Engineered Bacterial Outer Membrane Vesicles as Multifunctional Delivery Platforms

Ruizhen Li[1] and Qiong Liu[1,2]*

[1] Department of Medical Microbiology, School of Medicine, Nanchang University, Nanchang, China, [2] Key Laboratory of Tumor Pathogenesis and Molecular Pathology, School of Medicine, Nanchang University, Nanchang, China

Outer membrane vesicles (OMVs) are valued for their unique, convenient, and amendable functions. They are flexible, controllable nanoparticles, which can be modified in different ways, for use in a wide array of applications like adjuvants, vaccines, drug presentation, and fluorescence tracking. Attempts have been made to alter the nanoparticles in liposomes and bacterial ghosts using traditional methods. However, it has not been highly successful owing to certain unavoidable disadvantages. OMVs have unique advantages and are considered novel platforms for heterologous protein presentation. Although previous reviews have described this to some extent, owing to the rapid development in this field, there is a need for the regular update of the reviews. This review focuses on few novel applications of engineered OMVs, and redefines the significance of OMVs as novel multifunctional delivery platforms. Additionally, this review also supplements and provides an update on the OMV transformation methods.

Keywords: genetic engineering, multifunctional delivery platform, outer membrane vesicles (OMVs), LPS, ClyA, AT

INTRODUCTION

Outer membrane vesicle (OMV), naturally secreted by Gram-negative bacteria, is a spherical nanostructure with a diameter of 30–200 nm (**Figure 1**) (Liu et al., 2016). Bacteria can produce OMVs in solid and liquid media, or in biofilms and intracellular infections under *in vitro* conditions (Unal et al., 2011; Klimentová and Stulík, 2014; Caruana and Walper, 2020). The generation of OMVs is considered a stress response. Various stress factors, such as temperature, nutrient consumption, and exposure to harmful chemicals, may cause accumulation and aggregation of misfolded proteins in the periplasm. Packing these pressure products to the outer membrane and releasing them, OMVs are formed (Klimentová and Stulík, 2014). Quantitative analysis showed that *Escherichia coli* packages ~0.2–0.5% of outer membrane and periplasmic protein into OMV (Kesty and Kuehn, 2004). Approximately 1% of the outer membrane material could be incorporated into the vesicles of *Pseudomonas aeruginosa*, while the logarithmic phase culture of *Neisseria meningitidis* incorporates 8–12% of its total protein and endotoxin into vesicles (Devoe and Gilchrist, 1973; Bauman and Kuehn, 2006). Most of the components of OMVs are lipopolysaccharide (LPS), glycerophospholipids, and proteins including periplasmic proteins (Ellis and Kuehn, 2010; Underhill and Goodridge, 2012). The various components on OMVs serve different functions. LPS of OMVs gives its adjuvant function, which is useful for vaccine formulation, and some other antigen components that can be used for vaccine development. In addition, other components on OMVs also play roles that cannot be ignored. OMVs have been found to function as a multifaceted delivery system with intra- and inter-species

FIGURE 1 | (a) Showed that Cryo-EM imaging of OMVs (Liu et al., 2016). OMVs derived from the flagellin-deficient *S.typhimurium* were visualized using cryo-EM. The red arrows indicate the visible OMVs; **(b)** showed schematic diagram of OMVs secretion process.
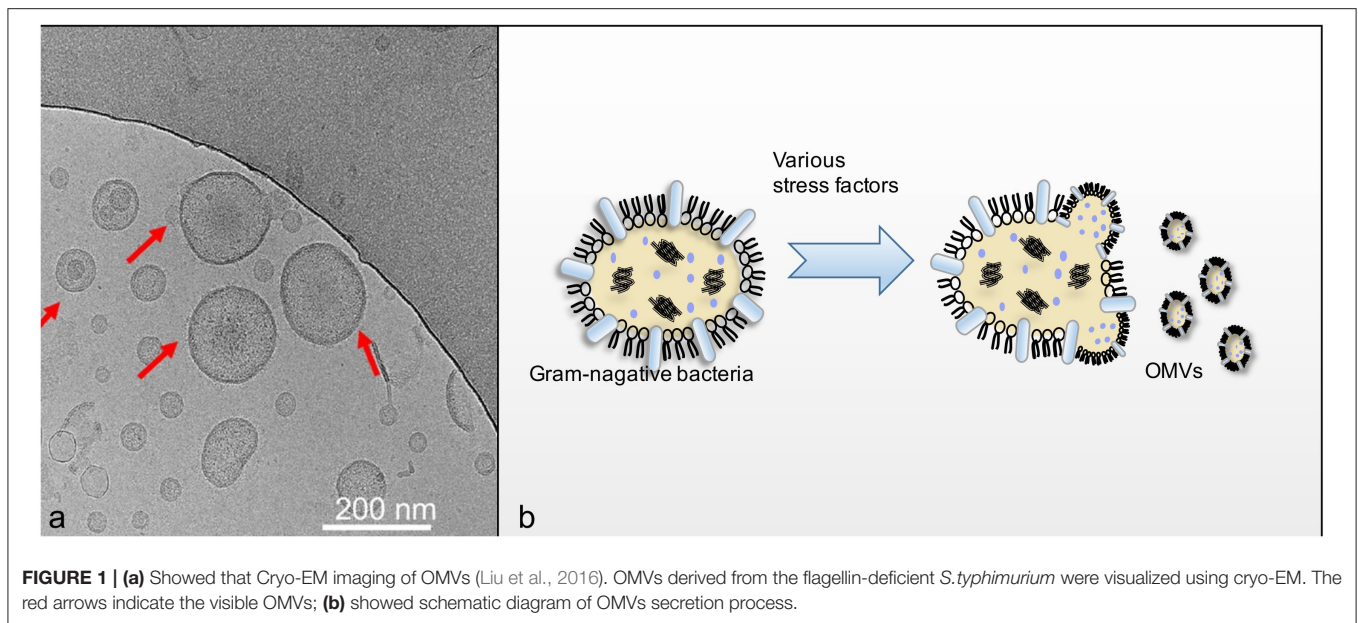
interactions through membrane proteins (Ellis and Kuehn, 2010). The OMVs produced by *Mycobacterium tuberculosis* carries the iron-carrier mold, mycomycin, which transports this essential nutrient over long distances in a hostile host environment (Prados–Rosales et al., 2014). Regarding the interspecies interactions of OMVs, multiple studies have indicated the use of OMVs by many bacterial pathogens to deliver virulent determinants to host cells, at proximal and distal sites, thereby impairing the host defense system and promoting infection. In contrast, OMVs from various mucosal pathogens show numerous pathogen-associated molecular patterns including LPS, lipoproteins, DNA, and RNA. Their interaction with the pattern recognition receptors (PRR) on host epithelial cells stimulates the production of cytokines and chemokines on the mucosal epithelial surface, their interactions with immune cells, and regulates pathology in a variety of ways (Yoon, 2016). The virulence factors contained in OMVs can be distributed over long distances, and also transferred onto the plasma membranes of the host cells (Ellis and Kuehn, 2010).

Schulz et al. (2018) characterized the morphology of OMVs by electron microscopy and found that OMVs are biocompatible with epithelial cells and differentiated macrophages (Schulz et al., 2018). They showed low endotoxin activity compared to control samples, indicating low acute inflammation potential. At the same time, OMVs also showed good biodegradability, and it has been proven that OMV in the lysosome can be completely degraded after 48 h of endocytosis (Kim et al., 2012). Bioengineering can be used to increase OMV output,

and may be used in conjunction with specific production processes to obtain a large number of well-defined, stable, and unified OMV particle vaccine products (van der Pol et al., 2015). Commercially available *N. meningitidis* serotype B OMV vaccine indicates the use of OMVs for disease prevention and treatment (Mirlashari et al., 2010; Collins, 2011; Santos et al., 2012). Currently, research has been focused on the use of OMVs in vaccine transformation platforms; different heterologous proteins can be efficiently added to achieve the use of such nanoparticles in multiple applications (Chen et al., 2010; Bartolini et al., 2013; Alves et al., 2015).

Although previous reviews have described OMVs in vaccine transformation platforms, since this is a rapidly growing area of research, the reviews need to be updated frequently (Gerritzen et al., 2017). This review mainly describes the applications of the most recently engineered OMVs, and gives an update on few novel engineering methods. Additionally, the advantages and disadvantages of the different engineering methods have been compared, and few prospective applications of the engineered OMVs have been described.

PURIFICATION, THE KEY STEP IN OMV USAGE IN VACCINE APPLICATIONS

Some bacterial nanoparticles have gradually gaining interest and are being developed with antigen-related functions. However, compared with OMV, the production process is more complicated and expensive, and it is difficult to ensure its safety in living organisms (**Table 1**) (Carita et al., 2018; Hu et al., 2019; Lee et al., 2019; Sánchez et al., 2020). Presently, there have been a variety of bacterial OMVs used in vaccine production attempts, such as *E. coli, P. aeruginosa, Shigella, Salmonella, Helicobacter pylori* (*H. pylori*)*, Campylobacter jejuni, Borrelia burgdorferi, Vibrio,* and *Neisseria* spp. (Bauman and

TABLE 1 | The advantages and disadvantages of OMVs and other bacterial small molecules.

Bacterially produced carriers	Particle size	Material properties	Advantages	Disadvantages	References
Liposomes	10 nm–hundreds nm	Phospholipid vesicles	More extensive research; High biocompatibility; Low immunogenicity	Drug leakage; Incomplete loading function; Cumbersome quality control system	Carita et al., 2018
Bacterial ghosts	40–200 nm	Empty envelopes of Gram-negative bacteria	High security	Low efficiency; Complex production; Unclear effects of human application	Hu et al., 2019
Biologically synthesized polyester	100–500 nm	Polyester particles	Ensure antigen integrity; High biocompatibility; Non-toxic metabolism	Complex purification; Unstable production; Difficult to control the particle size of the host cell	Lee et al., 2019
Inclusion Bodies (IBs)	50 nm–hundreds nm	Inactive protein particles	Dense structure, hard to hydrolyze; Complete biocompatibility; Highly expressed protein	Purification is complex; Hard to produce large batches; Complex and Inconsistent production	Sánchez et al., 2020
OMVs	30–200 nm	Outer membrane of Gram-negative bacteria	Clinical use; High biocompatibility; Good biodegradability; Large-scale production; Simple purification steps; Unified production process; Convenient antigen presentation platform	Multiple antigen presentation efficiency is not high	Kesty and Kuehn, 2004; Ellis and Kuehn, 2010

Kuehn, 2006; Chatterjee and Chaudhuri, 2011; Elmi et al., 2012; Schwechheimer and Kuehn, 2015; Turner et al., 2015; Elhenawy et al., 2016). In the making of vaccines of different strains, some components on OMVs (such as LPS) could affect the safety of the vaccine. Purification can remove some unnecessary or harmful components, thereby making OMVs more in line with needs. Purification is the first and the most important step in the use of OMVs and impurities can have a large impact, resulting in extremely unfavorable on experimental outcomes. Thus, the purification of OMVs using traditional methods has been summarized, and the latest approaches for the purification of OMVs using genetic modifications have been elaborated.

Purification of OMVs Using Traditional Methods

Traditional methods of OMV purification include ultracentrifugation, density gradient separation, precipitation, and gel filtration techniques (Kataoka et al., 2014; Klimentová and Stulík, 2014; Cecil et al., 2016; Kim et al., 2017a,b). Density gradient centrifugation and gel filtration are commonly used methods for OMV purification (Kulp and Kuehn, 2010), while ultracentrifugation and precipitation are the primary methods to obtain OMVs. Although ultracentrifugation methods have been widely used in the preparation of OMVs, this method has certain shortcomings. The primary problem is the complexity of the operation process. It is time consuming, and requires skilled labor, owing to the complexity of the process. In addition, the high cost of the purification equipment makes it difficult to commercialize. Although sedimentation is relatively inexpensive, it also has certain unavoidable disadvantages. The preparation of the salt solution required for precipitation is difficult, and the steps are complicated. Also, the proteins on the OMVs are easily damaged, thereby allowing for a negative impact

on experimental results (Qing et al., 2019). Additionally, considering the impact of LPS on the experiment, researchers have attempted certain special methods. Initially, detergent was added during the extraction process to attenuate LPS. However, studies have shown that LPS is a potential endotoxin, and acts as a powerful adjuvant for specific antigens (Mitra et al., 2013). Consequently, the complete removal of LPS from OMVs was required, rather than LPS attenuation with detergent, which proved unsuitable for the betterment of vaccine performance (Mitra et al., 2016). Therefore, researchers proposed the use of EDTA extraction (chelating agent) to obtain high quality OMVs, by accurately controlling the pH and collection point (van de Waterbeemd et al., 2012). However, this method demands stringent extraction conditions, and has a complex operation process. If the purification methods do not meet the research requirements, the development and application of OMVs in the biomedical field will be limited. Therefore, the development of a simpler and more feasible method is very crucial.

Genetic Engineering Used for Attenuating Toxicity of OMVs

Generally, major pathogenic Gram-negative bacteria can secrete OMVs and interact with the host through OMVs (Kuehn and Kesty, 2005). Although OMVs can be obtained by extraction with detergents, the improper use of chemicals can result in the loss of lipoproteins and enzymes, and their activity, which impairs the ability of OMVs to stimulate cross-protective immunity. In order to obtain OMVs with low toxicity and high safety, the genetic modification, and purification of OMVs has been proposed. Genetic modification is primarily aimed at the attenuation of the OMVs, and improvement of its yield. An established method is the knock out of the *msbB* gene, which regulates LPS in bacteria. The inactivation of *msbB* leads to a significant decrease

in the level of LPS endotoxin (Kim et al., 2009; Ranallo et al., 2010). This method has been widely used in the preparation of OMVs (Leitner et al., 2015). Latest research has combined a new modification scheme with the genetic modification of the *msbB* gene, and has helped to obtain good results. At the same time, the Dutch vaccine research institute found that the deletion of the *lpxL1* gene attenuated LPS toxicity while retaining the adjuvant activity required for the immune response (van der Ley et al., 2001; Fisseha et al., 2005; Koeberling et al., 2008). Engineered OMVs obtained from *Bordetella pertussis* expressing the *pagL* gene of *Bordetella bronchiseptica*, had lower endotoxin activity and better adjuvant performance. Attenuated OMVs are considered as prospective candidates in the development of novel vaccines targeting multiple antigens (Asensio et al., 2011).

Compared to the traditional methods, genetically engineered bacterial strains not only possess exclusively attenuated LPS, but also have the ability to induce pathogen-specific immune responses to OMVs, which is more suitable for the non-endotoxin conversion of OMVs (Shim et al., 2017). These pivotal genes attracted attention, and their knockout lead to a decrease in immunogenicity. Consequently, researchers have gradually started to explore more novel methods such as using non-pathogenic symbiotic bacteria as the source of OMVs, which can overcome the risks of gene knockout and render a certain degree of safety (Carvalho et al., 2019). Now, OMVs can be mass-produced through optimized purification methods, and for reference, Gerritzen et al. (2019) has summarized various purification methods of OMV (Gerritzen et al., 2019).

Genetic Engineering in the Increasing Production of OMVs

The Tol-Pal system continues to attract attention since its functioning affects the OMV production (Shrivastava et al., 2017). The five proteins of the Tol-Pal system includes three inner membrane proteins (TolA, TolQ, and TolR), a periplasmic protein (TolB), and a peptidoglycan-related lipoprotein (Pal) associated with the outer membrane proteins (Derouiche et al., 1995). The *tolB* mutant shows increased OMV release in many bacteria like *E. coli*, *Salmonella typhimurium*, and *H. pylori* (Deatherage et al., 2009; Nevermann et al., 2019). Interestingly, *tol* mutants can often be introduced into the bacteria of interest by means of electrotransportation (Turner et al., 2015). Additionally, the *rmpM* gene deletion mutant was found to have a loosely attached outer membrane, which increased OMV release (Maharjan et al., 2016). Theoretically, the combination of the *lpxL1* and *rmpM* mutations can address toxicity and resolve issues associated with the detergent-free OMV vaccines. Both gene mutations have been reported to have beneficial effects, making the detergent-free method a better alternative to the traditional detergent-based OMV purification methods (van de Waterbeemd et al., 2010). With gradual improvement in genetically engineered OMVs, multiple genes have been modified for the purification of OMVs. For example, few studies have attempted to incorporate a non-natural histidine amino acid. The sequence (His-tag) has been used to purify the bacterial OMVs by

affinity chromatography (Alves et al., 2015). Additionally, genetic engineering has also been used to produce a high-yield of bacteria lacking the *OmpA* gene, which has been widely used in OMV engineering (Moon et al., 2012; Kulp et al., 2015).

Studies in *E. coli* have shown that the stability of the envelope comes from three major cross-links: OmpA, LppAB, and the Tol-Pal complex (Mizushima, 1984; Yeh et al., 2010; Park et al., 2012). When there is a decrease in these cross-links, the yield of OMV increases, and vice versa. Thus, *S. typhimurium* mutants lacking OmpA, LppAB, Pal, TolA, or TolB show increased OMV production (Deatherage et al., 2009). Similarly, gene deletion can have a negative impact on immunogenicity. For example, the TolA gene can increase the resistance of *S. typhimurium* to bile (Lahiri et al., 2011). The *tolR* gene encodes proteins with exercise-related functions in *S. typhimurium* and *E. coli* (Santos et al., 2014). Deletion of these genes will inevitably have a negative impact on the function of OMVs, like reduced immunogenicity. Consequently, the question of balancing yield and the immune efficacy remains unsolved.

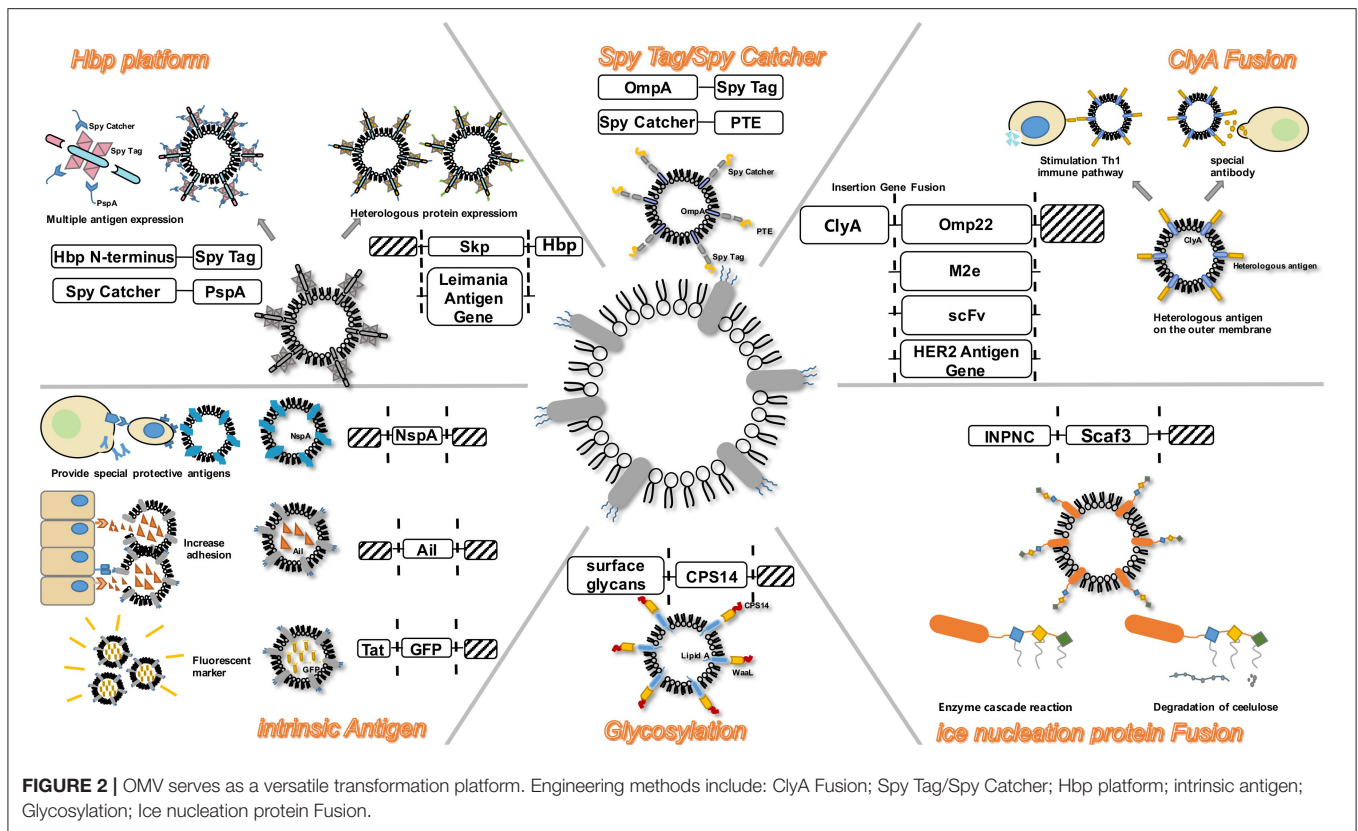# UPDATE ON THE APPLICATION OF OMV$_S$ AS ANTIGEN PRESENTATION PLATFORMS USING GENETIC ENGINEERING

Increasing evidence exists for the delivery of antigens by OMVs, to proximal host cells and distal host cells, which in turn stimulates the immune system and triggers immune response in the host (Shen et al., 2012). The antigens in OMVs can be distributed over long distances and are not damaged by dynamic physical and biochemical stresses. In addition, they can be transferred to the plasma membrane of the host cell (Sharpe et al., 2011; Yoon et al., 2011). Moreover, some transmembrane proteins on OMVs can be used as anchoring proteins, and play a role in presenting heterologous proteins in engineering transformation. To design a safer and a more efficient antigen delivery system, the naturally occurring OMVs have been modified by genetic engineering to obtain a multifunctional antigen delivery platform (Kim et al., 2009). By summarizing existing transformation methods, we hope to highlight the systematic processes of engineering OMVs (**Figure 2**).

Presentation of Heterologous Proteins

The modification of OMVs, in terms of the presentation of heterologous antigens, is an established method. Since this modification alters the communication between the cells and the bacteria to a certain extent, it affects the functionality of OMVs as vaccines. Most of the heterologous antigens are heterologous proteins.

Intrinsic Antigens

The expression of intrinsic antigens in OMVs is widely used, and is closely associated with the development of vaccines. The symbiotic bacteria *Neisseria gonorrhoeae* and *N. meningitidis* share a tendency to overproduce the bacterial outer membrane, leading to the formation and release of OMVs during growth.

FIGURE 2 | OMV serves as a versatile transformation platform. Engineering methods include: ClyA Fusion; Spy Tag/Spy Catcher; Hbp platform; intrinsic antigen; Glycosylation; Ice nucleation protein Fusion.

OMVs from the meningococcal and commensal intima have been reported to function as a vaccine against meningococcal disease (Mirlashari et al., 2010). The *nspA* gene has been shown to be highly conserved in a variety of meningococcal strains, and has been extensively studied as a potential antigen for vaccines (Lewis et al., 2010, 2012; Echenique–Rivera et al., 2011; Giuntini et al., 2011). However, this method is difficult to commercialize. Previous studies have reported that recombinant *E. coli* was not suitable for the expression of other meningococcal OMV vaccine components, owing to its ability to genetically up-regulate certain outer membrane proteins in meningococcus, and separately engineer OMVs (O'dwyer et al., 2004). Ail is an outer membrane adhesin and invasin from *Yersinia enterocolitica*. Following genetic engineering, the OMVs of the *E. coli* DH5α strain carrying Ail can be internalized in the eukaryotic cells. The presence of Ail enabled non-invasive strains to invade the CHO cells (Kesty and Kuehn, 2004).

Moreover, green fluorescent protein (GFP) fused with the Tat signal sequence, was secreted into the periplasm by the double arginine transporter (Tat) in the *E. coli* DH5α strain. Pronase-resistant fluorescence was detected in the vesicles of the Tat-GFP transformed strain, demonstrating the presence of GFP in intact vesicles (Kesty and Kuehn, 2004). The inclusion of GFP cargo increased the vesicle density, but did not bring about any morphological changes in the vesicles. More than 60 membrane proteins have been detected in the OMVs (Molloy et al., 2000; Tokuda and Matsuyama, 2004; Lee et al., 2007). These proteins continue to be identified

as anchoring proteins in the engineered OMVs. With further study of OMVs, more proteins with important functions will be identified.

Fusion of *ClyA*

Cytosolic A (*ClyA*), a 34-kDa cytosolic protein encoded by the *ClyA* gene (also known as *sheA* and *hlyE*), is located on the K-12 chromosome of *E. coli* (Westermark et al., 2000). Lately, researchers have favored the genetic fusion of *ClyA*. *ClyA* acts as a leading sequence and aids in the localization of heterologous proteins in the outer membranes where OMVs are secreted, resulting in the display of functional proteins on the surface of the OMVs (Kim et al., 2008). The gene for the heterologous protein expresses a recombinant outer membrane protein by fusion with the *ClyA* gene. Since *ClyA* is anchored to the outer membrane, the heterologous antigen can be expressed in an extracellular manner. Experiments on the heterologous protein presentation of *ClyA*-OMVs have been gradually extended. For example, expression of the Omp22 protein using the *E. coli* DH5α strain and the Fv fragment of the presenting antibody, the HER2 antibody, and the influenza virus M2e antibody, and so on (Kesty and Kuehn, 2004; Kim et al., 2008; Gujrati and Sangyong, 2014; Garrett Rappazzo et al., 2016; Huang et al., 2016).

Vesicles produced by bacterial pathogens promote the direct delivery of antigens into the host cells. In this regard, the recombinant *ClyA*-OMV increases the antigen delivery efficiency by 8-fold (Wai et al., 2003). Further research shows that the protein fusion on *ClyA* has different effects. Fusion with the

N-terminal of *ClyA* produces heterologous antigens that are inconsistent, while C-terminal fusions always produce well-displayed proteins which retain their biological functions (Kim et al., 2008). This may be due to the fact that the C-terminus of *ClyA* is closer to the outer surface compared to the distance between the N-terminus of *ClyA* and the outer membrane, and is therefore more likely to extend into the extracellular environment (Tzokov et al., 2006; Eifler et al., 2014). Additionally, the physiological state of *ClyA* also affects its localization in the OMVs. According to experiments, the redox state of *ClyA* is pivotal in regulating its localization in the OMVs (Kim et al., 2008).

AT Platform

Autotransport (AT) is a large virulence factor (usually over 100 kDa) secreted by Gram-negative bacteria. The AT system is simple, and is hypothesized to carry all information concerning the inner periplasm, and the outer membrane translocation of the protein itself. AT is synthesized as a large precursor protein containing three domains: first, the N-terminal signal peptide, which targets the protein to the Sec translocator and initiates the transfer through the intima; second, the passenger domain; third, the C-terminal beta-domain, which integrates into the outer membrane and plays a crucial role in passenger translocation into the extracellular space (Eifler et al., 2012). The AT system has been used as a transport vehicle for many heterologous fusion partners in Gram-negative bacteria (Kjaergaard et al., 2000; Junker et al., 2019). In most cases, variants of the *Neisseria* IgA protease and the endogenous *E. coli* AT AIDA-I are used to engineer antigens specifically for surface display (Junker et al., 2007). Two model antigens of *Leishmania* were successfully expressed using this method (Schroeder and Aebischer, 2009). Recently, the skp protein of ETEC has been successfully loaded into OMVs through AT, which fused with the glutathione-S-transferase (GST) epitope and combined with cholera toxin to prepare an ideal vaccine (Hays et al., 2018). OspA has been extensively studied as a vaccine component against Lyme disease (Wressnigg et al., 2014; Comstedt et al., 2015). The OspA antigen has been successfully expressed in OMVs but has not been exposed on the surface of OMVs. The improved method uses a meningococcal surface protein, fHbp, as the anchor protein. By connecting OspA to the N-terminus of fHbp, the strong immune response elicited by the engineered OMVs was successfully detected (Salverda et al., 2016; Zhang et al., 2016). Ovalbumin (OVA) hemoglobin protease has also been successfully expressed on the surface of engineered OMVs through the AT platform, and has been shown to have the ability to induce antigen-specific CD8 + T cell responses (Schetters et al., 2019). Unlike *ClyA*, the AT system can successfully present multiple antigens using a slightly different approach. Using the crystal structure of the AT passenger, five passenger side domains were selected and replaced with the *M. tuberculosis* antigen ESAT6 sequentially, while the β-helix core structure (β-stem) remained intact (Otto et al., 2005). The resulting Hbp-ESAT6 chimera was efficiently and stably secreted into the medium of *E. coli*. The Hbp-ESAT6 display variant was constructed by disrupting the cleavage site between the passenger and the β-domain, which maintains cell correlation and promotes efficient surface exposure of ESAT6 (Eifler et al., 2012). Compared to the *ClyA* fusion system, the AT system has multiple sites for the insertion of heterologous sequences that can simultaneously bind multiple antigens. This enables the formation of a multivalent recombinant live vaccine. This is of high value because multivalent vaccines are a key requirement for the prevention of infectious diseases such as tuberculosis (Aagaard et al., 2011). Importantly, since the passenger side domains have Hbp function, their replacement with antigenic proteins automatically eliminates potential toxic effects, making the proposed Hbp platform safe for antigen delivery (van Bloois et al., 2011). Thus, the AT system has been found to be more efficient, convenient, and safer than the engineered OMVs associated with *ClyA*.

Spy Tag/Spy Catcher System

The SpyCatcher is a genetically encoded protein designed to spontaneously form a covalent bond with its peptide partner SpyTag (Brune et al., 2016). Studies have demonstrated the ability of this bioconjugate system to deliver antigens, which can be linked to an antigen delivery system via common cell-linking proteins (Alves et al., 2015). It has been found that this system can be used as a modification of the autotransport system (Jong et al., 2010). In the AT system, although antigen delivery has been successfully accomplished, their density on the OMVs has been found to be affected (Jong et al., 2010). The SpyCatcher/SpyTag protein ligation technology can enzymatically attach the antigen to the high-density Hbp in OMVs, without being hampered by the membrane environment, as verified by recent experiments. This protein ligation system is based on the fibronectin of *Streptococcus pyogenes,* and forms an intramolecular isopeptide bond between the adjacent lysine and aspartate residues by an autocatalytic mechanism (Zakeri et al., 2012). The domain was split into a 13 amino acid (AA) peptide called the SpyTag, and a 138-AA fragment called the SpyCatcher, to create a SpyCatcher/SpyTag pair for coupling with heterologous proteins and autotransporters. When fused to different proteins, the SpyTag and the SpyCatcher recombine with the domain following mixing and form stable intermolecular covalent bonds under certain conditions (Fierer et al., 2014). Additionally, a similar structure, the orthogonal SnoopTag/SnoopCatcher system, can also couple multiple antigenic modules to Hbp by a sequential ligation strategy without affecting antigenic display (Veggiani et al., 2016). Spy ligation can be easily performed by gene fusion to the end of the Hbp display vector, and directional loading onto the OMVs. OMV display technology, combined with protein linkages, constitutes a plug-and-play system designed for customized antigen delivery. As a "mediating" protein system, the Spy Tag/Spy Catcher can also link other anchoring proteins such as OmpA, to deliver the corresponding antigen. This has been verified in the presentation of the active enzyme phosphodiesterase (PTE) (Alves et al., 2015). Therefore, the Spy Tag/Spy Catcher system can be used for modifying anchoring proteins, allowing engineered OMVs to perform antigen delivery more efficiently.

Ice Nucleation Protein

To display multiple functional proteins on OMVs, a trivalent scaffold (Scaf3) plasmid was constructed to express the protein of interest (Tsai et al., 2009). Park et al. (2014) used an enzyme scaffold to express the protein in bacteria, and immobilized it on the outer membrane using a truncated ice nucleation protein anchor. The plasmid pINP was constructed by amplification using a truncated ice nucleation protein gene from pPNC20 (Bae et al., 2002). High levels of INP-scaf3 were successfully detected on engineered *E. coli* OMVs, by binding Scf3 to INP by primers (Park et al., 2014). This provides a simple and convenient way to express different enzymes on OMVs. The assembled enzyme complex not only retains full activity, but also leads to the hydrolysis of cellulose, 23 times faster than the conventional enzymes. This method can thus be used as a simple platform for the efficient functioning of multiple enzymes as nano-biocatalysts (Park et al., 2014). The flexibility to design scaffolds using various specific binding domains allows for the display of unlimited number of functional proteins on OMVs.

Similar to the AT system, the enzyme scaffold is also a high-performance system that can simultaneously load multiple heterologous proteins. However, the AT system has certain inevitable shortcomings, like a truncated passenger structure used in the loading of multiple antigens (Jong et al., 2010). Similar to the AT system, the enzyme scaffold also binds the target protein in an orderly manner, forming a multi-functional system independently. Therefore, it is expected to demonstrate more applications.

Glycosylation Linkage

Glycosylation-modified OMVs (geOMVs), as seen in *Streptococcus pneumoniae* capsules and *C. jejuni* N-linked glycans, have been shown to produce a good immune response (Price et al., 2016). The geOMV platform utilizes the loose glycan structure of the O-antigen ligase. Specifically, they are delivered to the lipid A-core molecule in the inner membrane (Kaniuk et al., 2004; Han et al., 2012). First, glycans were customized using the initial glycosyltransferase, WecA, and a specific glycosyltransferase encoded on a plasmid. The flip enzyme Wzx subsequently moved the glycans to the periplasmic surface of the inner membrane, and the enzyme Wzy polymerized the glycan subunits into a high-molecular-weight form of the surface glycans. The WaaL ligase then attached the glycan to the lipid A-core molecule. Finally, these customized LPS molecules were transported to the outer membrane and flipped into the extracellular space through the Lpt protein complex (Kaniuk et al., 2004; Simpson et al., 2015). When bacteria formed the OMVs, LPS as well as proteins and lipids were packaged into OMVs, resulting in the formation of mature geOMVs. The *S. pneumoniae* capsules were expressed in *E. coli* cells lacking the relevant O antigen, and the relevant plasmid was constructed and introduced into *E. coli* to secrete the corresponding OMVs. The immunological potency was confirmed using animal experiments (Price et al., 2016). Glycosylated conjugate vaccines are valuable. However, technical challenges exist with respect to the attachment of glycan chains (Jones, 2005; Wacker et al., 2006; Bottomley et al., 2012).

The glycol-engineered *E. coli*-expressing OMVs can express the glycans required for binding to the vaccine, and thus can complement the current conjugate vaccines, particularly for serotypes corresponding to glycan structures that have not been resolved. The studies of glycans were less than those of proteins, lipids, and nucleic acids (Williams et al., 2018). The successful validation of glycosylated OMVs provides an inspiring platform for future engineered glycosylation efforts (Liang et al., 2014; Shimoda et al., 2017).

OTHER APPLICATIONS OF OMV$_S$ USING GENETIC ENGINEERING

In addition to presenting antigens for vaccine application, the transformation of OMVs has now embraced more functions, including drug delivery, bioimaging, nanogenerators, and new adjuvants (**Table 2**). OMVs are being developed for more applications due to their flexible retrofit characteristics.

Drug Delivery

In addition to serving as a platform for surface antigen presentation, the function of OMVs to encapsulate small molecule particles in the lumen for presentation is also of interest. Recent studies have found that OMVs have a great potential in the transport of small molecule drugs (**Table 3**). The method of loading drugs into the lumen of OMVs by electroporation has been proven. For relatively hydrophobic and positively charged small molecule drugs that easily interact with lipophilic membranes, passive diffusion can be used. However, for hydrophilic molecules, the bilayer lipid membrane may constitute an obstacle, in which case electroporation may be the method of choice (Gujrati et al., 2014) (**Figure 3**). Although OMVs can only encapsulate about 15% of KSP-siRNA, the amount is sufficient to exert cytotoxicity. This electrotransport method is convenient and easy to implement, and has been widely used in loading small molecular substances (Turner et al., 2015). Recently, scientists have tried to use bioengineering methods to successfully upload cancer treatment drugs on OMVs. OMVs from the non-pathogenic commensal bacteria were used to deliver keratinocyte growth factor 2 (KGF-2), a small molecule with the potential to treat inflammatory bowel disease (Baumgart and Sandborn, 2007). Adding intact KGF-2 OMVs to epithelial cell culture promoted cell proliferation and accelerated wound healing, in a safe manner (Carvalho et al., 2019). Melanin, a specific agent for photothermia therapy in cancer treatment, was presented through OMVs, and almost all the cancer cells in the animal model were destroyed (Gujrati et al., 2019) (**Figure 4**). Another loading method is the incorporation of drugs into OMVs during their biological generation through parental bacteria. Further to cancer treatment, drug delivery by OMVs has also been confirmed. Allan and Beveridge (2003) have demonstrated that the incorporation of gentamicin during the growth of *P. aeruginosa* PAO1 strains produced gentamicin-containing OMVs, which can deliver drugs to the target, *Burkholderia*

TABLE 2 | Application and performance of engineered OMVs.

Application	Lumen/outer membrane	Anchor protein	Results and efficacy	References
Nanopolymer bioreactor	Outer membrane	Ice Nucleation Protein (INP)	Provide synergistic cellulose hydrolysis; Increase glucose production by 23 times compared with free enzyme	Park et al., 2014
Multifunctional imaging and detection platforms	Lumen	None	Easily customize through genetic engineering; Create a wide range of applications including live cell imaging	Chen et al., 2017
Drug delivery	Lumen	None	Slowly release drugs under specific environmental conditions; Reduce non-specific toxicity caused by drug leakage into the circulatory system	Gujrati et al., 2014
Vaccine adjuvant	Lumen / Outer membrane	ClyA; Autotransport (AT); Ice nucleation protein; Glycosylation	Safe and effective vaccine platform	Kesty and Kuehn, 2004; Junker et al., 2007; Park et al., 2014; Price et al., 2016

TABLE 3 | Engineering OMVs delivering drugs and corresponding efficacy of diseases.

Bacterial strain	Disease	Drugs	Effect and characteristic	References
Bteroides thetaiotaomicron VPI-5482	Colitis	keratinocyte growth factor-2 (KGF-2)	Control colitis clinically and pathologically; Reduce colon atrophy and length reduction caused by treatment; Reduce epithelial damage and inflammatory infiltration	Carvalho et al., 2019
Escherichia coli strain that exhibits reduced endotoxicity toward human cells	Cancer	Therapeutic siRNA	Complete biodegradability; Very rigid and stable nanostructure; Reduced drug leakage and non-specific toxicity	Gujrati et al., 2014
Escherichia coli K12	Cancer	Naturally occurring melanin	Tumor growth is reduced by about 43%; High safety, non-toxic to normal cells	Gujrati et al., 2019
Pseudomonas aeruginosa PAO1 strain	Cystic fibrosis, Cepacia syndrome	Gentamicin	Thermodynamic stability and will not decompose in suspension	Allan and Beveridge, 2003
Streptococcus flexi serotype 5 (M90T)	Parasitic disease	Gentamicin	Drug stability	Kadurugamuwa and Beveridge, 1998

cepacia (Allan and Beveridge, 2003). This was also confirmed in *Shigella flexneri* OMVs (Kadurugamuwa and Beveridge, 1998).
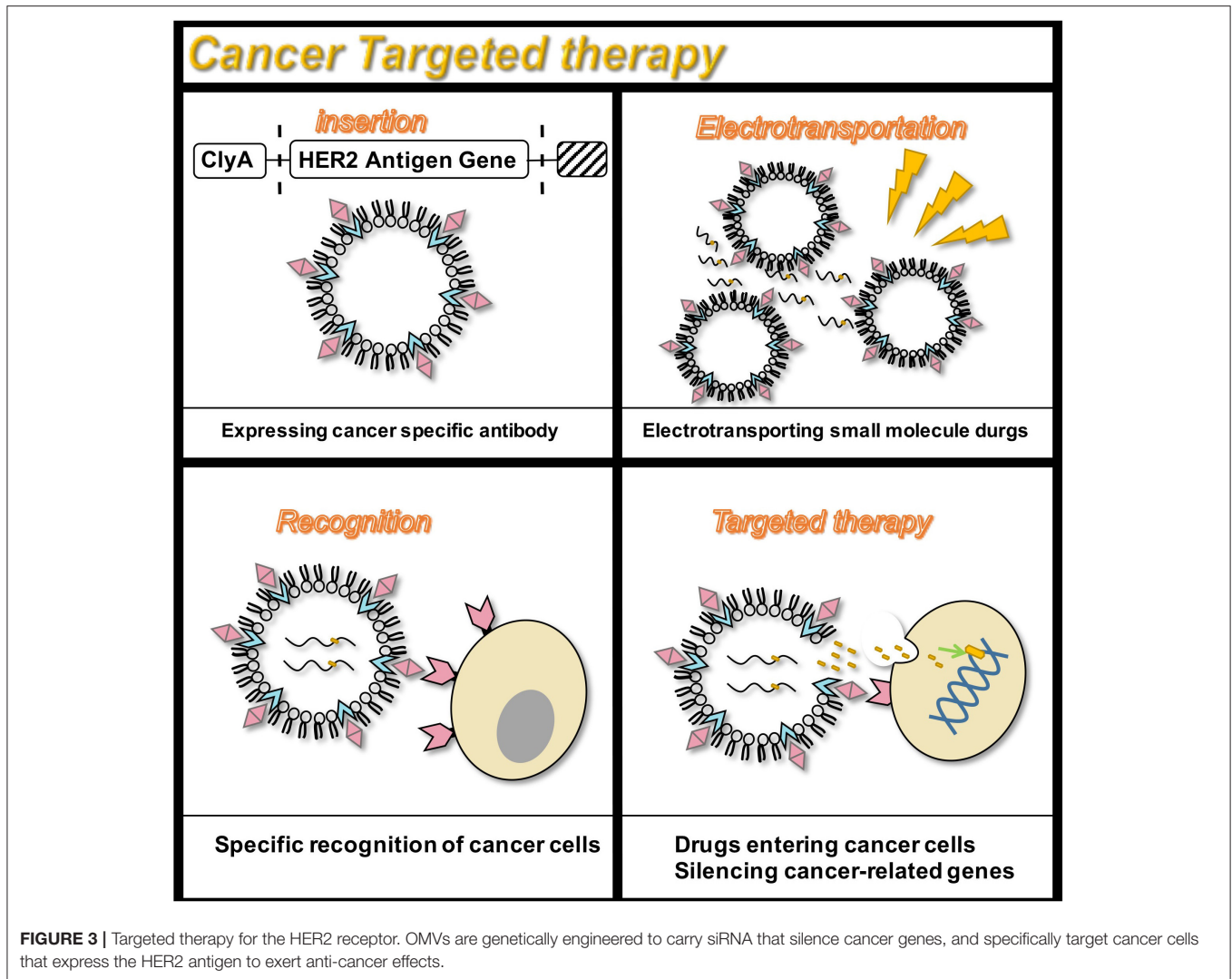
As a drug presentation tool, OMVs have the advantage of being small, stable, well-tolerated, and highly specific. OMVs can slowly release drugs under specific Physiological conditions, reducing non-specific toxicity caused by drug leakage into the circulatory system. In addition, this slow-release property can provide sufficient time for siRNA to reach its target cancer cells in areas with abnormal blood supply (Gujrati et al., 2014). OMVs have thermodynamic stability and will not decompose in suspension, while ordinary liposomes have the disadvantages of drug leakage and easy degradation of circulation. Although the packaging efficiency is not ideal, it is sufficient to exert drug efficacy.

Multifunctional Imaging and Detection Platforms

Complex, multi-antigen-bound engineered OMVs have begun to evolve and grow. Based on this technology, scientists have proposed a "one-pot synthesis" model (Chen et al., 2017).

According to the mutual binding between proteins, the nano-luciferase (Nluc) and the Z domain recruits antibodies which exists in OMVs at the same time (Tashiro et al., 1997). Experiments have confirmed the feasibility of imaging cancer cells by multifunctional OMVs (Ferreira et al., 2006). Currently, multifunctional OMVs can be customized with ease through genetic engineering, creating an almost unlimited combination of "capture" and "report," for a wide range of applications including live cell imaging (Chen et al., 2017) (**Figure 5**). At the same time, the photoacoustic imaging method has proven to be feasible to produce encapsulated biopolymer-melanin OMV (OMV Mel), using bacterial strains expressing the tyrosinase transgenes. The OMV Mel is generated under near-infrared light, and is suitable for imaging applications. The strong photoacoustic signal non-invasively monitors tumor-associated OMV Mel distribution *in vivo* (Gujrati et al., 2019). This experiment represents the rationale on the use of bioengineered OMVs as effective alternatives to synthetic particles in photoacoustic imaging, for image enhancement and photo-thermal applications (**Figure 4**).

OMVs have been reported to possess excellent sensitivity. When used in thrombin detection, the detection limit of

FIGURE 3 | Targeted therapy for the HER2 receptor. OMVs are genetically engineered to carry siRNA that silence cancer genes, and specifically target cancer cells that express the HER2 antigen to exert anti-cancer effects.

thrombin by OMVs was found to be 0.5 nm (Chen et al., 2017). Additionally, the preparatory method is simple, and a high yield of OMVs can be successfully obtained with ease only through a simple fermentation process. Furthermore, since more than 60 membrane-anchoring proteins have been detected in OMVs, a variety of modified enzymes can be presented by OMVs. Therefore, OMVs can flexibly modify electrochemical-based detection or colorimetric detection of enzymes. Owing to these advantages, OMVs can be applied in immunoassays, *in vivo* imaging, and cancer cell detection.

Nanopolymer Bioreactor

Multi-step enzyme pathways play a key role in cell metabolism. Enzyme cascade reactions are highly complex systems. A key feature of a highly efficient enzymatic pathway is the synergistic and spatial organization of the enzyme to ensure sequential transformation of the substrate (Agapakis et al., 2012). OMV is relatively stable in the surrounding environment and can be manufactured cost-effectively, and is considered to have

the potential of a bioreactor. A novel study by Park et al. (2014) identified a single protein scaffold based on the Coh-Doc interaction, which can be used to sequentially assemble multiple enzymes onto the surface of OMVs, thereby resulting in a nanoreactor capable of complex biocatalysis (Park et al., 2014) (**Figure 6**). By genetically engineering enzyme scaffolds into OMVs, scientists have succeeded in obtaining an enzyme system with an enzyme cascade. Recently, the expression of organophosphate hydrolase in *E. coli* OMVs has also been successful, and engineered OMVs have enhanced thermal stability and pH stability (Su et al., 2017). After 15 reaction cycles, at least 83% of the OMVs initial activity is retained. After 40 days, about 20–30% of initial activity is retained (Su et al., 2017). In addition, phosphotriesterase was displayed in OMVs through the outer membrane porin OmpA of *E. coli* (Alves et al., 2018).

In recent decades, various methods have been developed to create artificial multi-enzyme complexes, including OMVs, polyhydroxyalkanoate (PHA) particles, virus-like particles (VLP), enzyme-derived nanoparticles (EZPs), and

magnetosomes (Parlane et al., 2017; Yan et al., 2017; Wilkerson et al., 2018; Schmid-Dannertand and López-Gallego, 2019). However, in terms of its wider application, there are still some obstacles. For example, due to steric hindrance, the encapsulation rate of large proteins is low, and the substrate cannot fully enter the enzyme assembly and the vesicle shell (Wong et al., 2020). These nanoparticles are difficult to challenge for long-term storage and have poor biocompatibility. The enzyme system expressed through the outer membrane vesicles has been proven stable for long-term storage and has the potential to be used as a carrier for industrial production of enzyme systems.

As a Vaccine Adjuvant

An adjuvant is defined as a molecule that aids an antigen in eliciting an immune response. Adjuvants improve the immune response in people with poor immune response, expand cross-protection, induce a shift in the specific T cell-assisted response, and reduce the amount of antigen required for vaccine development (Maisonneuve et al., 2014). OMVs have been considered as potential vaccine adjuvants since their inception (**Figure 7**). However, due to the toxicity of wild-type LPS, such as flagellin, lipoprotein, and other OMPs, they may trigger allergic reactions such as excessive inflammation (Arigita et al., 2005; Thompson et al., 2005). Therefore, the

OMV endotoxins are removed manually following production. Another disadvantage is that the LPS-deficient OMVs generally exhibit lower immunogenicity compared to the immunogenicity of the wild-type OMVs containing wild-type LPS (Gnopo et al., 2017). Therefore, the appropriate modification of LPS is necessary to obtain an optimal balance between low-toxicity and high-immunogenicity. The latest method of LPS modification involves the synthetic assembly or genetic modification of bacteria (Arenas et al., 2010). Further, OMVs with *LpxL* gene deletion and *PagL* gene expression have been proven to possess good adjuvant ability and show significantly better abilities compared to that of the wild type (Arenas et al., 2010; van de Waterbeemd et al., 2010; Asensio et al., 2011). Further comparison has revealed that the *PagL* gene expression possesses higher endotoxic activity compared to that of the *LpxL* gene, which was also supported by animal studies. The *PagL* gene is considered to have a better adjuvant effect (Asensio et al., 2011).

Recent studies have shown that the adjuvant ability of engineered OMVs continues to attract the attention of researchers. Engineered OMVs carrying the Ail antigen have been shown to increase adhesion to the CHO cells, and non-invasive vesicles have been modified to invade the CHO cells (Kesty and Kuehn, 2004). Recent developments in vaccine research involve the use of more antigen profiles, expressing
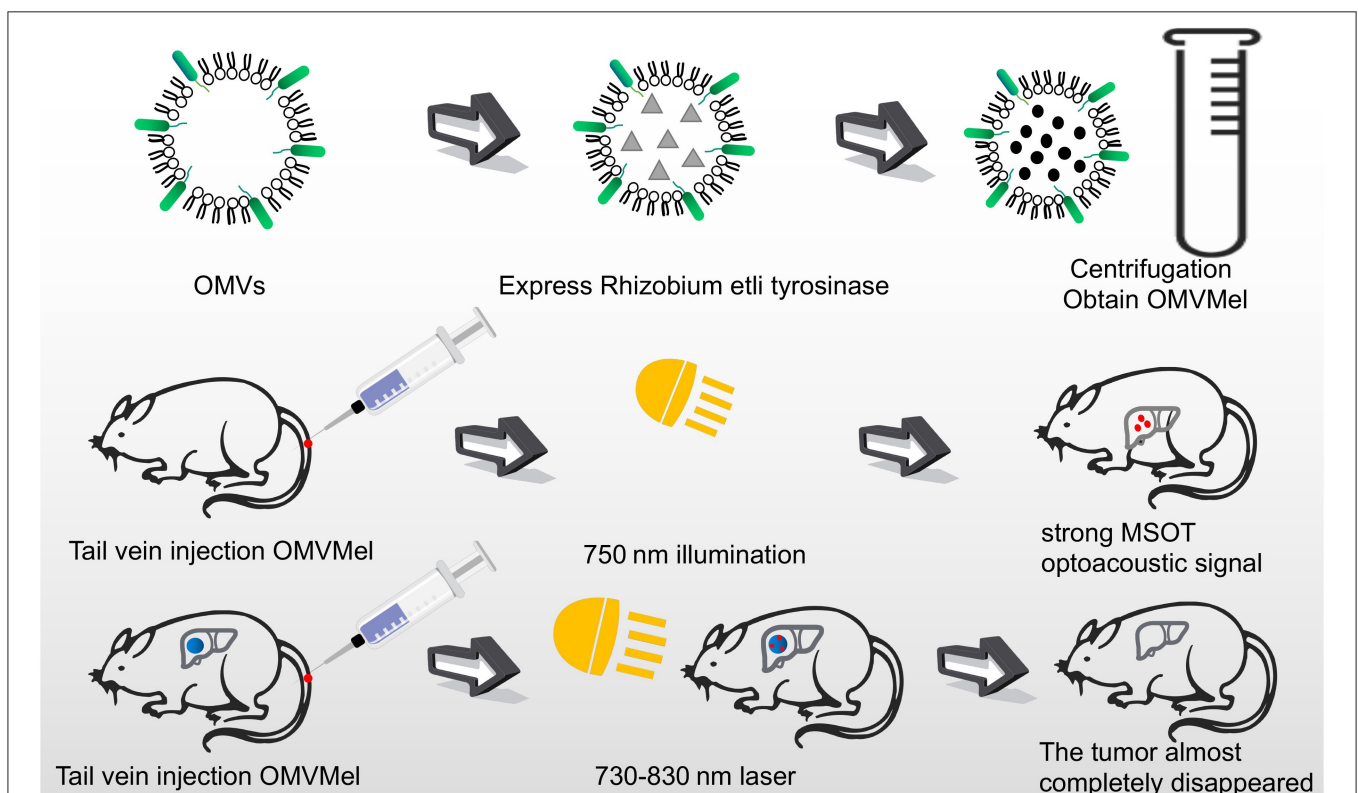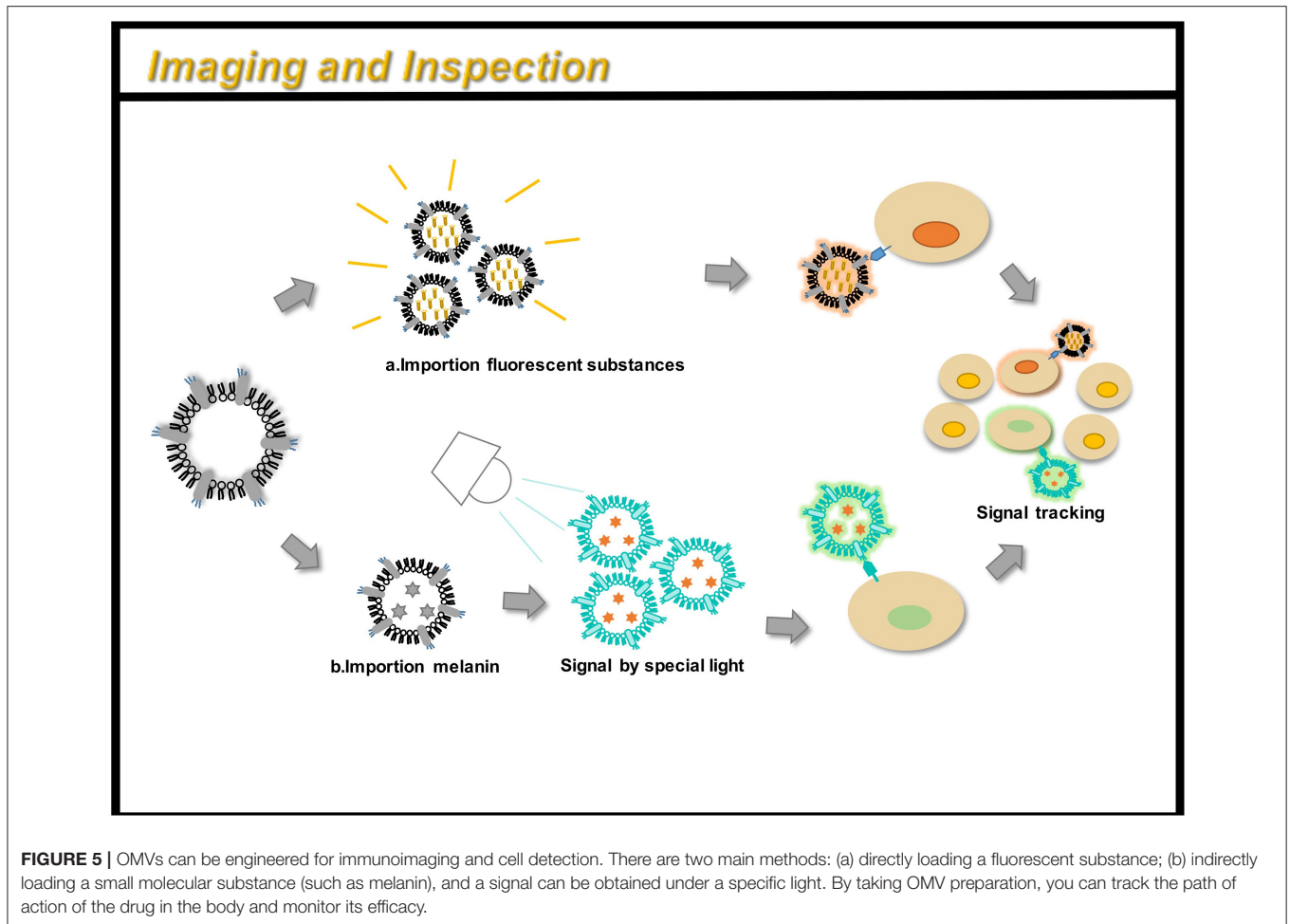

FIGURE 4 | OMVs loaded with melanin were injected into mice through the tail vein. After irradiating at 750 nm, a strong photoacoustic signal of MSOT was generated. The tumor cells were almost completely killed by laser irradiation at 730–830 nm for several minutes.

FIGURE 5 | OMVs can be engineered for immunoimaging and cell detection. There are two main methods: (a) directly loading a fluorescent substance; (b) indirectly loading a small molecular substance (such as melanin), and a signal can be obtained under a specific light. By taking OMV preparation, you can track the path of action of the drug in the body and monitor its efficacy.

mutant forms of HlaH35L, SpAKKAA, LukE, LukD/E, one of the two components of the interleukin Csa1A, and the iron binding protein, FhuD2, on meningococcal OMVs, have proven to possess good immunogenicity (Falugi et al., 2013; Gasanov et al., 2013; Spaan et al., 2017). The OMVs produced in *Salmonella* carrying pneumococcal protein antigens have shown promising results in a murine model (Kuipers et al., 2015). This allows glycans and proteins to synergistically enhance the immunogenicity of vaccines (Price et al., 2016). In contrast, the currently existing and the most successful antimicrobial vaccine glycoconjugates face multiple problems like complex preparation, slow development, insufficient safety, instability, and batch-to-batch variability. In addition, glycoconjugates do not contain multiple serotypes. OMVs can be genetically modified with flexibility and ease to produce geOMVs with the perfect balance between reduced toxicity and optimal adjuvant properties.

FUTURE RESEARCH DIRECTIONS

Compared to conventional adjuvants, OMVs show a clear advantage in terms of future development (Tan et al., 2018).

As a platform for the convenient implementation of genetic modification, OMVs have received attention in the field of precision medicine, in addition to a wide range of vaccine applications (Wang et al., 2019). For example, OMVs have become a potential choice for the targeted delivery of cancer drugs. As an easily engineered nanoparticle, OMVs can transport a variety of small molecules with different characteristics, thereby involving in important applications such as immunoimaging, enzyme-linked reaction polymers, etc. Further research will help to unveil the undiscovered aspects and future applications of the engineered OMVs.

Targeted Cancer Therapy Delivery Platform

Targeted therapy continues to receive attention owing to its fewer side effects. Bio-nanocarriers derived from a variety of natural resources like pathogens and mammalian cells have been identified to be well-suited for drug delivery (Yoo et al., 2011), and nanomaterials have been widely studied as drug delivery vehicles in the treatment of cancer (Petros and DeSimone, 2010; Shi et al., 2010). Designing OMVs to target specific cell types makes them an attractive treatment option. In addition, engineering OMVs offers several advantages. The outer
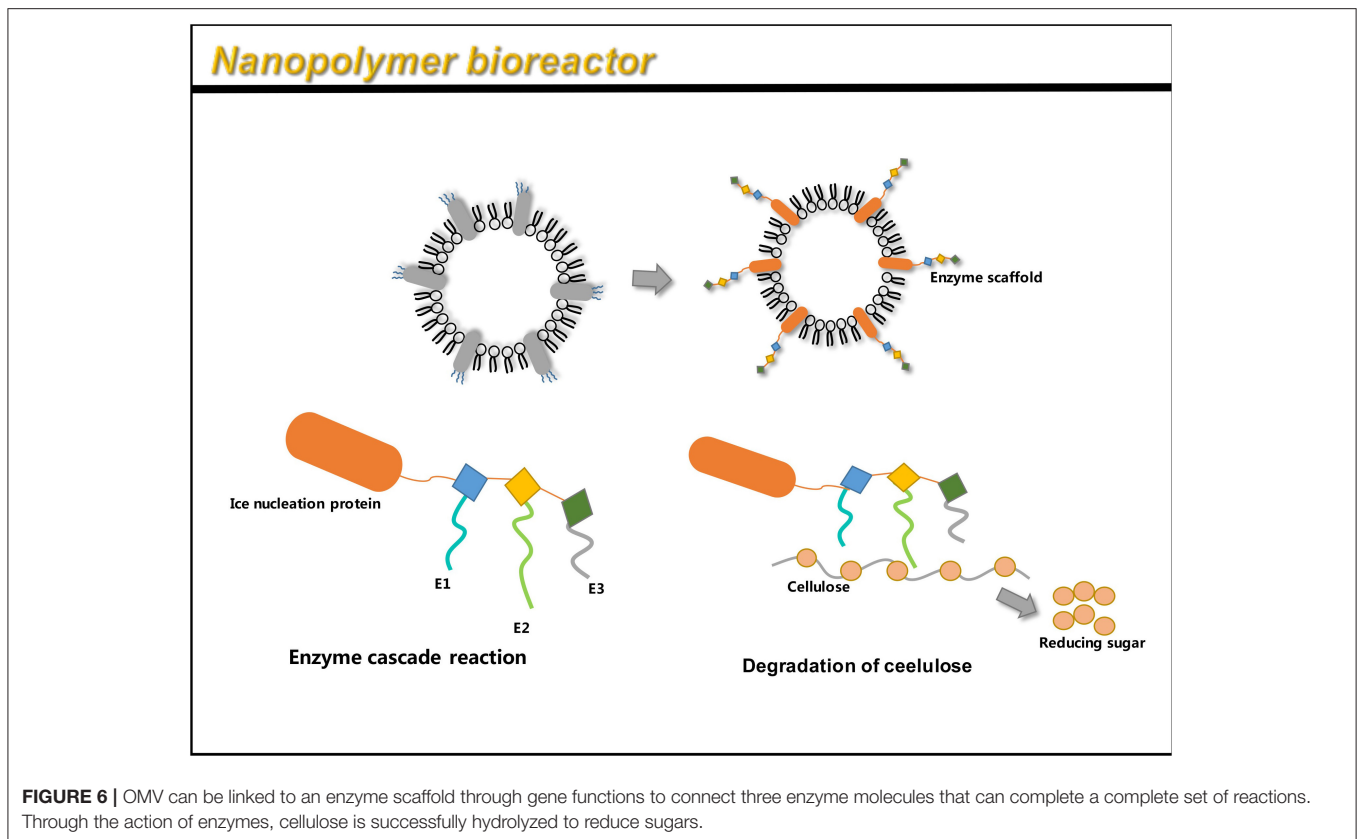
FIGURE 6 | OMV can be linked to an enzyme scaffold through gene functions to connect three enzyme molecules that can complete a complete set of reactions. Through the action of enzymes, cellulose is successfully hydrolyzed to reduce sugars.

membrane protects the OMV contents such as chemotherapeutic agents, from protease or nuclease degradation, thereby increasing the circulating half-life of the drug. Recent studies have successfully designed tumor-targeted OMVs carrying an anti-tumor siRNA (Gujrati and Sangyong, 2014; Gujrati et al., 2014). The transmembrane receptor HER2, chosen as the cell-specific target, was found to be overexpressed in a range of cancers, including breast, ovarian, and gastric cancers (Weaver and Cleveland, 2005). By binding HER2 antibody to ClyA, OMVs targeting cancer cells were obtained. *In vitro* studies by Gujrati et al. (2014) have demonstrated the ability of the siRNA-loaded OMVs to deliver siRNA in ovarian and breast cancer cell lines, subsequently inducing cancer cell death (Gujrati et al., 2014). Multiple forms of cancer treatment have been proven feasible. By presenting melanin, a substance with good light-to-heat conversion rate, a treatment can be achieved that can kill tumor cells by selectively irradiating the melanin-containing tissues, thereby inducing local heating (Liu et al., 2013). Animal studies have shown that almost all tumor cells in the body were eliminated. Furthermore, this photothermal therapy monitors the effect of local treatment by photoacoustic imaging (Gujrati et al., 2019). Additionally, recent studies have shown that engineered OMVs, which transport OVA hemoglobin protease using the AT system, have a mediatory function in the induction of antigen-specific CD8$^+$ T cell response (Schetters et al., 2019). CD8$^+$ T cells control viral replication and tumor growth. This property of OMVs also hints at its powerful potential in cancer treatment.

OMVs deliver heterologous proteins thus, acting as tools for delivering targeted drugs. Such vesicles are expected to be further developed in the near future. The advent of nanobiotechnology, and advances in genomics, immunology, microbiology, and vaccine needs, alone and in combination, have made OMVs a novel choice for precision therapies and targeted-drug therapies.

Multifunctional Delivery Platform

In addition to the widely known function of antigen presentation, OMVs have been used in a wide array of applications. For example, OMVs can be used as bioreactors for enzyme cascade reactions. By carrying anchor proteins which load multiple heterologous proteins such as ice nucleating proteins, OMVs can combine multiple related enzymes to complete a more complex set of biological reactions (Park et al., 2014). Additionally, engineered OMVs have also been used in the field of immune bioimaging. OMVs can be directly tagged with fluorescent substances that can be easily detected, such as GFP, and perform functions such as *in vivo* imaging and remote tracking (Chen et al., 2017). Indirect detection methods have also been confirmed. OMVs carrying melanin generate strong photoacoustic signals suitable for imaging applications under near-infrared light (Gujrati et al., 2019). This method has also proven to be therapeutically effective. For rendering OMVs
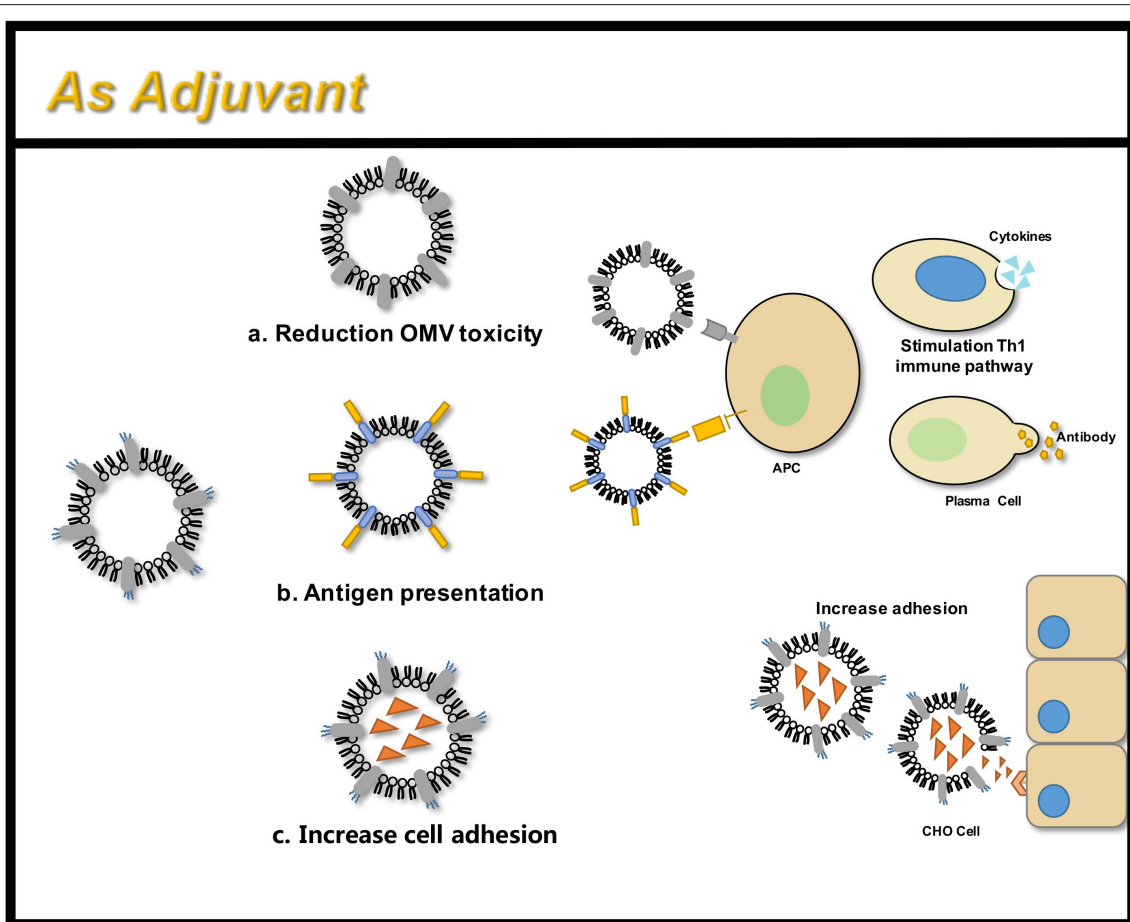
FIGURE 7 | OMVs function as an adjuvant through three main areas: **(a)** attenuated antigen stimulation; **(b)** presentation of heterologous antigens; **(c)** increased adhesion to tissue cells.

in various platforms, secure performance is a pre-requisite. In addition to existing methods for attenuating gene knockouts, the use of non-pathogenic symbiotic bacteria as a source of OMVs has also received attention (Carvalho et al., 2019). Reconstruction of these OMVs can easily produce engineered OMVs with high safety.

With further study of OMVs, it has been shown that this bio-nanoparticle has a variety of functions not limited to vaccine adjuvants. Relying on flexible genetic modifications, OMVs perform multiple functions by carrying small molecules with multiple functions. OMVs are expected to demonstrate more applications in the fields of immunology, diagnostics, and so on.

CONCLUSION

OMVs have attracted attention as a new engineering platform for vaccine development. Through research on the engineering of OMVs, we find that the applications of OMVs are not limited to vaccines. As a convenient bio-nanoparticle, OMV has the

potential to function as a multi-functional delivery platform, and thus can be applied in many fields such as immunology, clinical medicine, diagnostics, and biochemistry. However, there are still some problems in the application of OMVs: (i) the toxicity of LPS on OMVs is still a major concern, especially when applied in the human body; (ii) in biological engineering, OMVs load heterologous proteins and the efficiency of small molecular substances is not high; (iii) in large-scale production, the size of OMVs is not easy to control, and the difference between batches is large. At the same time, the lack of understanding of the exact assembly mechanism of OMVs leads to poor programmability of the particles. In future research, attention should be paid to other problems of modifying OMVs such as: (i) increasing the load rate; (ii) improving safety and reduce costs when large-scale production is required; and (iii) delineating the mechanism of OMVs germination for a better understanding of the mechanism of its functions. This review focuses on some of the latest advances in the application of engineered OMVs such as drugs for cancer treatment, immune imaging, etc. It is anticipated that the functions of OMVs may be better manipulated through genetic engineering in subsequent studies.

AUTHOR CONTRIBUTIONS

RL wrote the manuscript. QL and RL revised the manuscript for integrity and accuracy. QL approved the final version of the manuscript and takes responsibility for its content. All authors contributed to the article and approved the submitted version.

FUNDING

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