a functioning nanodisk. This was then successfully packed within a vault, and the drug remained within the disk during formation and packaging into vaults. As a model drug, all-trans retinoic acid (ATRA) was used. This drug has the potential to treat a variety of illnesses, including cancer or acne.[9,55–57] However, this drug also shows various side effects due to being teratogenic and sometimes causing retinoic acid syndrome.[9,58,59] It was possible to circumvent this problem by packing ATRA into vaults while being attached to nanodisks (see Figure 4). They retained their biological activity and were stably incorporated.[9,49] This example also shows what unique potential vaults have to improve drug delivery and reduce the side effect of many drugs.

From hydrophobic drugs to chemotherapeutics, all have been successfully incorporated into vaults and reduced potential side effects. Vaults can potentially become a promising delivery vehicle by conducting more research and improving the production of vaults.

Natures garbage collector

As already discussed in the previous chapter, vaults have been investigated regarding their application in different areas in the medical field, such as vaccines, tumour therapy or drug delivery. A new area where the specific characteristics of vaults have been explored is bioremediation.

Enzymes are essential molecules in our daily life. They are responsible for catalysing various biochemical and chemical reactions, which are essential to maintaining life. Not only are they breaking down macromolecules, but they are also responsible for building essential molecules.[60] These unique properties of enzymes can also be used for *in vitro* applications, mainly biodegradation and biosynthesis, like eliminating contaminants or producing renewable fuel.[21,61,62] One way to produce these biocatalysts is through fungi, including the commonly used enzymes laccase, manganese peroxidase, or cytochrome P450.[21,63–65] Live fungi have the ability to degrade contaminants, however, due to the harsh environment where those contaminants are usually found, these cells lose their function, leading to high operational costs.[21,66,67] When enzymes are isolated from fungi, this problem can be circumvented, as these proteins can be less vulnerable.[21,68] Furthermore, some of those enzymes exhibit a high redox potential, which makes them able to degrade even persistent contaminants.[21,69,70] Besides their ability to degrade pollutants, fungal enzymes can be used for various other applications, such as additives in baking products or detergents.[21,71–73] Despite having all these advantages and potential applications, fungal enzymes still exhibit some disadvantages. Even though they are more robust compared to fungal whole cells, if conditions are harsh, the isolated enzymes can denature, and their affinity to the substrate as well as their activity, can be diminished.[21,74] Therefore, different immobilization techniques have been explored in order to circumvent this problem. An overview can be found illustrated in Figure 5. On one hand, this technique enhances the interaction between enzyme and substrate but it also increases the stability and reusability of those enzymes, which subsequently lowers the overall cost.[21,75–77] There are several different ways of immobilizing enzymes. Besides the conventional materials like glass, polymers or activated carbons, nanomaterials have been investigated as potential immobilization substrates.[16,21]

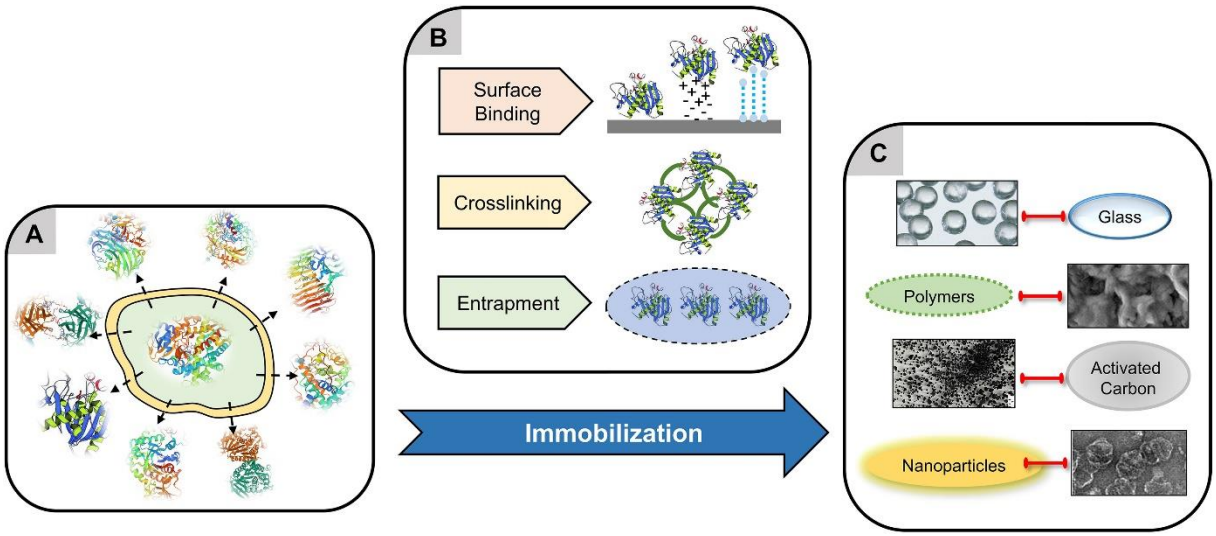

FIGURE 5: Methods to immobilize enzymes. (a) fungi produce intracellular and extracellular enzymes (b) different methods of binding the enzyme to the surface (c) many materials as immobilization supports have been studied and developed (from ref [21])

There are three different techniques for enzyme immobilization: surface binding, crosslinking and entrapment. Initially, environmental engineers focused on large supports like glass or activated carbon. In order to covalently immobilize enzymes on glass, the surface was first modified with functional groups, such as carboxyl groups or linkers.[21,78,79] The covalent bond formed between the surface and the enzyme prevents the enzyme from leaking into the environment.[21,80] However, this binding can also lead to a reduced activity or even inactivation of the active site.[21,81] In recent years, nanomaterials, such as nanogels or protein nanocages (PNC), as functional materials came into focus.[21,82] PNCs are biocompatible, and their production does not release any contaminants into the environment during production. An example of a PNC are vaults, which are found in almost all eukaryotes.[21,83] The hollow interior of recombinant vaults makes them an ideal candidate for packaging and protecting enzymes from proteases or fluctuating environmental factors.[20] By fusing the INT domain to the protein of interest, the enzyme can be directed and immobilized in the vault's interior.[7,11,20]

As already mentioned, enzymes can be used for biological remediation to clean contaminated soil or water. Enzymes are not as sensitive to their environment as microbial whole cells, and there is no risk of potentially releasing pathogens into the environment. One well studied enzyme with great potential for biological remediation is manganese peroxidase (MnP). It is a glycoprotein and a Class II peroxidase, which uses H_2O_2 as an electron acceptor to catalyze enzyme reactions and hydroxylations.[21,84,84] MnP is able to oxidize a variety of phenolic compounds through different steps beginning with a reaction with H_2O_2, forming a Fe^{4+} oxo-porphyrin radical complex. [21,85] Besides phenolic compounds, MnP is also able to oxidize a variety of other contaminants, such as polycyclic hydrocarbons and azo dyes.[20,86,87] As covalently binding an enzyme to a surface often leads to lower efficiency and higher Michaelis half-saturation constant (Km) values, a very promising alternatives are vaults.[20,81,88] Due to its compact shell, it can prevent conformational changes of packaged enzymes and can also act as a shield against potentially harmful contaminants. Furthermore, vaults can be synthesised naturally, in comparison to other alternatives, where several reaction steps and harsh chemicals are necessary. This gives vaults a further advantage compared to other applications, as this could be a potential way of producing a green, cost- and energy-efficient biological remediation process. In a study by Wang et al., an INT fusion MnP was successfully incorporated into vaults, with the enzymatic activity remaining.[20] At first, MnP-INT was expressed in Sf9 cells, which was secreted into the supernatant (sMnP-INT). The incorporation of sMnP-INT into the vault's interior and intact vault structures was confirmed via negative stain TEM. As the next step, the kinetics of the MnP-INT, as well as the packaged MnP-INT, were investigated by using an ABTS peroxidation assay. When ABTS is oxidized, its product exhibits a strong absorbance at 420 nm, so the rate of change in absorbance can determine the enzymatic activity. sMnP-INT was able to oxidize ABTS, showing that the INT domain did not affect the enzyme's activity. Furthermore, when packaged into vaults, the sMnP-INT was still able to exhibit a significant change in absorbance, showing an enzyme activity of about 80%.[20] This is a very promising result, as other packing approaches were only able to reach 30%.[89] As a next step, the ability of vaults to prevent thermal inactivation was investigated.

Free sMnp-INT, MnP as well as packaged sMnP-INT were exposed to 20, 30 or 40 °C for 1h hour. Afterwards, the residual activity of the enzyme was investigated with the ABTS oxidation assay. At 20 °C, only sMnP-INT lost 20% of its activity. At 30 °C,

sMnP-INT was able to preserve 75 °C of its activity, compared to native MnP with only 45 %. Even at 40 °C, where both the native MnP as well as the sMnP-INT lost their activity, the sMnP-INT packaged within vaults was still able to retain 16 % of its activity. These results show the great potential of vaults to protect enzymes from degradation while retaining their activity. Even though those results are already very promising, in order to show enzyme packaged enzymes are a viable option for biological remediation, further experiments were necessary.[20]

For that, phenol was used as a model, as it is a commonly found pollutant and is degraded by MnP through biochemical reactions.[90] When incubated for 24 h, all three enzyme variants, native MnP, sMnP-INT and sMnP-INT in vaults, were able to degrade phenol, with sMnP-INT-vaults having the best performance with 98% reduction of the initial concentration, compared to the native peptide that only achieved 87%. These results highlight that enzymes packaged into vaults are a very promising system for biological remediation.[20]

These results inspired scientists to further investigate vault packaged enzymes for enzymatic bioremediation. Wang et al. performed follow-up studies, where they encapsulated MnP again in recombinant vaults.[25] This time, the degradation of bisphenol A (BPA) and some of its analogues were tested. BPA is one of the most commonly used chemicals in the paper, plastic and food packaging industry. However, it is also a very prevalent endocrine disrupting compound (EDC) with a substantial impact on reproductive health.[18,19,91] Therefore, structural alternatives to BPA, such as bisphenol F (BPF) or bisphenol AP (BPAP), are now in use. However, due to their structural similarities to BPA, these alternatives still exhibit endocrine disrupting activity, which is why finding a way to eliminate these contaminants is so important.[92,93] As demonstrated in Figure 6, the study by Wang et al. revealed that the vault packaged MnP was able to remove around 98% of the BPA in the solution, compared to the native MnP, which only achieved around 30% removal.[25] Similar results were also shown with the structural alternatives of BPA. When investigating the removal of BPF, vault packaged MnP achieved 89% removal, compared to the native MnP, which only achieved 64.4%.[25] But the most exciting result of this study was that when using vault packaged MnP, the reproductive toxicity of the BPS decreased dramatically due to less toxic side products.[25] These results show that when packaging MnP inside vaults, the enzymatic stability increases and the toxicity of the bisphenols and their side products decreases.

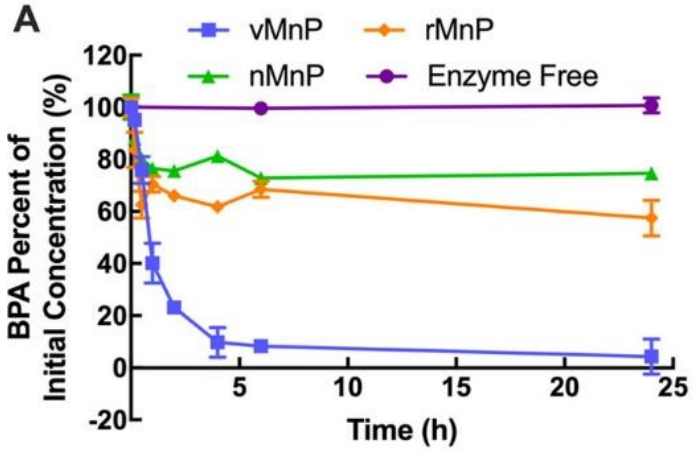

FIGURE 6: Removal of BPA was enhanced when MnP was packaged within vaults. Vault packaged MnP managed to degrade nearly 100% of BPA within 24h (blue) in comparison with native (green) and recombinant MnP (orange) which only achieved 30-40%. (from ref [25])

The immobilization technique was improved by Wang et al. by embedding pre-encapsulated vaults in silica.[24] Silica has been used in other techniques to immobilize enzymes.[82] However, the harsh processing conditions required to embed the enzyme in silica lead to a significant loss of activity and leaching during long-term storage.[74,82,94] Wang et al. managed to develop a vault nanocapsule-induced biomimetic silicification process, achieving little to no protein activity loss of the incorporated protein. Furthermore, the proteins could be extensively reused and showed minimal protein leaching.[24] Wang et al. incorporated mCherry, green lantern and MnP via fusion with the INT domain into vaults. Subsequently, the packaged vaults were exposed to silica precursors, as they acted as a nucleation site for the condensation of silica, leading to the formation of vault/silica composites. Both fluorescent proteins, mCherry and green lantern exhibit 100% fluorescence intensity when embedded in this composite. Similar results were also shown with MnP, which retained 78% of its initial activity and showed superior reusability, with 90% activity left after 30 reuses.[24]

These results highlight what amazing potential vaults have regarding the stabilization of proteins. With improving immobilization techniques, like the vaults embedded in silica, this technology shows great potential in the field of enzymatic bioremediation.

Conclusion & Future Perspective

Recombinant vault nanoparticles have incredible potential in their application as delivery vehicles for therapeutics as well as an immobilization technique for the biodegradation of environmental contaminants. Promising results have been shown when vaults were applied as a delivery vehicle for hydrophobic drugs as well as an adjuvant for vaccines.[49] Furthermore, vaults have been shown to successfully enhance the stability of immobilized enzymes, leading to a significant reduction and detoxification of contaminants in wastewater.[25] Their hollow structure makes them ideal for packaging different substances inside, protecting them also from the harsh outside environment.

However, more investigations have to follow regarding their application as a delivery vehicle. In order to use vaults as therapeutic delivery vehicles in humans, more tests are needed to demonstrate the lack of toxicity and sustained biodistribution. Furthermore, a scale-up and GMP manufacturing process will need to be established. Additionally, more tests concerning their structure, stability, composition and purity have to follow, as this is required by the FDA and EMA for the approval of new nanomaterials.[95]

Preliminary *in vitro* results of different therapeutic applications of vaults showed promising results. As an adjuvant, vaults packaged with the Fc binding Z peptide successfully induced protective immunity and reduced the bacterial burden and excessive inflammation in a *Chlamydia muridarum* infection.[40] Furthermore, hydrophobic drugs that usually show poor solubility as well as poor pharmacokinetic and pharmacodynamic were successfully incorporated in vaults without losing their biological activity.[53] More research on other potential applications of vaults should follow. Nowadays, gene therapy is getting more and more attention. It would be interesting to load DNA or RNA into vaults. Gene therapy often fails due to the lack of protection of the labile DNA or the insufficient targeting to cells or tissue.[96,97] The currently commonly used viral vectors for the delivery of DNA often lead to off-target effects due to non-specific gene transfer to a cell other than the targeted cell.[96,97] Several techniques have been proposed to circumvent this off-target effect. However, some drawbacks, like poor biocompatibility or low efficiency, still remain.[98] Vaults have been shown to protect their cargo from potential outside harm, and it is possible to add a C-terminal peptide extension to the MVP to target the vault to a specific cell type.[14] Exploring vaults as a possible delivery vehicle of DNA or RNA is very tempting. By fusion with the INT domain, DNA or RNA could be safely incorporated into vaults and, for example, could be used to sequester siRNA.

Another exciting area to explore is the controlled release of drugs with vaults. For that, the exact mechanism behind the interaction between the INT domain and MVP has to be elucidated. It seems that materials packaged inside vaults may get released gradually, it is crucial to know the exact release mechanism to achieve a controlled and regulated release of packaged material. Especially if vaults are used in therapeutic applications, the exact extent of the release mechanism must be known to foreshadow possible toxicities or off-target effects due to premature release. Furthermore, if the exact mechanism of the interaction between the INT domain and MVPs is known, drug delivery vehicles with controlled drug release can be designed.

Concerning enzymatic bioremediation, it would be fascinating to explore several different enzymes and even a combination of different enzymes to further improve the effectiveness of this already promising platform. Furthermore, other applications

besides the biodegradation of wastewater should be explored. In a recent paper, it was revealed that some fungal enzymes have the ability to degrade micro- and macroplastics.[99] It would be interesting to explore the potential application of vaults as a stabilizing agent for those enzymes to achieve a cheap and green process to remove micro- and macroplastics.

More studies should follow on the use of MnP, laccase and lignin peroxidase (LiP), as those are commonly studied enzymes in biodegradation.[21] A possible combination of those enzymes should be explored to make this application even more versatile. Furthermore, more different contaminants should be tested, including a mixture of contaminants, to evaluate the effectivity of enzymes immobilized in vaults. Similar to the application of vaults as a delivery vehicle, further experiments regarding scale up and batch to batch variability have to be performed.

This review highlights the great potential of vaults. Since the discovery of vaults, a huge advancement has been made to explore vaults as delivery vehicles for pharmaceutical materials as well as an immobilizer of enzymes in the field of enzymatic bioremediation. If further research toward reducing toxicity and scale-up is made, vaults should have a bright future as a versatile and functional material.

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Abbreviations

ATRA	all-trans retinoic acid
BPA	bisphenol A
BPAP	bisphenol AP
BPF	bisphenol F
DC	dendritic cells
EDC	endocrine disrupting compound
FcR	Fc immunoglobulin receptor
INT	MVP interaction domain
Km	Michaelis half-saturation constant
LiP	lignin peroxidase
LRP	lung resistance-related protein
MnP	manganese peroxidase
MOMP	major outer membrane protein
MRP	MDR-related protein
MVP	major vault protein
NDI	nanodisk
Pgp	P-glycoprotein
TEM	transmission electron microscopy
TEP 1	telomerase-associated protein 1
VPARP	poly-(ADP-ribose) polymerase
vRNA	vault-associated RNA

Layman's Summary

Vaults are small particles found in almost all eukaryotic cells. After discovering that it is possible to produce empty particles, a wide range of different applications opened up for those unique structures.[1,2] At first, different therapeutic applications were investigated. As vaults are present in all human cells, those naturally occurring capsules were tested as a vaccine delivery vehicle.[12] Their small size prevents them from being trapped in the kidney or liver, their large interior space makes them ideal for packaging a vast number of proteins, and their outer shell gives their interior protection against the potential harmful environment.[13] Vaults were successfully applied as a delivery vehicle for proteins able to prevent *Chlamydia trachomatis* infection in mice.[15]

However, those unique particles are not limited to therapeutic applications. They were successfully applied as a way to immobilize enzymes to improve the degradation of contaminants in wastewater. Enzymes are essential molecules in our daily lives. They are responsible for breaking down and building essential molecules in our bodies.[60] Those special properties can also be applied for applications beyond our bodies. They can also be used for the degradation of contaminants or producing renewable fuel.[21,61,62] Enzymes have been successfully applied as a way to eliminate contaminants in wastewater. However, many scientists have investigated alternatives to improve their effectiveness due to high operational costs and them losing their function due to the harsh environment.[21,66,67] They immobilized enzymes on different surfaces, which improved their stability. However, their activity was reduced due to the interaction between the enzymes and the substrate.[21,81] Materials like protein nanocages have been explored as alternatives for enzyme immobilization in recent years. Vaults are part of those protein nanocages and have been successfully applied for the elimination of contaminants.[21,83] Manganese peroxidase (MnP), an enzyme able to oxidize a variety of contaminants, was successfully incorporated into vaults. The enzyme was able to retain its activity and was able to remove 98% of the contaminant, in comparison with the native enzyme, which only achieved 87%.[20] Further investigations showed that not only were the contaminants eliminated, but they also dramatically reduced the toxicity of the side products of the contaminant.[25]

These examples show the great potential of vaults in different areas. From therapeutic applications to enzymatic wastewater cleaning, there lies great potential in those unique particles. This review highlights the great potential and explores more examples for the application of vaults.