Wilfred Chen ORCID iD: 0000-0002-6386-6958

Yu Lei ORCID iD: 0000-0002-0184-0373

Outer Membrane Vesicles (OMVs) Enabled Bio-applications: A critical review

Yikun Huang[1], Mu-Ping Nieh[2], Wilfred Chen[3*], Yu Lei[1,2*]

[1]Department of Biomedical Engineering, University of Connecticut, Storrs, CT 06269, USA

[2]Department of Chemical and Biomolecular Engineering, University of Connecticut, Storrs, CT 06269, USA

[3]Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, DE 19716, USA

Corresponding authors: Yu Lei and Wilfred Chen

yu.lei@uconn.edu

wilfred@udel.edu

Abstract

Outer membrane vesicles (OMVs) are nanoscale spherical vesicles released from Gram-negative bacteria. The lipid bilayer membrane structure of OMVs consists of the similar components as bacterial membrane and thus has attracted more and more attention in exploiting OMVs' bio-applications. Although the endotoxic

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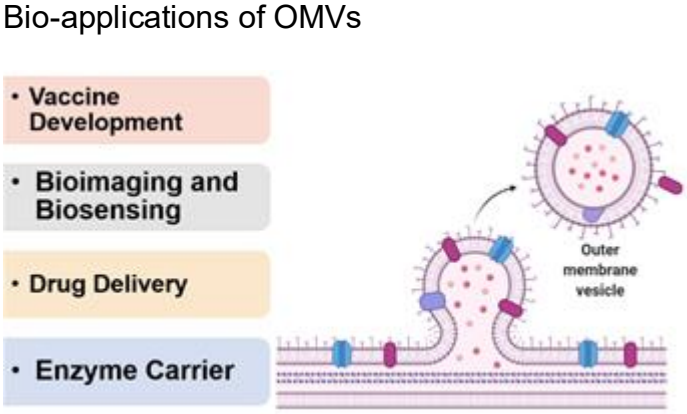
lipopolysaccharide on natural OMVs may impose potential limit on their clinical applications, genetic modification can reduce their endotoxicity and decorate OMVs with multiple functional proteins. These genetically engineered OMVs have been employed in various fields including vaccination, drug delivery, cancer therapy, bioimaging, biosensing, and enzyme carrier. This review will first briefly introduce the background of OMVs followed by recent advances in functionalization and various applications of engineered OMVs with an emphasis on the working principles and their performance, and then discuss about the future trends of OMVs in biomedical applications.

Graphical Abstract



Keywords

Outer membrane vesicles, drug delivery, bioimaging, immunoassay, vaccine, immunotherapy, enzyme carrier

1. Introduction of OMVs

Since the first discovery and characterization of outer membrane vesicles (OMVs) in 1967, these Gram-negative bacteria derived vesicles have been extensively studied for

a half century (Chatterjee & Chaudhuri, 2012; Pathirana & Kaparakis‐Liaskos, 2016). OMVs are spherical nanoparticles with lipid bilayer structure and in a size of 20-250 nm. These vesicles contain the similar components as bacteria, including phospholipids, β-barrel proteins, lipopolysaccharide (LPS), lipoprotein, DNA, etc., since they are formed from cell envelop of bacteria (Schertzer & Whiteley, 2012; Shin, Gedi, Kim, & Lee, 2019). Understanding the biogenesis and biological functions of OMVs is paramount of importance since it confers new pathways on the application of OMVs (Schwechheimer & Kuehn, 2015). In general, the formation of OMVs can be described as the detachment of outer membrane bleb (Qing et al., 2019). Although it has been studied for decades, there is no universal explanation toward the formation mechanism of OMVs (van der Pol, Stork, & van der Ley, 2015). As for the biological functions of OMVs, it has been found that OMVs participate in and/or mediate several bacterial physiologies (Cecil, Sirisaengtaksin, O'Brien-Simpson, & Krachler, 2019). First, OMVs are used in long distance delivery of signaling components to achieve the communication among bacteria, environment, and other bacteria community (Kulp & Kuehn, 2010). Additionally, OMVs booster the bacteria pathogenesis due to the participation of biofilm formation and delivery of virulence factors (Begić & Josić, 2020). Furthermore, OMVs can help bacteria survive in the harsh environment. For example, OMVs can protect bacteria in antibiotic existing condition by transferring antibiotic resistance genes or acting as decoys to bind with antibiotics (Ünal, Schaar, & Riesbeck, 2011).

OMVs have been widely used in vaccine development due to their well-documented biological functions and components. The first OMVs containing Serogroup B Meningococcal vaccine has been approved by the regulation agency and applied in disease prevention (van der Pol et al., 2015). In recent years, recombinant DNA

technology further accelerated the research of OMVs in biomedical and other fields. With the capability to decorate OMVs with heterologous functional materials, these vesicles are not only used as the antigen, but also served as the carrier and adjuvant in vaccine development. OMVs can be further used to deliver pathogens from other species like Gram-positive bacteria or virus as well. Besides antigens, OMVs are decorated with targeting ligands for drug delivery.(V. Gujrati et al., 2014) In cancer therapy, the immunotherapeutic ability of OMVs potentially enhances the performance of traditional chemotherapy drug, which opens a new avenue for cancer therapy.(Kuerban et al., 2020) Furthermore, imaging reporters such as luciferase for bioluminescence imaging and melanin for optoacoustic imaging, are employed in the functionalization of OMVs. With the help of those reporters, OMVs can be applied for non-invasive *in vivo* imaging and thermal therapy.(Q. Chen, Rozovsky, & Chen, 2017; V. Gujrati et al., 2019) Moreover, OMVs can be loaded with enzymes to complete cascade reaction for cellulose hydrolysis and environment decontamination.(Alves et al., 2015; Park, Sun, Liu, DeLisa, & Chen, 2014) According to the discussion above, OMVs exhibit a great potential in biomedical and biochemical fields. Their bio-applications could be further advanced through decoration of the OMVs with heterologous materials or encapsulation of therapeutic molecules, enabled by genetic modification technologies and other functionalization methods. In this review, we aim at providing a systematic overview of OMVs enabled bio-applications. Recent advances in various bio-applications of engineered OMVs are discussed based on the difference of applications, ranging from vaccination, drug delivery, cancer therapy, bioimaging, biosensing, to enzyme carrier (Figure 1). The review will conclude with and discuss about the future trends of OMVs in biomedical applications.

2. Functionalization of OMVs

Although the intrinsic biological features make OMVs attractive as a unique platform in bio-applications, further functional modification is required prior to a broader practical application. For example, OMVs can be loaded with small molecules or siRNA and serve as the carrier in drug delivery (Sihan Wang, Gao, & Wang, 2019). The lipid bilayer membrane structure of OMVs can act as a barrier to protect the loading materials from decomposition in circulation system and prolong the half-life. Additionally, ligands or proteins that recognize the biomarkers in lesions can be functionalized on OMVs surface to achieve targeted delivery (V. Gujrati et al., 2014). In this section, we present two major functionalization methods that have been applied to OMVs. One relies on the conventional, physical or chemical approaches, while the other counts on genetic engineering. In some application of OMVs, both genetic and physical functionalization methods are employed jointly to accomplish multifunctionalities of OMVs. Table 1 summarizes the advantages and disadvantages of different OMVs functionalization methods.

Physical functionalization is commonly used for loading drugs or small molecules into the OMVs lumen. Incubation is the simplest way to load a desired cargo into OMVs by adding the cargo material during bacteria culturing or after OMVs isolation. Huang et al. used the incubation method to load antibiotics into OMVs by direct addition of antibiotics into the culture mixture (W. Huang et al., 2020). However, this method requires large amount of loading material as well as high stability of loading material in the incubation environment. Also, the loading capacity and efficiency are typically low or uncontrollable (Somiya, Yoshioka, & Ochiya, 2017). Besides the incubation method, other physical methods such as

electroporation, sonication, and extrusion, can be applied in OMVs functionalization as well (Ayed, Cuvillier, Dobhal, & Goreham, 2019; Q. Chen et al., 2019). Taking electroporation method as an example, this method has been widely used in loading exogenous material into extracellular vesicles (Johnsen et al., 2016), which can be adapted to encapsulate therapeutic molecules into OMVs. Researchers have loaded OMVs with gold nanoparticles and therapeutic siRNA by using electroporation (Ayed et al., 2019; V. Gujrati et al., 2014). Besides physical methods, chemical coupling reactions are also used in the OMVs functionalization. 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride/ N-hydroxysuccinimide (EDC/NHS) reaction is the one of the classic coupling reactions to realize bioconjugation. This reaction is usually applied for OMVs labeling by mixing OMVs with the dye NHS-ester (Jang et al., 2015). From the discussion above, one can see there are various options for OMVs functionalization via physical or chemical methods. Each method possesses different merits and weaknesses and relies on different working principles. Therefore, the suitable method should be selected, depending on the chemical and biological properties of materials, which are used. However, further purification is generally required after functionalization, which might limit the applications of aforementioned physical and chemical methods in OMV functionalization.

With the help of recombinant DNA technology, genetically engineered *E. coli* can also serve as a micro-factory to effectively produce OMVs with decorated heterologous proteins. Genetic engineering methods possess obvious advantages over other methods in OMVs functionalization with diverse proteins. First, this method is more environment-friendly and cost-effective compared with other methods. Decorating material is not required since it can be produced during cell culturing

through introducing the heterologous DNA into bacteria. In addition, large-scale production of functionalized OMVs can be cost-effectively achieved through facile bacterial culturing. After harvesting OMVs, further purification is not required. Moreover, the functionality of OMVs can be pre-determined and designed with high-level controllability and preciseness. Finally, genetic engineering method enables the placement of the required modification material in either exterior or interior of OMVs using different expression strategies, which provide flexibility to specific tailored applications.

Besides aforementioned direct decoration methods, natural binding systems are also introduced onto genetically engineered OMVs for further protein-protein interaction based functionalization, where high affinity between the binding pairs is used to modify OMVs with target materials (van den Berg van Saparoea, Houben, Kuijl, Luirink, & Jong, 2020). Chen et al. developed a multifunctional OMV using the dockerin-cohesin pair (Q. Chen et al., 2017). In this study, a scaffold structure with three dockerin domains was expressed on the OMVs surface. The target molecule, such as green fluorescence protein (GFP), was tagged with the cohesin domains and then anchored on OMVs surface through dockerin-cohesin interactions. Other binding systems, such as SpyTag/SpyCatcher system, are also used to develop a versatile OMVs-based platform for environment decontamination (Alves et al., 2015). The use of these binding pairs can overcome the limitation of direct modification via recombinant DNA technology (e.g., the size of protein, the number of proteins, and the expression efficiency). In addition, the decorated functional proteins can be replaced without re-engineering of OMVs. Moreover, multiple binding domains can be displayed on the same OMVs to accomplish multi-functionalization of OMVs. In

following sections, recent progress in various OMVs enabled bio-applications is systematically discussed.

3. OMVs enabled bio-applications

3.1 OMVs application in vaccination

The most common application of OMVs is for vaccination (M. Gerritzen et al., 2018; M. J. Gerritzen, Martens, Wijffels, van der Pol, & Stork, 2017). In 2013, the first MenB vaccine contains OMVs component was approved for human use by the European Medicines Agency (EMA). U.S. Food and Drug Administration (FDA) also approved the use of OMVs to carry MenB vaccine in 2015 (van der Pol et al., 2015). Additionally, other genetically modified *N. meningitidis* OMVs vaccines with enhanced protection effect are tested and under clinical trials (Holst et al., 2009). Compared with bacteria, these vesicles exhibit significant advantages in vaccine development. First, considering subcutaneous or intramuscular injection as the administration methods of vaccines, transport of antigens from tissue to lymphoid organs is critical in determining vaccine efficacy (Bachmann & Jennings, 2010). The small size of OMVs allows them to access to lymph vessels easily, thus achieving high antigen transport efficiency. In addition, since OMVs are produced by bacteria, they contain the same pathogen-associated molecular patterns (PAMPs) as the bacteria and thus can induce the immune response and establish long-term immune memory (Kulp & Kuehn, 2010). Although OMVs have great potential to be used in vaccine technology, there are still some challenges to be addressed, including high immunogenicity induced by endotoxin, low coverage caused by strain variation, etc. (van der Pol et al., 2015). Currently, genetically engineered OMVs with decreased endotoxicity and combination of several antigens have become a powerful platform to

resolve aforementioned challenges and facilitate the development of OMVs based vaccines (Bachmann & Jennings, 2010). Besides native antigens from bacteria, the foreign antigens can be captured in the lumen of nonpathogenic vesicles via vesiculation process or be decorated on the surface through bacterial membrane protein (Lee et al., 2020). In the bioengineered OMVs vaccine, OMVs are used not only as antigen delivery vehicles, but also as adjuvants to enhance the immune response. Putnam and co-works first proved such concept of using genetically engineered OMVs as vaccines (D. J. Chen et al., 2010), in which a poorly immunogenic antigen (GFP) was fused with a pore-forming hemolytic protein (Cytolysin A) in *E. coli* OMVs. The native protein ClyA also served as platform to enhance the immunogenicity of the antigen and simplify the protein purification from cell culture system. Remarkable amount of anti-GFP antibody was observed in response to the OMV-GFP immunizations, especially when comparing ClyA-GFP fusion protein to pure GPF treatment. They demonstrate the first example of poorly immunogenic subunit antigen vaccination using bioengineered OMVs as adjuvants and carrier. One prominent advantage of bioengineered OMVs based vaccine is the reduction of the cost associated with antigen purification in traditional subunit vaccines with enhanced immune-stimulating activities. Following this proof-of-concept study, more reports were published, in which OMVs containing heterologous pathogens induced immune response against the target antigens (Tan, Li, Huang, & Liu, 2018). Besides decorating heterologous antigens on OMVs, native proteins can be removed from OMVs to enhance immunogenicity of antigen through genetic modification. Matthias et al. reported outer membrane protein (OMP)-deficient OMVs vaccines against invasive serogroup B meningococcal (MenB) disease (Matthias et al., 2020). Comparing with vaccine relying on wide type OMVs, the

OMP-deficient OMVs evoked broadly cross-reactive bactericidal antibodies and enhanced the ability to against heterologous strains.

Cancer vaccine is a new therapeutic strategy using patient's own immune system to against tumor by immunotherapy (Wen, Umeano, Kou, Xu, & Farooqi, 2019). Also, some cancer vaccines, like human papillomavirus (HPV) vaccines, contain cancer-causing viruses, which can help individuals create long-term memory of immune response and thus prevent cancer development (Christianson, Wodi, Talaat, & Halsey, 2020; Winter et al., 2020). Recently, OMVs have attracted great interest as a new toolkit in cancer vaccine development(Zhang, Fang, Li, Huang, & Liu, 2019). Foreign antigens, especially tumor antigens, can be easily displayed on OMVs through genetic modification. In addition to single antigen, multiple heterologous proteins are able to be decorated on OMVs for multivalent anti-tumor vaccine. Wang et al. recently developed therapeutic tumor vaccine using OMVs containing human papillomavirus type 16 early protein E7 (HPV16 E7) to treat tumor and HPV infections (Shijie Wang et al., 2017). Comparing with other HPV vaccines, the HPV16 E7 protein displayed OMVs vaccine is capable to treat established HPV infections. Increased interferon-gamma (IFN-R) and IFN expressing CD4+ and CD8+ cells were found in trx-E7 OMVs injected animals. Furthermore, significant suppression of grafted TC-1 tumors in animals demonstrates that OMVs are a potential candidate of cancer vaccine for antigen delivery. Other than carriers and adjuvants in cancer vaccine, OMVs can also be employed as the antigen in cancer vaccines. Zhang et.al have summarized the cancer treatment using bacterial vectors in preclinical or clinical study (Zhang et al., 2019). Since OMVs are released by bacteria and contain similar components, these bacterial extracellular vesicles exhibit great potential for developing OMVs based cancer vaccine.

3.2 OMVs application in cancer therapy and drug delivery

Weakened bacteria was used to treat cancer patients in the early of 1890s (Patyar et al., 2010). However, the safety issue associated with bacterial components has been a great concern that limits the clinical practice of bacteria mediated cancer therapy (Hirayama & Nakao, 2020). In 1997, Bermudes's group demonstrated that *Salmonella* can be used as a novel anticancer vector with tumor targeting function (Pawelek, Low, & Bermudes, 1997). It was discovered that deletion of *msbB* gene in *Salmonella* can reduce its side effects in immunotherapy. Using *msbB*-mutant *Salmonella* can protect the bacteria-treated animal from tumor necrosis factor α (TNFα)-mediated septic shock, which was stimulated by lipid A (Low et al., 1999). Although animal study showed impressive anti-tumor effect, the phase I clinical trial of *Salmonella*-mediated antitumor failed due to its insufficient tumor inhibition (Mi et al., 2019). However, these seminal studies provide solid ground for employing engineered OMVs in cancer immunotherapy for improved efficiency. As discussed in previous section, OMVs have been widely used as antigen or adjuvant in vaccination. Beyond the use of engineered OMVs in vaccination, the OMVs can also be employed as anti-tumor agents to treat cancer through immunotherapy. OMVs share the similar composition as bacteria because they are released from bacteria. In addition, OMVs secreted by genetically engineered attenuated bacteria could have reduced endotoxicity. Consequently, the attenuated OMVs can replace bacteria in cancer immunotherapy.

Recently Kim et al. reported that OMVs derived from attenuated *E. coli* stain can be used as therapeutic agents to treat cancer and exhibit excellent tumor inhibition (O. Y. Kim et al., 2017). The gene encoding for lipid A acyltransferase (msbB) in *E. coli* was

inactive to minimize adverse effects associated with bacterial endotoxin liposaccharide. As shown in Figure 2a, significant fluorescence signal was observed at the tumor site 12 hr after administration of attenuated OMVs. Additionally, increased cytokines IFN-γ and CXCL10 around tumor tissue were observed in tumor-bearing mice, accompanying with significant tumor inhibition (Figure 2b). This study demonstrates that systematically administrated OMVs can accumulate in tumor site and induce interferon-γ dependent antitumor immune response. Furthermore, health conditions of OMVs injected animals were monitored and no apparent adverse effect was found. Taken together, genetic modification of OMVs provides a potential solution to address the safety issue in OMVs-mediated cancer immunotherapy.

Long distance transportation and communication is an important function of OMVs in natural environment (Bomberger et al., 2009). Consequently, OMVs can also be used as the delivery vehicle in drug delivery. OMVs are able to enhance pharmacokinetics and pharmacodynamics of drug loaded in OMVs lumen by protecting the drug from degradation and extending blood circulation time (V. B. Gujrati & Jon, 2014). With the help of genetic engineering, OMVs can be decorated with specific ligands for targeting delivery and thus minimize the side effects. In addition, as aforementioned, attenuated OMVs are an effective immunotherapeutic reagent that can evoke strong anti-tumor response for cancer treatment without notable adverse effects. Therefore, loading chemotherapy or radiotherapy drugs into OMVs could improve the anti-tumor performance and obtain effective cancer treatment. Furthermore, large scale production of engineered OMVs are more cost effective and environmentally friendly, compared to synthetic nanoparticles (Scorza et al., 2012). These unique features and merits of engineered OMVs accelerate the use of OMVs as a new class of drug delivery platform.

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The first engineered OMVs based target drug delivery platform was developed by Jon and co-workers (V. Gujrati et al., 2014). In this study, a transmembrane reporter, human epidermal growth factor receptor 2 (HER2), was selected as the biomarker for targeting due to its overexpression in various types of cancers (Figure 3). The antiHER2 affibody was decorated on OMVs surface via genetic modification. To minimize the adverse effect, *E. coli* with reduced endotoxicity was employed to produce OMVs. Electroporation was further employed to load anti-tumor siRNA into purified antiHER2 affibody modified OMVs. The siRNA was labeled with red fluorescence dye to track the bio-distribution of OMVs. Animal models with xenografted tumor were used to test the tumor targeting/therapy efficacy of the as-developed functional OMVs. Significant accumulation of these vesicles in tumor tissue was observed after 24 h of treatment. Additionally, compared with the control group, which was treated with PBS buffer, the $Affi_{HER2}OMV^{siRNA}$ treated group exhibited significant tumor growth inhibition (Figure 3 b and c). However, the anti-tumor drug was not loaded into OMVs via genetic engineering methods, which may suffer from the low loading amount of drug. This is the first study that OMVs are applied as a potential drug delivery vehicle for targeted therapy in cancer treatment, which stimulates more research activities in OMVs enabled cancer therapy.

Besides the use as drug delivery vehicle, integration of anti-tumor OMVs with other therapeutic agents could be more effective in cancer treatment. In this platform, OMVs not only are employed as the delivery vehicle, but also can elicit anti-tumor response and inhibit tumor growth. Chen *et.al* designed a functional OMV-coated polymeric micelles structure for drug delivery and cancer therapy (Q. Chen et al., 2019). In this study, OMVs from attenuated *Salmonella* were used as the delivery platform due to the anti-cancer efficiency of *Salmonella* and their reduced

endotoxicity. In addition, both tumor targeting property and enhanced permeability and retention (EPR) of OMVs provide additional benefit to targeting drug delivery. After purification, OMVs were functionalized with polyethylene glycol (PEG) to further decrease the immunogenicity and enhance colloidal stability. However, tumor targeting effect of OMVs could be affected by PEG modification. A tumor targeting ligand, Arg-Gly-Asp (RGD), was thus decorated on OMVs surface to improve the targeting ability and blood circulation of OMVs. Tegafur, a prodrug of 5-FU, was selected as the tumor therapy drug for the delivery system. Tegafur loaded micelles were coated with surface functionalized OMVs to form the sophisticated structure for targeted delivery and tumor treatment. Besides the targeting role of OMVs, OMVs-mediated anti-tumor response helps anti-tumor drug loaded in OMVs lumen become more effective as well, since 5-FU becomes effective after immune activation (Nagasaki et al., 2010). The animal experiments show that the combination of chemotherapy and OMVs-mediated immunotherapy can greatly boost the cancer treatment efficacy. Moreover, after systematic administration, these micelles loaded drug also generate robust antitumor immune memory and show "vaccine-like" protective effect. This study illustrates the potential of bioengineered OMVs to act as both anti-tumor booster and delivery vehicle in cancer therapy.

In another practice, Kuerban et al. used OMVs secreted from attenuated *K. pneumonia* as drug delivery vehicles for non-small-cell lung cancer treatment (Kuerban et al., 2020). An effective and widely used chemotherapy drug Doxorubicin (DOX) was selected for tumor therapy (Tacar, Sriamornsak, & Dass, 2013). To evaluate the performance of DOX-OMVs and compare the delivery efficiency of OMVs and liposomes, tumor-bearing animals were treated with different therapeutic agents including OMVs, free DOX, DOX-OMVs, and DOX loaded liposomes (DOX-

LIPO), respectively. Obvious tumor growth inhabitation was observed in DOX-OMVs and DOX-LIPO treated animals, as compared to free DOX treated animals and the control group. In addition, animals injected with DOX-OMVs exhibited faster anti-tumor effect than the group injected with DOX-LIPO. The enhanced anti-tumor performance in DOX-OMVs is attributed to the immune response induced by OMVs. Significant increase of TNF-a and IL-6 in serum was found in the subsequent experiment, which demonstrates that OMVs can elicit the cytokine-mediated anti-tumor effect. This study further demonstrates the applicability of bioengineered attenuated OMVs as drug delivery vehicle in cancer therapy.

As systematical administration of OMVs is able to induce immune response, the subsequent immune activities can be used for drug delivery and enhance accumulation in tumor site. Li et al. designed a chemotaxis-driven delivery of nano-pathogenoids for photothermal therapy (PTT), which relied on phagocytosis and chemotaxis of neutrophils (Li et al., 2020). Macrophages in blood circulation, especially neutrophils, are a type of leukocytes that engulf and digest foreign antigens such as bacteria, virus, nanoparticles, etc. (De Filippo et al., 2013). Beside phagocytosis, macrophages are prone to accumulate at inflamed sites, like the inflammation induced by certain cancer treatment(Nywening et al., 2018). In this study, nanoparticles for PTT were loaded into *E. coli* expressed OMVs to form nanopathogenoids (NPNs). Notably, neutrophils are the target for NPNs rather than tumor. After uptake by phagocytes, the NPNs were delivered to tumor site due to the accumulation of neutrophils around inflammation area. Increased nanoparticle accumulation in tumor site was validated in animal experiment. In addition, xenografted tumors in animals were completely eradicated by the subsequent photothermal therapy. This study provides a new strategy to increase delivery

efficiency using the pathogen-mimicking nano-vesicles and hitchhiking circulation neutrophils.

Recently, needle-free administration method has been explored for OMVs based drug delivery system. The needle-free drug administration can protect the patient from transmission of bloodborne infectious diseases (Dicko et al., 2000; Simonsen, Kane, Lloyd, Zaffran, & Kane, 1999). Huang et al. developed an antibiotic loading OMVs using the antibiotic efflux phenomenon (W. Huang et al., 2020). The results show that OMVs can enhance the antibiotic stability and bacteria killing efficiency. Moreover, the drug loaded OMVs can be administrated orally and exhibited strong bactericidal abilities in intestine. This seminal study provides a new pathway for OMVs based drug delivery system towards more friendly clinical applications.

3.3 OMVs application in bioimaging

Bioimaging techniques play important roles in cancer treatment as they can not only enable to diagnose cancer in the early stage but also evaluate the treatment efficacy (Gu et al., 2019). Like functionalized synthetic nanoparticles, OMVs with imaging reporters and targeting ligands are candidates for bioimaging. Gujrati et al. reported a bioengineered OMVs for contrast enhancement in optoacoustic imaging and photothermal tumor therapy (Figure 4a) (V. Gujrati et al., 2019). Biopolymer-melanin, which is able to generate strong optoacoustic signals upon near-infrared irradiation, is selected as photoabsorber and encapsulated in OMVs (Stritzker et al., 2013). Endotoxic *E. coli* strain with inactivation of *msbB* gene was used to produce the bioengineered OMVs encapsulating biopolymer-melanin (OMV^{Mel}) to avoid systemic side effects, which are associated with bacterial endotoxin lipopolysaccharide. In animal experiment, passive targeting and accumulation of

OMVs in tumor site was observed after systemically administration of OMV^{Mel} due to improved permeability and retention effect. The spatio-temporal biodistribution of OMV^{Mel} was monitored noninvasively in tumor bearing animals with success. Besides optoacoustic imaging application, melanin decorated OMVs can also be employed for cancer therapy because of their photothermal and cytokine-mediated effects. Under pulsed Near-Infrared (NIR) light, OMV^{Mel} can produce localized heat and thus inhibit tumor growth. Melanin loaded bioengineered OMVs provide an effective bio-nanoparticle based theranostic platform that can be simultaneously employed for tumor imaging and therapy.

Inspired by protein expressing on both exterior and interior of natural OMVs, Chen et al. developed a bioengineered multifunctional OMVs with both antibody recognition element and scaffold structure for biosensing (Q. Chen et al., 2017). In this study, the small antibody binding domain, Z-domain, was displayed on OMVs surface using ice nucleation protein (INP) as an anchor. A tri-functional scaffold structure with three orthogonal cohesin domains was inserted between INP and Z-domain to avoid poor binding between antibody and Z-domain due to the steric hindrance from bacteria surface glycoproteins. Moreover, the natural affinity between dockerin-cohesin pair can help achieve further tailored functionalization of OMVs by mixing bioengineered OMVs with dockerin-tagged proteins. As a demonstration, the multifunctional OMVs with Z-domain were further modified by dockerin-tagged GFP for immunofluorescence imaging. GFP decorated OMVs (GFP-OMVs) were added to fixed HeLa cells stained with anti-MUC1 antibody for interaction between Z-domain and antibody. Compared to the control group (no anti-MUC1 antibody treatment), bright green fluorescence was observed from the cells stained with GFP-OMVs

(Figure 4b), indicating that engineered OMVs can be employed for immunofluorescence imaging and cancer cell detection.

Bioluminescence (BL) imaging is another powerful imaging method comparing with fluorescence imaging. Since excitation is not required, BL based imaging can avoid the common issues encountered in fluorescence imaging, including autofluorescence, phototoxicity, and photobleaching (J. Kim & Grailhe, 2016). Our recent study reported the *in vivo* bioluminescence kinetics of genetically engineered OMVs through non-invasive imaging (Figure 4c) (Y. Huang, Beringhs, et al., 2019). The structure of OMVs was the same as the previous one which contains both nano-luciferase (Nluc, bioluminescence reporter) and Z-domain (antibody binding domain). Nluc-furimazine is a novel bioluminescence system with remarkable stability and enhanced emission light (England, Ehlerding, & Cai, 2016; Gibbons, Luker, & Luker, 2018). To explore the application of OMVs in non-invasive bioluminescence imaging, different animal models were used to study the *in vivo* behavior of bioluminescence generated by Nluc loaded OMVs. In the first model, both OMVs and substrate were injected subcutaneously to observe effect from absorption and reaction, while in the second model, after subcutaneous administration of OMVs, the substrate was injected intravenously to mimic the real *in vivo* imaging applications. A mathematical model was developed to theoretically quantified the Nluc-furimazine reaction and absorption observed from the bioluminescence decay during experiments. The model has successfully described the *in vivo* experimental results and established the correlation between bioluminescence signal and enzymatic reaction and OMVs absorption. The bright bioluminescence emission observed during animal experiment indicates the great potential of engineered OMVs in bioimaging applications.

3.4 OMVs application in immunoassay

Our group further developed a bioluminescence-based immunoassay for immunoglobulin G (IgG) detection using OMVs with co-expressed bioluminescence reporter and antibody binding domain (Y. Huang, Liu, Chen, Nieh, & Lei, 2019). As aforementioned, a highly effective bioluminescence enzyme Nluc is expressed in OMVs, while a small antibody binding domain (Z domain) is decorated on OMVs surface. As shown in Figure 5, the genetically engineered multifunctional OMVs can replace the detection antibody in the traditional enzyme-linked immunosorbent assay (ELISA) for IgG detection. Z-domain on OMVs surface can specifically bind IgG with high affinity in the detection system. Also, multiple Nluc luciferase on OMVs can generate strong bioluminescence for signal amplification. Z-domain is an engineered domain from IgG binding protein staphylococcus protein A (SpA) (Tashiro et al., 1997). Unlike other native domains from protein A, Z-domain only shows strong affinity to fragment crystallizable region (Fc region) of IgG (Jansson, Uhlén, & Nygren, 1998). OMVs only bind with the analyte and do not bind with immobilized capture antibody during the detection due to the unique characteristic of Z-domain. The detection results show that this bioluminescence-based immunoassay for IgG detection has good reproducibility. Moreover, this new OMVs-based IgG immunoassay has a similar limit of detection as commercial IgG ELISA kit and shows a better performance than many other IgG assays relied on different detection methods. The OMVs with co-expressed imaging reporter and recognition element can be cost-effectively and eco-friendly mass produced through cell culturing, which is better than chemical conjugation-based strategy. More importantly, the recognition element can be replaced via genetic modification or using bioconjugation pairs such

as cohesin-dockerin pairs. This study provides a new strategy to develop low-cost and effective immunoassay using engineered OMVs.

Engineered multifunctional OMVs were also used in immunosensor for thrombin detection (Q. Chen et al., 2017). A direct assay structure was employed by coating thrombin-containing samples to 96-well plate directly. After binding between detection antibody and thrombin, the OMVs modified with antibody binding domain and reporter were added to the system to bind with anti-thrombin antibody. The OMVs-based direct assay for thrombin detection shows a high sensitivity with a limit of detection as low as 0.5 nM. The performance of the Nluc-Z-domain conjugation was also evaluated in the experiment. Bioluminescence emission from bioengineered OMVs is 10-fold higher than that using Nluc-Z-domain conjugates, which is attributed to the presence of multiple copies of Nluc in individual OMVs (compared to 1 copy in each Nluc-Z-domain conjugate). These studies open a new avenue for OMVs in biosensing applications.

3.5 OMVs application as enzyme carrier

Microbial enzymes have been widely used in food and medicine industries for centuries (Singh, Kumar, Mittal, & Mehta, 2016). These biomacromolecules from bacteria or other live domains have attracted great interest because of their fast processing, low energy requirement, eco-friendly, and low cost (Ovais et al., 2018). Although microbial enzyme is powerful technique with various advantages, the nature characteristic of enzyme, like low stability, or sensitive to environment conditions, still limits the application of microbial enzymes (Woodley, 2006). Therefore, an efficient platform or vehicle for enzyme loading or immobilization without enzyme activity reduction is highly required. OMVs are great candidates for microbial enzyme

packing due to their nanostructure and low-cost production (Turner, Dean, & Walper, 2019). Also, OMVs can be used to improve the enzyme stability and accomplish cascade reactions. These bacterial nanoparticles can be decorated with enzymes via genetic engineering. The vesicle membrane can protect enzymes loaded in vesicles from denaturation. Additionally, using OMVs as the carrier for enzymes can also simplify the purification procedures by decorating other binding domains. Thus, the enzyme loaded OMVs can work as the nano-bioreactors in various fields.

3.5.1 Enzymatic cascade reaction for cellulose hydrolysis

Although OMVs have been successfully functionalized with heterogeneous protein such as GFP and β-lactamase (Bla), via genetically engineering tools, it is still challenging to modify OMVs with multiple functional proteins through direct co-expression of multiple functional proteins. Inspired by the cellulosome on anaerobic bacteria surface, Chen and co-workers developed multi-enzyme functionalized OMVs for cellulose hydrolysis and methanol oxidation (Park et al., 2014). An artificial scaffold structure is displayed on OMVs surface for positional assembly of enzymes. As shown in Figure 6a, a native bacterial membrane protein, ice nucleation protein (INP), was used as the anchor to accomplish the expression of a trivalent scaffold. After isolation, OMVs were incubated with different cell lysates that contain dockerin-fused cellulases. Different enzymes were sequentially assembled on the surface of OMVs through Coh-Doc interactions between cohesion domains and their specific dockerin molecules (AT for an endoglucanase fused with DocT from *C. thermocellum*, EC for an exoglucanase fused with DocC from *C. cellulolyticum*, and BF for a β-glucosidase fused DocF from *R. flavefaciens*). The performance of enzyme assembled OMVs was tested and compared with free enzymes. Compared to free AT

and free AT/EC mixture, 1.7-fold and 4.5-fold enhancement in sugar production were observed in AT decorated OMVs and both AT and EC co-assembled OMVs, respectively. Moreover, it was found that the cellulose hydrolysis rate of tri-enzyme assembled OMVs is 23-fold faster than the non-complexed enzymes, which is attributed to the improved enzyme proximity and cooperative action. As a proof of principle, this work demonstrates that bacterial membrane vesicles have great potential to be used as scaffolding platform to assemble multiple enzymes for high-performance enzymatic cascade reaction.

3.5.2 Environmental decontamination

Recently, Alves et al. reported a strategy that applies OMVs as the enzyme packaging platform for environmental decontamination (Alves et al., 2015). Phosphotriesterase (PTE) from *Brevundimonas diminuta* was encapsulated in the outer membrane through the SpyCatcher/SpyTag (SC/ST) bioconjugation system. PTE is an enzyme that can decompose organophosphates via hydrolysis reaction (Ghanem & Raushel, 2005). To achieve the decoration, the membrane protein OmpA was chosen as the membrane tethering protein because of its high expression level in bacteria membrane. As shown in Figure 6b, a bioconjugation system (SypCatcher/SpyTag) was employed to accomplish the combination of OmpA and PTE. Briefly, one subunit domain (SypCatecher) from fibronectin-binding protein was fused to the PTE, and the other one (SpyTag) was fused to OmpA. The natural affinity between SypCatcher and SpyTag creates a linkage between PTE and OmpA to fabricate enzyme loaded OMVs. Long term storage stability of bare PTE, PTE-SC, and PTE loaded OMVs were studied and tracked under different temperature conditions (Alves, Turner, Medintz, & Walper, 2016). PTE in OMVs remained high activity after 14 days at various

temperatures, compared to bare PTE and PTE-SC. The results indicate that packing enzyme in OMVs is an effective strategy to maintain the enzyme activity and improve enzyme stability for long term storage. Additionally, the effects of freeze-thaw cycles and lyophilization on the stability were also studied since these two procedures play an important role in long term storage. After four freeze-thaw cycles, the PTE activity in OMVs is 9.3-fold and 3.3-fold higher than in PTE-SC and PTE, respectively. It should be noted that after lyophilization, dramatic changes of enzyme activity were found in bare PTE and PTE-SC, while PTE in OMVs remained high activity level (over 60%). Although the mechanism is still unclear, this study demonstrates that OMVs can effectively protect the enzyme from denaturation under temperature variations and thus improve the long-term storage stability. PTE loaded OMVs were further applied in environmental decontamination. (Alves et al., 2018) Enzyme activity of free-PTE and lyophilized PTE loaded OMVs were conducted for environmental remediation. Lyophilized PTE encapsulated in OMVs exhibited better performance than the fresh free-PTE in almost all buffered environmental water samples tested. Although the decontamination activity of PTE loaded OMVs is not as good as free-PTE for non-buffered samples, it is worth noting that PTE loaded OMVs was lyophilized and free-PTE only remain a little of enzyme activity after lyophilization. Also, lyophilized PTE-OMVs exhibited similar performance in either buffered or non-buffered samples. The consistent activity and high stability offer great potential for decontamination of various environmental water sources. Furthermore, the activity of OMV-PTE was also tested on contaminated solid surfaces. These vesicles can effectively eliminate contaminants on painted and glass surface. PTE-encapsulated OMVs for decontamination applications demonstrate that bacterial

vesicles can improve the enzyme stability, thus serving as an excellent enzyme carrier.

In addition to improving enzyme stability, to recover enzyme-loaded OMVs after use is another major challenge limiting its broader applications (Stotzky & Norman, 1961). Ultracentrifugation is the most widely used method for OMVs isolation (Klimentová & Stulík, 2015). However, ultracentrifugation is expensive and labor-intensive due to the strict requirement on equipment and operators (Yu et al., 2018). Su et al. designed a novel bioengineered OMVs, enabling simplified and cost-effective recovery (Su, Tabañag, Wu, & Tsai, 2017). In this study, both cellulose binding module (CBM) and organophosphorus hydrolase (OPH) are decorated on the OMVs surface (Figure 6c). OPH was displayed on OMVs surface via ice nucleation protein, to decompose organophosphorus pesticides (Richins, Kaneva, Mulchandani, & Chen, 1997), while cellulose binding domain was used to achieve effective recovery of OMVs via high affinity between cellulose binding domains and cellulose. Significant enhancement in paraoxon degradation was observed in OPH and CMB co-expressed OMVs, compared to the free OPH. After binding with cellulose particles, the bioengineered OMVs were recovered by gravity precipitation for reusability study. Only 15% reduction in paraoxon degradation rate was observed, indicating the high reusability of recovered OMVs with co-expressed OPH and CMB. Moreover, using CBM decorated OMVs as the carrier for OPH can not only enhance enzyme activity and reusability, but also improve the enzyme activity in a wide pH and temperature range. These features endow engineered OMVs with great promise in environment decontamination.

4. Conclusion and future trends

Bacteria derived nano-vesicles (OMVs) have attracted great interests in biomedical and other fields. Driven by the same components as bacteria, the OMVs have been applied in vaccine development. The first OMVs based vaccine against *N. meningitidis* has been approved for clinical use. With the help of recombinant DNA technology, OMVs containing heterologous proteins or ligands can be constructed and produced using genetically engineered bacteria. These vesicles serve as the carriers for antigens or therapeutic molecules. Besides directly producing functional OMVs through cell culturing, natural protein-protein binding pairs are also used to further functionalize OMVs. Employing the protein-protein binding pairs and multi-domain scaffold structure, genetically engineered OMVs can severe as a multifunctional platform for a range of bio-applications, including targeted drug delivery, bioimaging, and enzyme carriers for cascade reactions. In addition, with the help of protein-protein binding pairs, the functional entity on OMVs can be easily swapped according to the requirement of different applications. Another intriguing possibility is the ability to display proteins onto both the exterior and interior of OMVs. This unique capability can be exploited for the encapsulation of different drug targets and enzymes for cell-specific treatment and imaging. This is an underexploited area that will likely attract more attention in the future.

Although OMVs have been widely used in different research, some challenges still limit the practical applications of OMVs. First, enriched endotoxins and virulence factors were found in OMVs due to the natural functions in bacterial pathology. The presence of endotoxin, like lipopolysaccharide, on OMVs limits their clinic applications due to the adverse effects. To solve this challenge, researchers developed

new class of OMVs with reduced endotoxins via genetic engineering and then used them in cancer therapy (Watkins et al., 2017). Although the safety of OMVs with reduced endotoxins has been proved in animal models, more *in vivo* studies are still required to accelerate the clinical applications of OMVs in human. Furthermore, ultracentrifugation that is commonly used in OMVs harvesting and purification is time-consuming and costly and requires advanced equipment and skilled personnel. Sometimes, ultracentrifugation could result in low yield due to the loss of desired products (Yu et al., 2018). Therefore, new OMVs separation/purification methods, which are capable of continuously separating OMVs from culture medium with a high yield and purity, should be developed for large scale manufacturing of OMVs. Membrane bioreactor with separation function or engineering purification tags on OMVs could be two potential solutions. In addition, other purification methods based on reversible phase separation such as those relied on the use of elastin-like-polypeptides (ELPs) (Q. Chen et al., 2015; H. Kim & Chen, 2016; Liu, Tsai, Madan, & Chen, 2012) can be incorporated to enable faster and simpler recovery. Although we focus on recent progress of OMVs enabled bio-applications in this review, including drug delivery, cancer therapy, immunosensing, bioimaging, enzymatic cascade reaction, and environment decontamination, these nano-vesicles also have great potential for other applications.

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interpreted as necessarily representing the official policies, either expressed or implied, of Industrial Solutions or UConn.

DATA Availability statement

The data that support the findings of this study may be available from the corresponding authors upon reasonable request.

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FIGURES

Figure 1. Overview of OMVs enabled bio-applications in vaccination, drug delivery, bioimaging, biosensing, enzymatic cascade reactions, and environmental decontamination. (Created with BioRender.com)

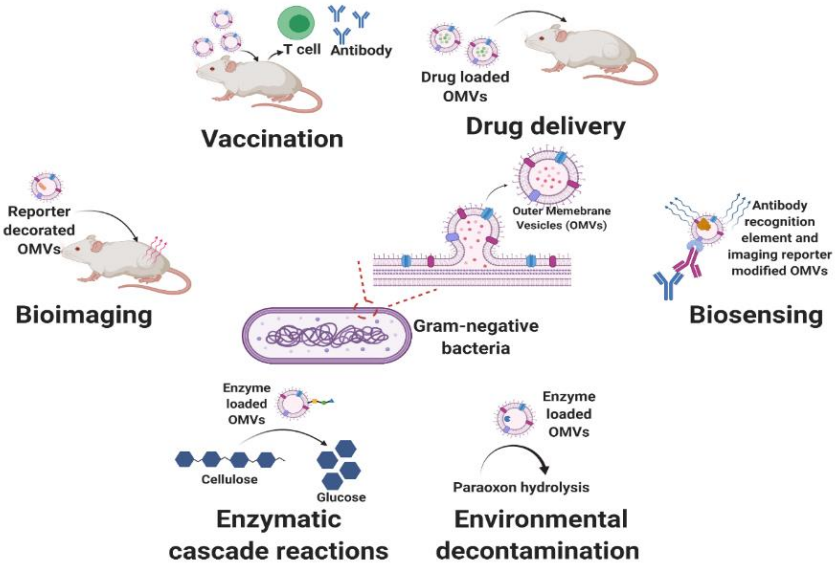

Figure 2. (a) Targeting of *E. coli* Δ*msbB* OMVs (*E. coli* W3110 *msbB* mutant-derived OMVs) to tumor tissues after systemic administration. Cy7 control and Cy7-labeled *E. coli* Δ*msbB* OMVs (Δ*msbB* Cy7OMVs) were systematically injected to BALB/c mice bearing CT26 tumor cells. For control, Δ*msbB* Cy7OMVs were also injected to healthy BALB/c mice with no tumor. Whole body distributions of the injected Cy7OMVs were observed using *in vivo* imaging spectrum at 12 h after injection. (b) Tumor volume of mice bearing CT26 murine colon adenocarcinoma measured after

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E. coli Δ*msbB* OMV treatments with various amounts (total n = 14 mice per group, two independent experiments). *E. coli* Δ*msbB* OMVs were injected intravenously four times from day 6 with 3 days intervals. (O. Y. Kim et al., 2017)

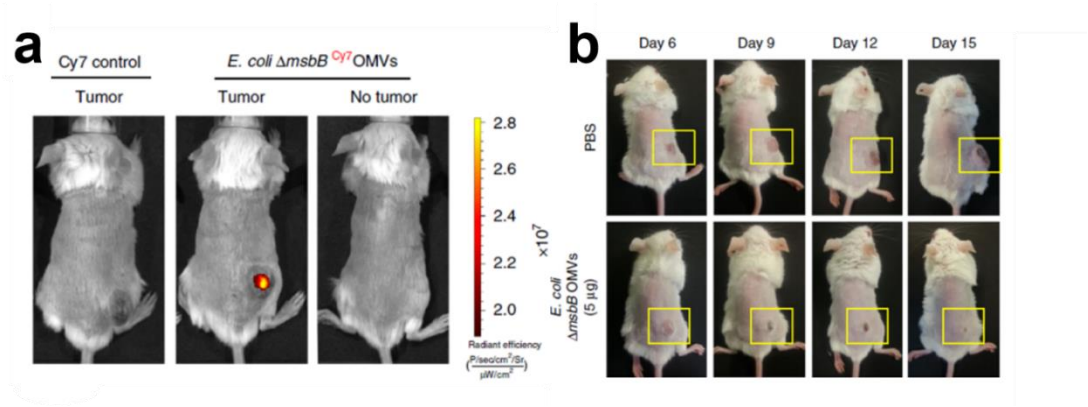

Figure 3. (a) Schematic representation of OMVs expressing HER2-specific affibody ($Affi_{HER2}OMV$), purified after vesiculation from the parent bacteria and further loaded with siRNA-TAMRA ($Affi_{HER2}OMV^{siRNA\text{-}TAMRA}$) using an electroporation method. (b) Tumor growth inhibition (TGI) by the engineered OMVs was monitored in HCC-1954 xenografts. Mice were treated intravenously (~4 μg siRNA per dose) on alternate days till day 22. $Affi_{HER2}OMV^{siRNA}$ exerted potent antitumor effects compared to all controls (**$p < 0.01$); each value represents the mean ± SD (n = 5 mice/group). (c) Dissected tumor tissues obtained from each group are shown, demonstrating differences in size.(V. Gujrati et al., 2014)

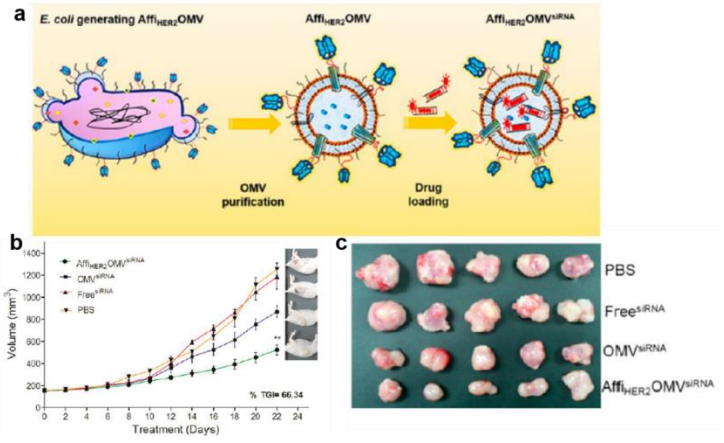

Figure 4. (a) Schematic representation of OMV^{Mel} generation (V. Gujrati et al., 2019). OMV^{Mel} was produced by spontaneous budding of outer cellular membranes with entrapped melanin; (b) Detection of HeLa cells with GFP-decorated OMVs in

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the presence or absence of anti-MUC1 antibodies. Scale bar = 10 μm (Q. Chen et al., 2017). GFP was loaded onto the OMVs using the displayed cohesin domain and antibody was recruited by the displayed Z-domain; (c) *In vivo* bioluminescence kinetics study using genetically engineered multifunctional OMVs (Y. Huang, Beringhs, et al., 2019). Nanoluciferase was genetically anchored to the inner membrane while antibody was recruited as described in (b).

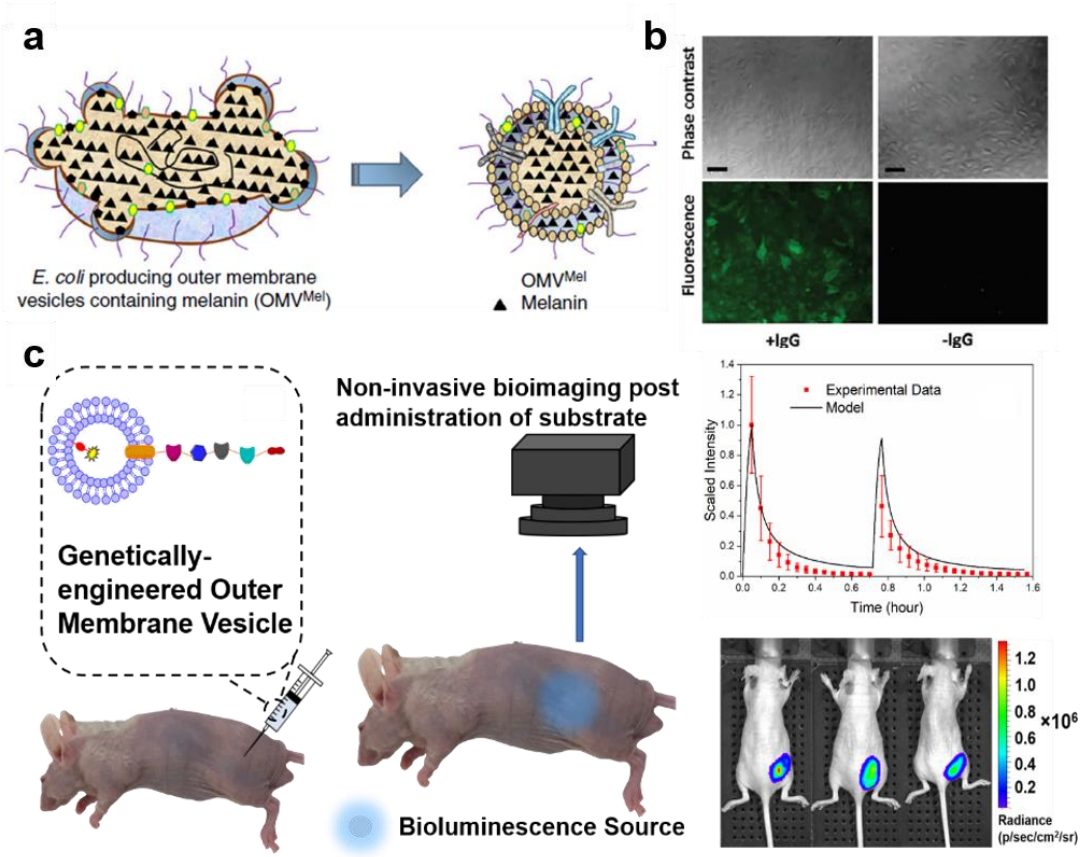

Figure 5. Bioluminescence based immunoassay for IgG detection using genetically engineered multifunctional OMVs. (a) Schematic representation of immunoassay and multifunctional OMVs structure. A bioluminescence reporter (Nluc) and antibody

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recognition element (Z-domian) was decorated on OMVs. (b) IgG detection based on IVIS imaging.(Y. Huang, Liu, et al., 2019)

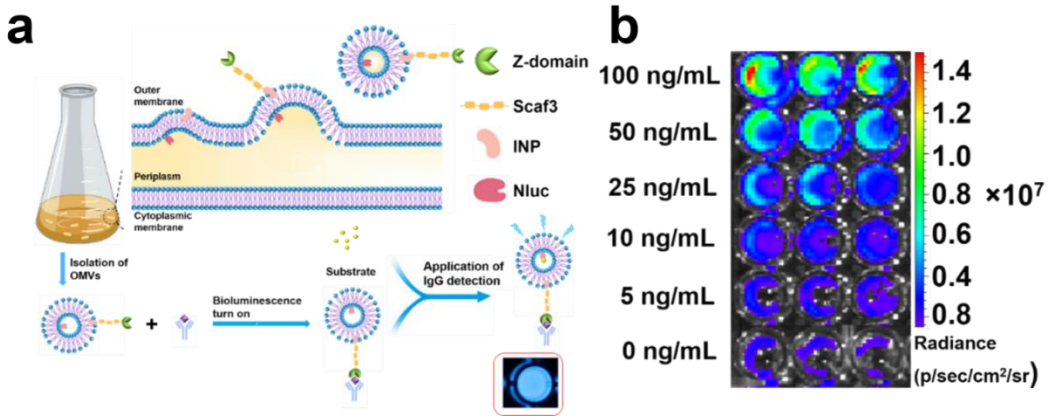

Figure 6. (a) Functional assembly of multiple enzymes on engineered OMVs. A trivalent scaffold containing three orthogonal cohesin domains, DocC (from *C. cellulolyticum*), DocT (from *C. thermocellum*) and DocF (from *R. flavefaciens*), and one cellulose-binding module, is displayed onto OMVs using the ice nucleation protein (INP) anchor. The specific interaction between each cohesin-dockerin pair enables the sequential assembly of three dockerin-tagged cellulases (E1, E2 and E3) onto the OMVs at their corresponding position (C, T or F).(Park et al., 2014); (b) Crystal structures for the proteins utilized in the biorthogonal membrane conjugation of PTE for packaging into outer membrane vesicles: OmpA, PTE, SpyTag, and SpyCatcher (Protein Data Bank: 2GE4, 1PTA, 4MLI, 4MLI, respectively). Three separate OmpA-SpyTag fusion constructs were synthesized: C-terminal (C), N-terminal (N), and an internal (I) OmpA loop fusion. Pictured above is a schematic representation of N-terminal OmpA-SpyTag and PTE-SpyCatcher forming an isopeptide bond at the outer membrane surface of the bacteria. This membrane fusion facilitates incorporation of the PTE within the OMVs that are released from the bacteria surface due to the directional insertion of OmpA into the bacterial

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membrane.(Alves et al., 2015); (c) Schematic illustration of the design, production, and recovery of engineered OMVs. Genetically engineered *E. coli* was employed to produce OPH and CBD decorated OMVs, which could be used for paraoxon degradation and recycled via cellulose precipitation.(Su et al., 2017)

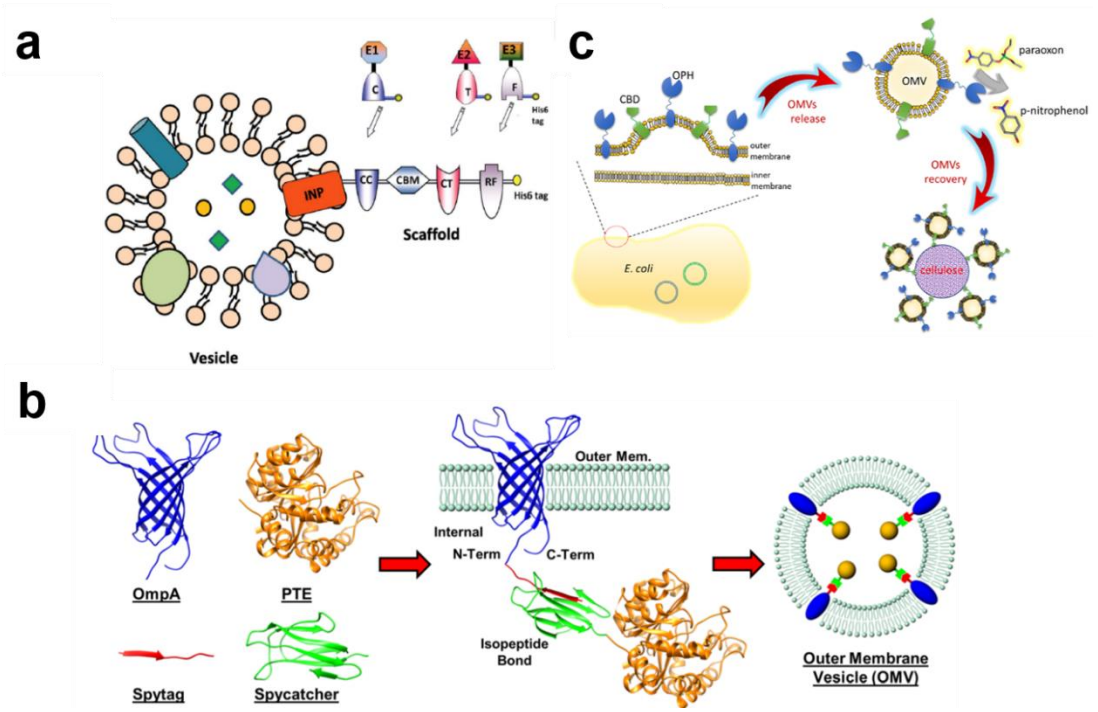

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Table 1. OMVs Functionalization Methods

Methods		**Advantages**	**Disadvantages**
Physical Methods	Incubation, electroporation, sonication, and extrusion	Easy operation, Loading small therapeutic molecules	Require further purification, Low efficiency in some methods
Chemical Methods	EDC/NHS coupling reaction	Stable covalent bond, Easy operation	Purification required after reaction
Genetic Methods	Direct decoration, Indirect bioconjugation using SpyTag/SpyCatcher system or dockerin-cohesin pair	Environment friendly, Large scale production feasible, Pre-determined decoration site, Multifunctionality	Efficiency depending on expression, Suitable for limited materials