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Rational design and engineering of polypeptide/protein vesicles for advanced biological applications

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Synthetic vesicles have gained considerable popularity in recent years for numerous biological and medical applications. Among the various types of synthetic vesicles, the utilization of polypeptides and/or proteins as fundamental constituents has garnered significant interest for vesicle construction owing to the unique bio-functionalities inherent in rationally designed amino acid sequences. Especially the incorporation of functional proteins onto the vesicle surface facilitates a wide range of advanced biological applications that are not easily attainable with traditional building blocks, such as lipids and polymers. The main goal of this review is to provide a comprehensive overview of the latest advancements in polypeptide/protein vesicles. Moreover, this review encompasses the rational design and engineering strategies employed in the creation of polypeptide/protein vesicles, including the synthesis of building blocks, the modulation of their self-assembly, as well as their diverse applications. Furthermore, this work includes an in-depth discussion of the key challenges and opportunities associated with polypeptide/protein vesicles, providing valuable insights for future research. By offering an up-to-date review of this burgeoning field of polypeptide/protein vesicle research, this review will shed light on the potential applications of these biomaterials.

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1. Introduction

All forms of life, from simple bacteria to complex multicellular organisms, are enclosed in membrane structures.[1,2] These membranes act as barriers, separating the interior of the cell from the external environment, and are essential for maintaining cellular processes. Vesicles, which are compartmentalized structures capable of encapsulating substances, represent the fundamental membrane structure found in all living organisms.[3] Vesicles fulfill crucial roles in various biological processes, such as cell signaling,[4] protein synthesis,[5] and cell-to-cell communications.[6] Drawing inspiration from natural vesicles, there has been a significant increase in interest surrounding the design and construction of synthetic vesicles.[7] These synthetic counterparts have been utilized for diverse applications in biology, chemistry, and medicine, including drug delivery vehicles,[8,9] biosensors,[10,11] bioreactors[12] and protein synthesis.[13] Synthetic vesicles, capable of compartmentalization, spatial organization, and mediating inter-molecule communications, can ultimately function as artificial cell platforms.

Various components, including lipids and polymers, are utilized in the construction of synthetic vesicles. In our body and nature, the cell membrane consists of a phospholipid bilayer. As a result, artificial lipid vesicles, called liposomes, have been extensively studied since the 1960s due to their exceptional biocompatibility.[14] However, engineering liposomes presents certain challenges, such as the limited tunability of their complex functionalities, primarily stemming from the short molecular weight of lipid molecules.[15] To overcome these challenges associated with liposomes, long molecular weight amphiphilic block copolymers have been employed as building blocks for constructing polymersomes. Polymersomes offer advantages such as high chemical versatility and stability. Nonetheless, practical biological applications still face hurdles due to the relatively lower, as well as reduced fluidity and permeability, compared to natural membranes.[15]

There is growing interest in utilizing polypeptides as building blocks for synthetic vesicle construction. Polypeptides, which consist of long chains of amino acids linked by peptide bonds, are one of the most well-known and prominent biomacromolecules. Their remarkable biocompatibility,[16] appropriate biodegradability,[17] and tunability[18] have prompted polypeptide vesicles for various biological applications. Furthermore, there is an increased demand for research focused on integrating functionally folded proteins into vesicles.[19,20] Proteins, complex biomacromolecules formed through the folding of one or multiple polypeptide chains, possess specific biological functions that are not easily replicated by other molecules. Consequently, the incorporation of proteins into synthetic vesicles has attracted massive attention for advanced biological applications, including drug delivery vehicles[21] and artificial cell platforms.[22,23]

This review offers valuable insights into the state-of-the-art technology and scientific progress concerning the development of polypeptide and protein vesicles. We present a comprehensive summary of recent research developments on polypeptide/protein vesicles, focusing on their potential for advanced biological applications. We highlight the widely used preparation techniques of building blocks and fabrication methods of polypeptide/protein vesicles and explore their prospective applications in various fields.

2. How can building blocks be synthesized?

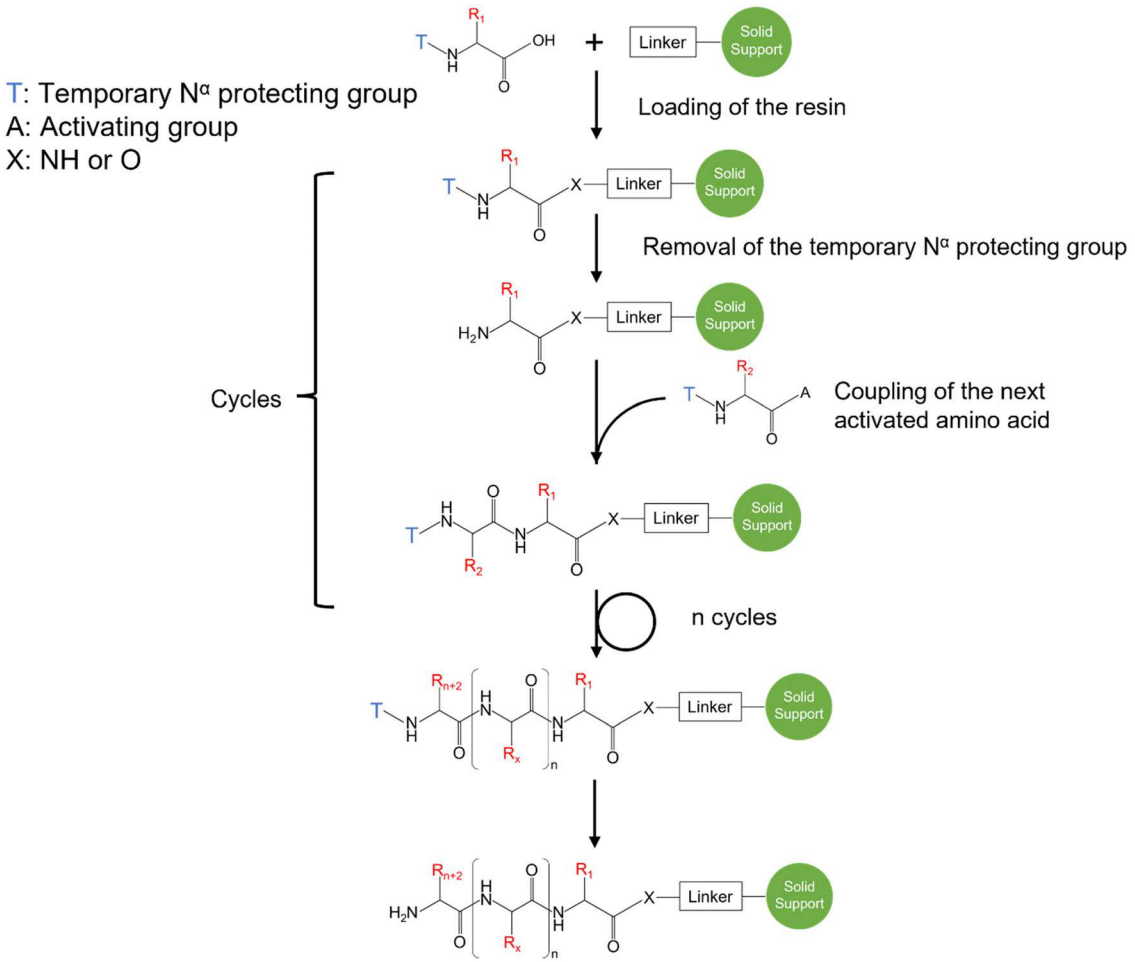
In order to employ polypeptides and proteins as the main constituents of the vesicle structure, it is imperative to design and precisely produce well-defined structures. Researchers have developed various methods for synthesizing and engineering polypeptides and/or proteins within laboratory settings, aiming to utilize them as building blocks for vesicles. This section highlights several commonly used methods for preparing polypeptides and proteins.

2.1. Solid-phase peptide synthesis

Solid-phase peptide synthesis, initially developed by Merrifield in 1963,[24] is a widely used method for polypeptide synthesis.[25] This approach offers several notable advantages including simplified isolation and purification, ease of automation, and high efficiency.[26] Solid-phase peptide synthesis involves a stepwise elongation reaction of polypeptides that are covalently linked to solid beads. The process entails repetitive deprotection and coupling steps, as illustrated in Scheme 1.[25]

The solid beads utilized in solid-phase peptide synthesis typically consist of polymeric resins that are functionalized with linker molecules. These linker molecules serve to facilitate peptide synthesis. The amine group of the linker and peptides is protected by specific groups, which prevents side reactions during peptide bond formation. Additionally, certain amino acid side chains, prone to reactions during coupling steps, require their own protecting groups to prevent undesired reactions. Importantly, these protecting groups must remain intact throughout all coupling reactions and should not be sensitive to deprotecting agents for the amine groups of the linker and peptide. Deprotecting agents are employed to remove the protecting group of the linker, allowing the carbonyl group of the first amino acid to form a peptide bond with the solid support. Subsequently, deprotecting agents are used to remove the protecting group from the amine group of peptides, facilitating coupling. Coupling reagents, also known as carboxylic activators, play a crucial role in increasing coupling rates, preventing racemization or undesired side reactions, and preserving the configurational integrity of polypeptides. Once the peptide assembly is complete, the peptide is cleaved from the resin.

Significant advancements have been achieved in the development of novel linker molecules, enhancing the efficiency of solid-phase peptide synthesis.[27] Efforts are also underway to explore new protecting groups that offer improved selectivity and compatibility with deprotecting agents.[28,29] Furthermore, the optimization of coupling rates and reduction of side



Scheme 1 Principles of solid phase peptide synthesis.[25] This scheme is created based on ref. 25 with permission. Copyright 2006 Springer Nature.

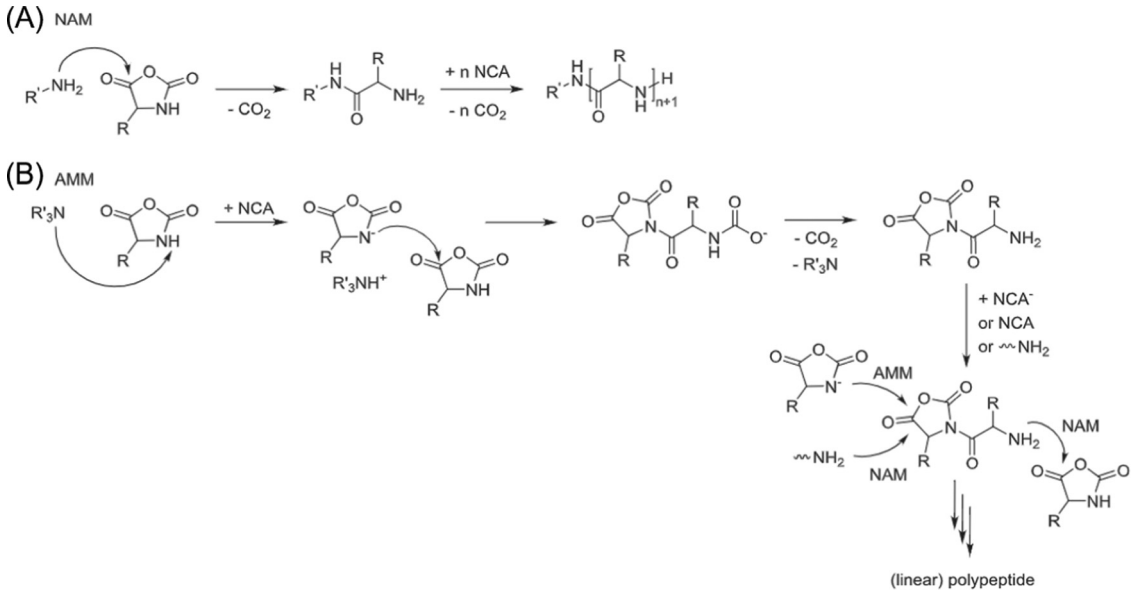
reactions are being pursued through advancements in coupling reagents and reaction conditions.[30] Automation and robotics have played a crucial role in the development of efficient and high-throughput methods, resulting in enhanced overall productivity.[31] These continuous improvements aim to refine the solid-phase peptide synthesis technique, enabling the synthesis of complex polypeptides with heightened precision and efficiency.[32] However, the challenge of sustainable production remains, primarily due to the use of hazardous solvents and explosive coupling agents.[33] Hence, ongoing research efforts are dedicated to addressing these challenges by developing solid-phase peptide synthesis in an aqueous phase,[34,35] with the ultimate goal of mitigating the environmental impact.

2.2. Ring opening polymerization of α-amino acid-*N*-carboxyanhydride (NCA) monomers

Although the solid-phase peptide synthesis method is a powerful and versatile tool for producing polypeptides with high purity and efficiency, it still presents challenges when attempting to directly produce long polypeptides. Incomplete deprotection and coupling steps often lead to unavoidable truncation, thereby creating extreme complications with difficult amino acid sequences.[36] In comparison, the ring-opening polymerization of α-amino acid-*N*-carboxyanhydride (NCA) offers a facile and cost-effective approach for synthesizing high molecular weight synthetic polypeptides.[37] This method has been initiated using various nucleophiles and bases, including amines, alkoxides, and transition metals. The choice of nucleophile depends on the specific NCA, target molecular weight, and desired polymerization rate.

There are two distinct pathways involved in NCA polymerization: the 'normal amine' mechanism (NAM) and the 'activated monomer' mechanism (AMM).[38] In NAM, ring-opening polymerization results in the linear growth of polypeptide (Scheme 2A). This mechanism is more favored when initiators are less basic than the nucleophilic groups in the NCA. On the other hand, AMM occurs through the deprotonation of NCA, leading to the formation of nucleophilic NCA anions (Scheme 2B). These NCA anions act as nucleophiles and can react with other NCA molecules or NCA anions, forming NCA dimers or undergoing condensation reactions. This pathway leads to the synthesis of polypeptides with high molecular weight and dispersity.

Recent advancements in the field of ring-opening polymerization of NCA have focused on developing novel initiators, optimizing reaction conditions, and exploring controlled polymerization techniques.[39,40] These efforts aim to enhance



Scheme 2 Mechanisms of ring opening polymerization of NCA monomers: (A) normal amine mechanism and (B) activated monomer mechanism.[38] Adapted with permission from ref. 38. Copyright 2017 Elsevier Ltd.

control over polymerization, achieve precise molecular weight control, and enable the synthesis of well-defined polypeptide architectures like block copolypeptides and star-shaped polypeptides.[41,42] However, achieving precise control over polymerization kinetics, side reaction management, and stereochemistry control remains complex.[43] Researchers have explored diverse monomers,[44,45] catalysts,[46] reaction conditions[47] to address these challenges. These advancements have significant implications for applications in drug delivery,[48] tissue engineering,[49] and biomaterials,[50] where the tailored structures and properties of synthetic polypeptides are crucial.

2.3. Protein engineering with recombinant technology

Both solid-phase peptide synthesis and ring-opening polymerization of NCA are widely used polymerization processes for polypeptide synthesis. However, these methods have certain limitations, such as wide dispersity, side reactions, and restricted molecular weight because they are fundamentally based on polymerization principles.[28,51] In contrast, recombinant DNA technology involves the use of restriction enzymes, ligases, and various laboratory techniques to engineer genes encoding proteins.[52] The engineered genes are inserted into a plasmid, which is a circular DNA molecule capable of independent replication within a cell. Subsequently, this engineered plasmid can be transformed into a host cell, such as *E. coli*. The host cells containing the engineered plasmid can then produce the desired polypeptide with a specific sequence and narrow size distribution (Fig. 1).[53]

The incorporation of amino acids into polypeptides using recombinant technology has traditionally been limited to the twenty naturally occurring amino acids. However, the development of the amber suppression method has allowed for the incorporation of non-natural amino acids at specific positions within recombinant proteins.[54] This advancement enables more sophisticated engineering of recombinant proteins with additional functionalities, such as photo-induced cross-linking,[55] site-specific proteolysis,[56] or the introduction of fluorescent groups,[57] without hampering the overall protein functions.

By utilizing the versatility and precision offered by recombinant DNA technologies, it becomes possible to engineer proteins with tailored properties and functionalities, specifically suited for vesicle construction. This includes the incorporation of specific peptide sequences or domains that promote self-assembly, enhance membrane stability, or facilitate specific interactions with other biomolecules. While recombinant DNA technologies are powerful tools for synthesizing polypeptides, challenges such as protein misfolding, aggregation, and degradation need to be addressed.[58] Researchers have focused on solving these issues through various approaches, including altering expression strains,[59] controlling cell strain growth,[60] and optimizing expression conditions.[61] These efforts aim to enable the precise and high-yield synthesis of diverse proteins using recombinant technologies for applications in biology and pharmaceuticals.

The integration of recombinant protein technology and vesicle engineering will enable the development of advanced synthetic vesicles with enhanced biological properties and tailored functionalities, applicable in diverse fields including drug delivery, diagnostics, and nanotechnology. We will discuss the recent developments in utilizing recombinant proteins for vesicle construction in the following section.

3. How are polypeptide/protein vesicles assembled?

Synthetic vesicles are typically formed through the self-assembly of amphiphiles, which are composed of the hydrophilic and

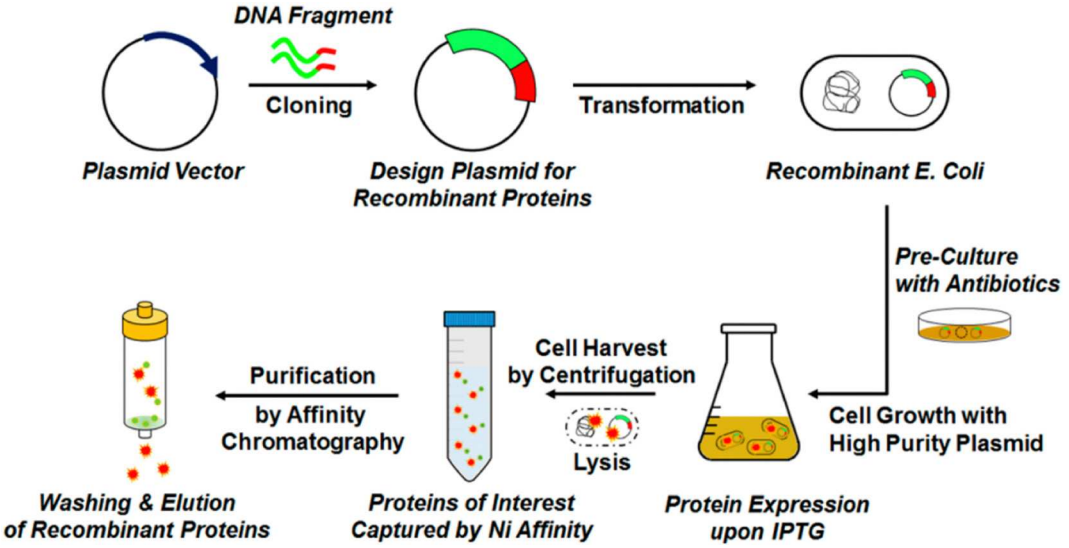


Fig. 1 Schematic image of the recombinant protein expression.[53] Adapted with permission from ref. 53. Copyright 2016 American Chemical Society.

hydrophobic components in appropriate ratios, in a suitable solution. Various techniques have been developed and explored for preparing vesicles using traditional amphiphilic building blocks such as lipids and polymers.[62,63] In this section, we provide an overview of the techniques used to fabricate polypeptide- and protein-based vesicles, along with relevant examples.

3.1. Self-assembly in aqueous solution

The self-assembly of amphiphiles in an aqueous solution is a straightforward method for creating synthetic vesicles.[64] The formation of self-assembled structures in aqueous solutions depends on the packing parameter (P) of the amphiphilic building block molecules, $P = \nu/a_0 l_c$,[65] where ν is the hydrophobic volume, a_0 is the hydrophilic area, and l_c is the hydrophobic chain length. To achieve vesicle formation through self-assembly, the packing parameter of amphiphiles needs to fall within a specific range ($1/2 \leq P \leq 1$). A study by the Mastrobattista group demonstrated the self-assembly of an amphiphilic oligopeptide composed of alanine, valine, and leucine residues in the hydrophobic domain, and glutamic acid residues in the hydrophilic domains, resulting in vesicle formation.[66]

Other polypeptides, such as longer polypeptides or full-size globular proteins, can also self-assemble into polypeptide-based vesicles. Lecommandoux and coworkers reported the reversible self-assembly of zwitterionic diblock copolypeptides into peptide vesicles (Fig. 2A).[67] Glutamic acid and lysine residues were utilized as building blocks for the vesicles, which were prepared through ring-opening polymerization. Polypeptide vesicles were formed upon the dissolution of the diblock copolypeptide in an acidic or basic aqueous solution. The neutralization of glutamic acid and lysine residues in the acidic or basic conditions led to the formation of secondary conformations, such as α-helical structure, resulting in reduced water-solubility. The decrease in water-solubility leads to an increase

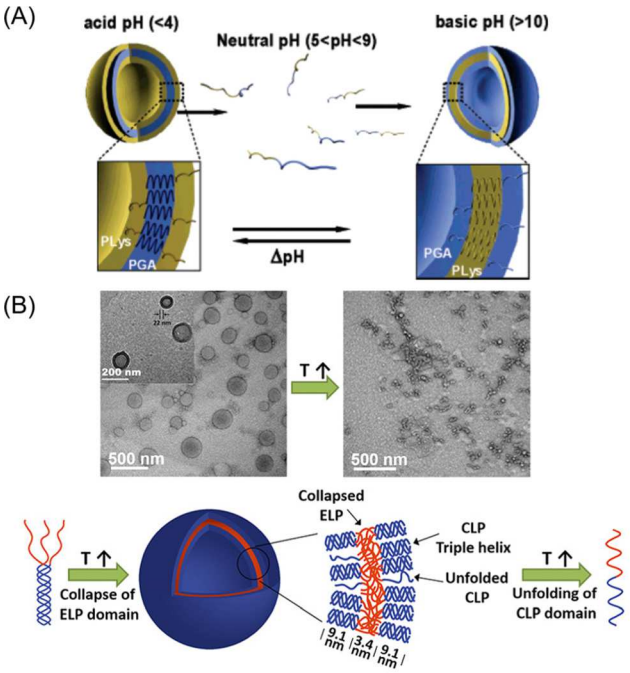


Fig. 2 Self-assembly of amphiphilic polypeptides into vesicles. (A) Schematic image of the self-assembly of diblock copolymer poly(L-glutamic acid)-*b*-poly(L-lysine)[67] Adapted with permission from ref. 67. Copyright 2005 American Chemical Society. (B) TEM images and schematic illustration of thermally induced vesicle formation from the ELP-CLP conjugates.[71] Adapted with permission from ref. 71. Copyright 2015 American Chemical Society.

in hydrophobicity, which drives the self-assembly process. The neutralized polypeptide block forms the vesicle membrane, while the other hydrophilic block forms the shell. In addition, Yoon *et al.* demonstrated the self-assembly of proline-rich polypeptide into polypeptide vesicles.[68] The author emphasized that the rigid nature of polyproline helix allows for hydrophobic

interactions between polyproline rods, leading to vesicle formation in an aqueous solution. They also explored the potential of the self-assembled vesicles for drug delivery applications by conjugating a highly charged peptide (*i.e.*, Tat cell-penetrating peptide) with a polyproline domain as a hydrophilic coil.

The self-assembly of elastin-like polypeptides (ELP) containing building blocks has been extensively studied due to their thermo-responsive properties.[69,70] Luo and Kiick reported the self-assembly of ELP and collagen-like polypeptide (CLP) conjugates (ELP-CLP) into vesicles structure based on their thermo-responsive behavior (Fig. 2B).[71] The ELP exhibits a lower critical solution temperature (LCST) behavior, becoming hydrophobic above the transition temperature.[72] CLP forms stable triple helix structures below its melting temperature. The ELP-CLP conjugated trimers are formed due to the triple helix structure of CLP, and these conjugates self-assemble into vesicles above the ELP transition temperature.

Additionally, researchers have developed folded functional proteins that can also self-assemble into vesicle structures, forming protein vesicles, through recombinant protein technology. Park and Champion reported the thermally triggered self-assembly of recombinant fusion proteins into vesicles that display folded proteins on their surface.[19] They employed ELP as the thermo-responsive domain serving as hydrophobic blocks and fluorescence model folded proteins (*e.g.*, mCherry and enhanced green fluorescence protein (eGFP)) as hydrophilic blocks. These ELP and folded proteins are linked using the leucine zipper domain, which exhibits high binding affinity. The packing parameter of these amphiphilic fusion proteins can be tuned by controlling temperature and salt concentration in the self-assembly solutions.[73,74]

Utilizing self-assembly for the creation of polypeptide- and protein-based synthetic vesicles offers several advantages because self-assembly allows for the spontaneous formation of vesicles without the need for complex synthesis techniques. Polypeptides and proteins with precisely controlled compositions enable the rational design of vesicles with tailored properties for specific applications. The incorporation of functionally folded, bioactive proteins within the vesicle structure through biocompatible self-assembly can also enhance their versatility and potential for diverse biomedical and nanotechnological applications.

3.2. Co-solvent method

Some polypeptide building blocks are not readily soluble in aqueous solutions due to their larger hydrophobic blocks. In such cases, the co-solvent method can be employed for vesicle assembly.[75] This technique involves the use of organic solvents that can dissolve both hydrophobic and hydrophilic blocks, along with aqueous solvents that can dissolve only hydrophilic blocks. By removing the organic solvents from the mixed solution by dialysis or evaporation, vesicles can be formed.

Deming *et al.* demonstrated the self-assembly of block copolypeptides composed of oxidized methionine, leucine, and phenylalanine into vesicles using the solvent replacement technique (Fig. 3A).[76] The authors dispersed lyophilized block copolypeptide in tetrahydrofuran (THF) and added a small amount of water to the solution. The mixed solution was then dialyzed against water, which resulted in the removal of THF and the formation of polypeptide vesicles. Similarly, self-assembly of recombinant polypeptides into vesicles can be induced *via* solvent replacement techniques. For example, Hammer *et al.* reported the expression of oleosin mutants through recombinant technologies and their assembly into vesicle structure (Fig. 3B).[77] Oleosins consist of hydrophilic segments at both ends and a hydrophobic block in the middle. The authors introduced organic solvents containing oleosins into water, which resulted in the formation of oil-in-water emulsions. As the organic phase evaporates, the local concentration of oleosins increases at the interface, leading to spontaneous vesicle formation. In this example, the ratio of hydrophobic and hydrophilic blocks dictates the vesicle self-assembly process.

4. What applications can polypeptide/protein vesicles be used?

The development of techniques for polypeptide/protein synthesis and assembly has enabled a wide range of biological

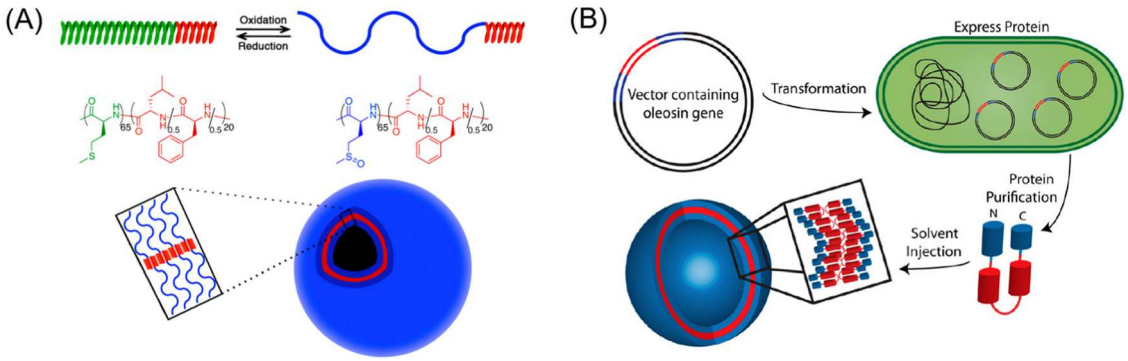


Fig. 3 Polypeptide-based vesicles formed by the co-solvent method. (A) Schematic illustration of the structure of block copolypeptide and vesicles.[76] Adapted with permission from ref. 76. Copyright 2013 American Chemical Society. (B) Schematic illustration of the expression process of recombinant oleosin protein mutants and formation of vesicles.[77] Adapted with permission from ref. 77. Copyright 2012 The National Academy of Sciences.

applications for polypeptide/protein vesicles. Significant efforts have been dedicated to achieving compartmentalization, spatial organization, and intermolecular communications within synthetic polypeptide/protein vesicles. These advancements aim to enable sophisticated biological applications that closely mimic the functionalities of living cells. In this section, we provide a summary of the diverse applications of polypeptide/protein vesicles, including drug delivery and artificial cell platforms, along with relevant examples.

4.1. Drug delivery applications

Drug delivery systems have been actively investigated to provide precise control over drug localization and dosage within the body. Synthetic vesicles have garnered significant attention as promising drug delivery vehicles due to their capacity to encapsulate water-soluble drugs within the lumen and accommodate water-insoluble drugs within the membrane.[9] Various candidates for drug delivery vehicles, including liposomes[78] and PEG–protein conjugates,[79] have been extensively studied and commercialized as drug delivery vehicles. Recently, there has been significant interest in exploring polypeptide/protein vesicles as drug carriers due to their potential tunability, which is derived from polymeric molecular structure and biocompatibility.[21] In particular, researchers have attempted to utilize stimuli-responsive polypeptides, such as pH-responsive or light-responsive polypeptides, to construct stimuli-responsive polypeptide vesicles that enable the controlled release of encapsulated drugs at specific target sites. In the following subsection, we introduce several examples of external stimuli-responsive polypeptide vesicles, along with the mechanisms behind drug release and how to design materials.

4.1.1. pH-responsive polypeptide/protein vesicles. The pH-responsive drug release has been widely studied due to the weak acidic conditions near cancer cells. Thus, pH-responsive design is considered an effective strategy for delivering drugs to the target tumor sites. Among amino acids, there are several pH-responsive ones, such as glutamic acid, lysine, or histidine, which are often incorporated into polypeptide vesicles to confer pH-responsiveness.[67,80–84] The pH directly influences the stability of these polypeptide vesicles since protonation or deprotonation dictates the solubility of polypeptide. For example, Johnson *et al.* synthesized a triblock copolypeptide, poly(ethylene glycol) methyl ether acrylate-*b*-poly(L-lysine)-*b*-poly(L-histidine) [p(PEGA)-*b*-p(Lys)-*b*-p(His)], and formed pH-responsive vesicles (Fig. 4A).[81] At neutral pH, histidine blocks show hydrophobicity and form a bilayer vesicle membrane. However, when the pH value is reduced to 5.5, typical of the extracellular tumor environment, histidine residues undergo protonation, reducing their hydrophobicity and lowering the stability of the vesicles. The authors demonstrated that the release rate of encapsulated doxorubicin in acidic conditions is faster than in neutral conditions, indicating the potential of these polypeptide vesicles for pH-responsive drug delivery. Additionally, Bellomo *et al.* synthesized a poly(L-lysine)-*b*-poly(L-leucine) block copolypeptide and formed vesicles under basic conditions.[82] Under acidic conditions, protonation of lysine blocks increases the hydrophilicity of lysine blocks and disrupts the helix structures of leucine-rich blocks due to the electrostatic repulsion, leading to the disassembly of vesicles and release of encapsulated Fura-2 dye. Champion group reported pH-responsive protein vesicles by incorporation of histidine into the elastin-like polypeptide sequences (Fig. 4B).[84] Histidine-incorporated protein vesicles disassemble in acidic conditions due to the protonation of histidine residues. Protonated histidine increases the hydrophilicity of elastin-like polypeptides, which are utilized as hydrophobic blocks, and results in the disassembly of vesicles. The application of protein vesicles for drug delivery was confirmed by the same group in the previous study.[21]

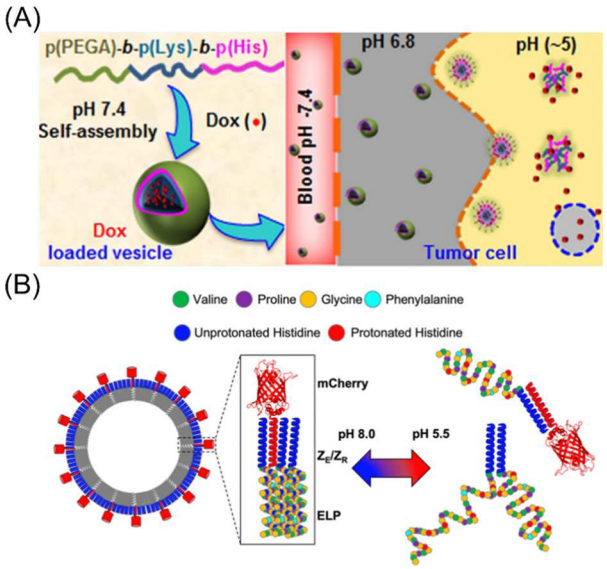


Fig. 4 Polypeptide-based vesicles for drug delivery applications. (A) Schematic image of targeted delivery of an anticancer drug (doxorubicin) under a weakly acidic environment, achieved through pH-responsive polypeptide vesicles.[81] Adapted with permission from ref. 81. Copyright 2015 American Chemical Society. (B) Schematic illustration of pH-induced disassembly of protein vesicles, designed by protonation of histidine under acidic conditions.[84] Adapted with permission from ref. 84. Copyright 2022 American Chemical Society.

4.1.2. Light-responsive polypeptide/protein vesicles. Light has garnered attention as a stimulus because of its remote applicability and high spatiotemporal precision. To confer light-responsiveness to polypeptides, light-sensitive molecules, such as spiropyran,[85] coumarin,[86] and *O*-nitrobenzyl ester,[87] are incorporated into the polypeptide side chain. Among these light-sensitive chemical structures, photo-cleavage molecules have shown potential for application in drug delivery systems owing to their controlled release capabilities. Liu *et al.* synthesized poly(S-(*O*-nitrobenzyl)-L-cysteine)-*b*-poly(ethylene glycol) (PNBC-*b*-PEG) by adding light-sensitive *O*-nitrobenzyl ester to the cysteine side chains, and fabricated light-sensitive polypeptide vesicles (Fig. 5A).[88] UV irradiation at 365 nm caused degradation of the nitrobenzyl group, leading to a decrease in vesicle size and eventually the formation of polypeptide aggregations. Furthermore, the authors demonstrated that the release of the anticancer drug doxorubicin can be controlled

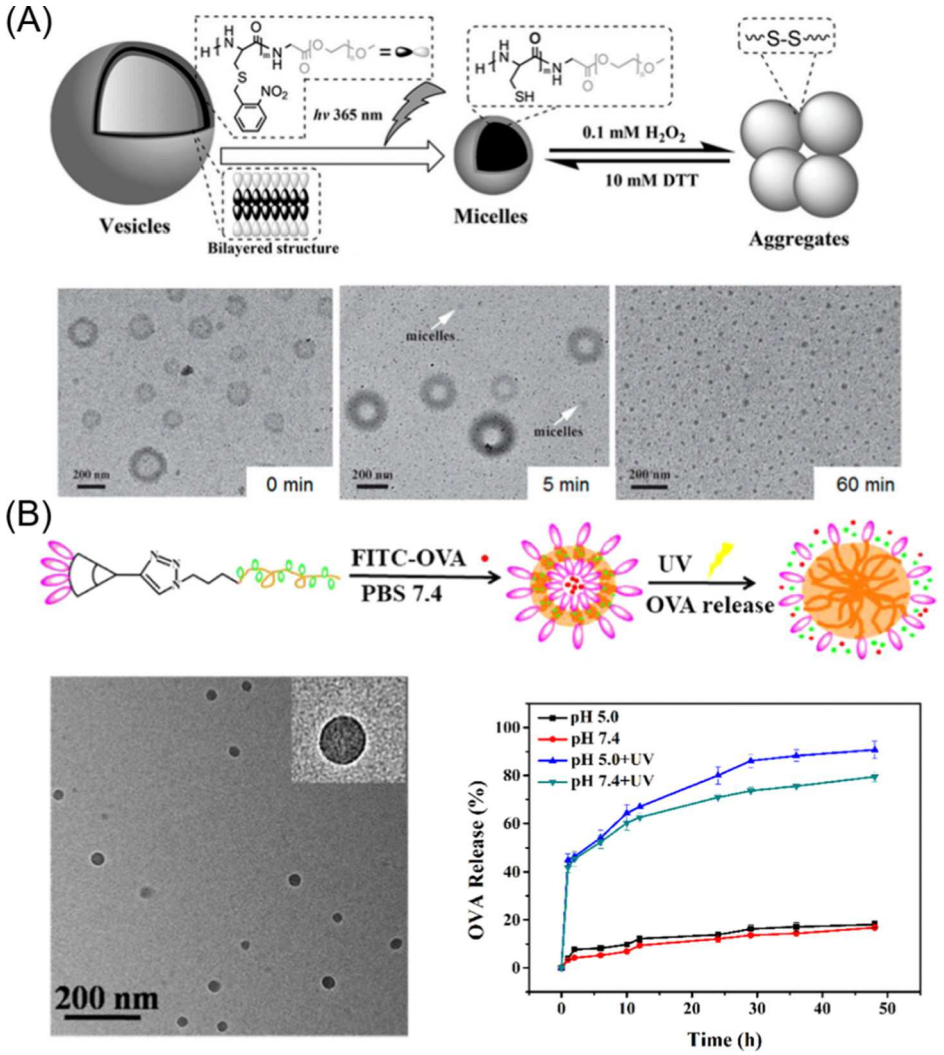


Fig. 5 (A) The disassembly process of UV-light responsive polypeptide-based vesicles triggered by UV irradiation (top) and TEM images of the vesicles (bottom left), micelles (bottom middle), and aggregates (bottom right).[88] Adapted with permission from ref. 88. Copyright 2014 Wiley-VCH Verlag Gmbh & Co. KGaA, Weinheim. (B) Fabrication steps of the light-sensitive OVA-loaded polypeptide vesicles (top), TEM image of OVA-loaded vesicles (bottom left), and OVA release profiles at pH 5.0 and 7.4 with/without UV irradiation (bottom right).[89] Adapted with permission from ref. 89. Copyright 2020 American Chemical Society.

through UV irradiation. Similarly, Song *et al.* synthesized photo-responsive polypeptide-glycosylated dendron amphiphile (PGDAs) and fabricated ovalbumin (OVA) loaded polypeptide vesicles (Fig. 5B).[89] Photo-sensitive *O*-nitrobenzyl groups were conjugated to lysine residues. UV irradiation at 365 nm induced disassembly of the polypeptide vesicles under acidic conditions, attributed to the increased hydrophilicity of protonated lysine side chains. The authors also demonstrated the release of OVA through UV irradiation in macrophages.

4.2. Artificial cell applications

Artificial cells are engineered particles designed to replicate one or more of the biological functions of living cells. Recently, various biomaterial platforms, including emulsions,[90] colloidosomes,[91] coacervates,[92] and vesicles,[53] have been extensively investigated and developed as artificial cell models. Among these platforms, vesicles have received significant attention due to their resemblance to the structure of living cells. Polypeptides/proteins play an indispensable role in mimicking the functions of living cells since they are essential macromolecules used by all organisms to sustain life. Consequently, polypeptide/protein vesicles offer distinct advantages for artificial cell applications, such as inter-molecular communications or signal transduction, which can be mediated by specific peptides or proteins.[93] Despite the numerous benefits of polypeptide/protein vesicles, research on developing artificial cells based on polypeptide/protein vesicles is still in the early stages and requires further active investigation. Herein, we introduce a few recent research efforts to develop artificial cell models based on polypeptide and protein vesicles.

4.2.1. *In vitro* transcription/translation (IVTT). One of the most important functions of cells is the synthesis of proteins,

which occurs through transcription and translation processes. *In vitro* transcription/translation (IVTT) refers to the production of proteins using biological materials without cell systems.[13] IVTT agents typically include cell extract, DNA templates, cofactors, substrates, and energy sources.[94] Encapsulating IVTT agents within polypeptide/protein vesicles have been actively studied for artificial cell research.[95] For instance, Vogele *et al.* demonstrated that IVTT of membrane peptides inside polypeptide vesicles can increase the vesicle size through the direct incorporation of membrane peptide into the vesicle's membrane (Fig. 6A).[96] The authors constructed ELP vesicles and encapsulated IVTT agents to produce the membrane peptide building blocks. The production of membrane-constituting peptides within the vesicles led to the incorporation of membrane building blocks into the membrane, resulting in vesicle growth. Sharma *et al.* also explored similar strategies to induce artificial cell growth.[97]

In addition to the direct incorporation of membrane building blocks from the vesicle lumen, other strategies have been developed to induce the growth of vesicles. For example, Frank *et al.* fabricated giant polypeptide vesicles within the size range of 1 μm to 100 μm using amphiphilic ELP through a solvent evaporation method (Fig. 6B).[70] These ELP vesicles encapsulated IVTT agents, specifically the fluorogenic RNA aptamer dBroccoli, within the vesicle lumen. The authors prepared two types of ELP vesicles, one without RNA polymerase or DNA templates, to allow the IVTT reaction to occur inside the vesicles upon fusion. The synthesis of the fluorogenic RNA aptamer induced an osmotic imbalance between the inside and outside of the vesicles, which resulted in fusion events and subsequent vesicle growth.

4.2.2. Bioreactors with functional proteins on the membrane. Proteins exhibit specific functions that arise from their folded three-dimensional structures, which are not easily replicated by other molecules. Owing to these specific functions and their biocompatibility, the incorporation of proteins into synthetic vesicle membranes has garnered attention for artificial cell applications. The Mann group reported the formation of micro-compartments using amphiphilic protein–polymer conjugates, which they referred to as 'proteinosomes'.[20] These proteinosomes can encapsulate water-soluble molecules and display enzyme proteins on their surface, making them

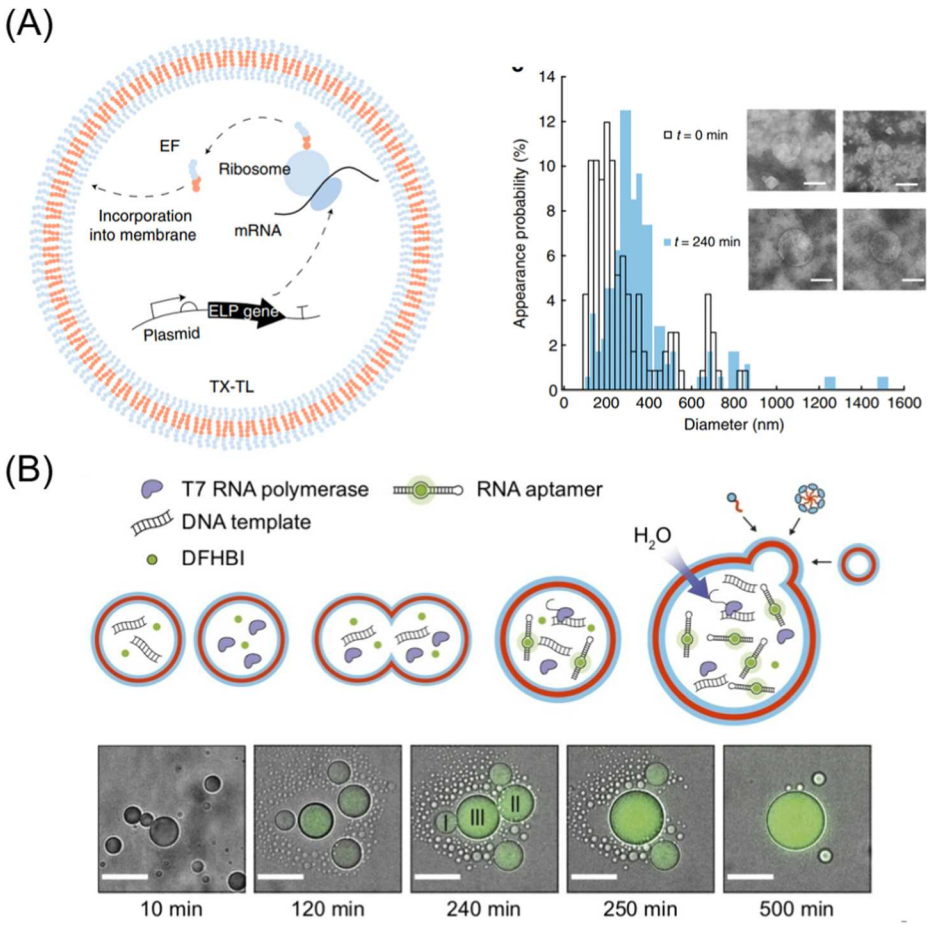


Fig. 6 (A) Schematic image of IVTT agents encapsulated ELP-based vesicles and the incorporation of membrane proteins into the membrane (left). Size distribution of ELP-based vesicles at the beginning of IVTT and after 240 min measured by TEM. (right)[96] Adapted with permission from ref. 96. Copyright 2018 Kilian Vogele *et al.* (B) Schematic image of ELP vesicles fusion and expression of dBroccoli RNA aptamers (top) and microscopy image of growing ELP vesicles (bottom)[70] Adapted with permission from ref. 70. Copyright 2020 Tobias Pirzer *et al.*

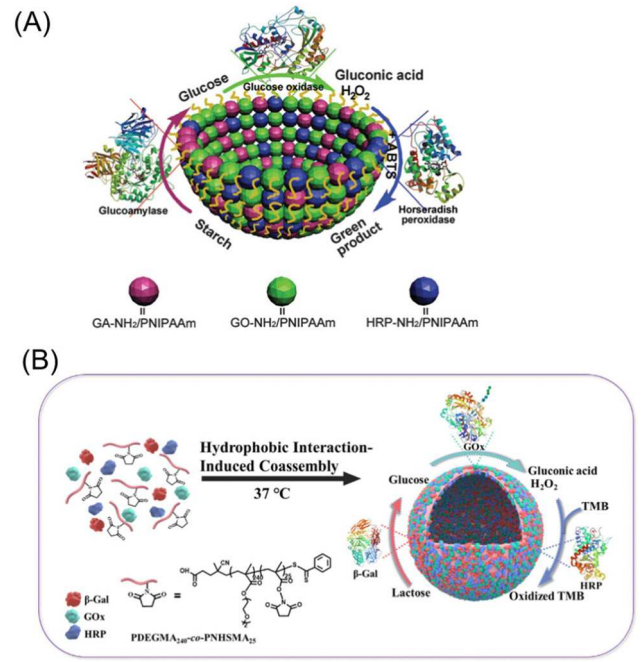


Fig. 7 (A) Schematic image of a proteinosome membrane-mediated enzyme cascade reaction.[98] Adapted with permission from ref. 98. Copyright 2014 The Royal Society of Chemistry. (B) Schematic image of preparation of multienzyme proteinosomes and enzyme cascade reaction.[99] Adapted with permission from ref. 99. Copyright 2021 Elsevier.

potential platforms for artificial cells. Continuing their work, protein-displayed vesicles were developed by replacing the proteins with several enzymes, creating compartments with enzyme-mediated cascade systems (Fig. 7A).[98] Enzyme cascade reactions are essential features that occur within living cell systems. The authors demonstrated that an equimolar mixture of three different enzyme–polymer conjugates, glucoamylase (GA), glucose oxidase (GO), and horseradish peroxidase (HRP), can form multi-enzyme-displayed proteinosomes and undergo an enzyme cascade reaction resulting in the production of a final green, fluorescence product. Li *et al.* also fabricated proteinosomes with three different enzyme sets (*e.g.*, β-gal, GOx, and HRP) through hydrophobic interactions between the polymer block (poly(di(ethylene glycol) methyl ether methacrylate) (PDEGMA) and the enzymes (Fig. 7B).[99] These enzyme-displayed vesicles have been considered potential platforms for artificial organelles to build artificial cells.

5. Conclusion & future perspective

Throughout this review, we have primarily summarized the research outcomes regarding polypeptide-based vesicles, focusing on the preparation of building blocks, fabrication methods, and applications. We highlighted two polymerization-based methods for polypeptide synthesis, solid phase peptide synthesis and ring opening polymerization of NCA, employed for polypeptide synthesis. With the emergence of recombinant technologies, polypeptide synthesis techniques have been expanded, allowing researchers to directly design the synthesis of proteins from cells. We have provided an overview of the characteristics of each technique and introduced examples of how these methods were utilized in polypeptide production for vesicle fabrication. We have also discussed common methods for vesicle assembly, including self-assembly and co-solvent methods, highlighting their principles and practical examples. Due to their biocompatibility and tunability, polypeptide/protein vesicles have been actively investigated for various applications, such as drug delivery and artificial cell platforms. (Fig. 8) Polypeptides incorporating stimuli-responsive modules hold promise as drug delivery vehicles, enabling the controlled release of drugs at target sites. Given that proteins possess specific biological functions that are challenging to replicate

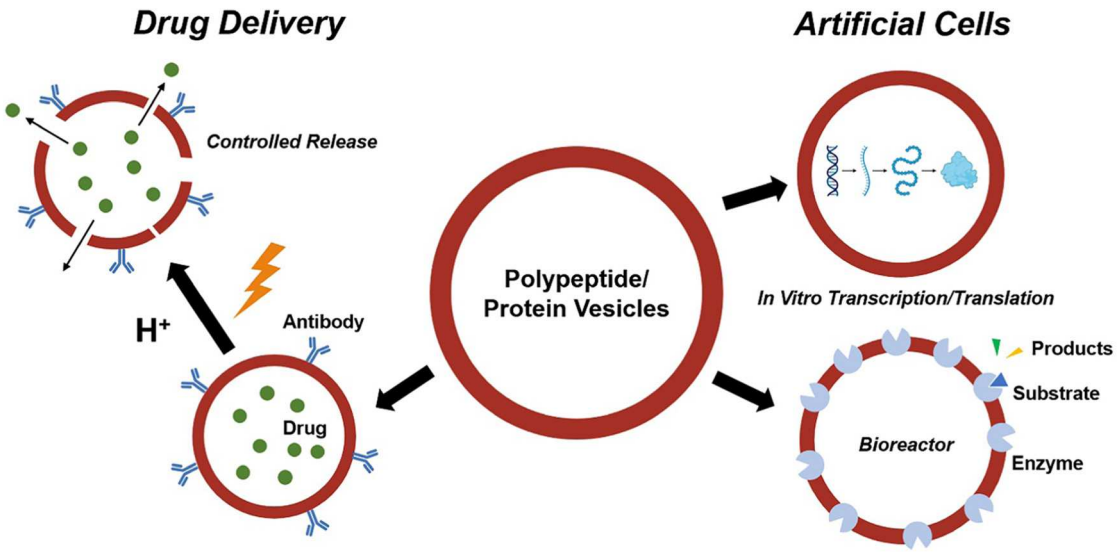


Fig. 8 A schematic image of biological applications of polypeptide/protein vesicles.

with other molecules, polypeptide- or protein-based vesicles represent an innovative platform for the hierarchical assembly of proteins into vesicle structures towards artificial cells. As engineering techniques of polypeptides, proteins, and fabrication methods continue to advance, the application fields of polypeptide/protein vesicles will expand. Therefore, gaining a comprehensive understanding of the engineering strategies employed in vesicle fabrication is of great significance. This review would provide a valuable summary for researchers investigating polypeptide- and protein-based vesicles for biological applications.

Conflicts of interest

The authors declare no competing financial interest.

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