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REVIEW

Polymersomes for Therapeutic Protein and Peptide Delivery: Towards Better Loading Properties

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Abstract: Therapeutics based on proteins and peptides have profoundly transformed the landscape of treatment for diseases, from diabetes mellitus to cancers, yet the short half-life and low bioavailability of therapeutic proteins and peptides hinder their wide applications. To break through this bottleneck, biomolecules-loaded polymersomes with strong adjustability and versatility have attracted more and more attentions recently. Loading proteins or peptides into polymersomes is the first but extremely important step towards developing high-quality formulation products. However, increasing protein and peptide loading content is quite challenging due to the inherent nature of self-assembled vesicle formation mechanism and physiochemical characteristics of biomacromolecules. This review highlights the potential of polymersomes as the next-generation therapeutic proteins and peptides carrier and emphatically introduces novel approaches and recent progress to achieve satisfactory encapsulation capability of polymersomes for proteins and peptides. On the one hand, with the help of intermolecular interactions, such as electrostatic, lipid–protein, and hydrophobic interactions, the drug loading could be significantly improved. On the other hand, loading improvement could be attained through innovation of preparation methods, ranging from modified traditional film hydration techniques to the novel phase-guided assembly method.

Keywords: protein, peptide, polymersomes, vesicles, drug loading

Introduction

Therapeutic proteins and peptides are playing an increasingly important role in treating cancers, diabetes, cardiovascular diseases, and other disorders.[1] Both protein and peptide are composed of amino acids, but they are artificially differentiated based on their amino acid units, molecular weights, and spatial conformations.[2] Generally, those with molecular weights greater than 5000 Da are defined as proteins, while those between 500 and 5000 Da are assigned to peptides.[3] Compared to small-molecule drugs, therapeutic proteins provide a larger contact surface area. This greater surface area improves binding specificity and lowers the risk of off-target effects.[4] Furthermore, owing to the presence of abundant reactive amino acid residues, therapeutic proteins and peptides are highly amenable to modifications such as fusion, glycosylation, lipidation, and phosphorylation.[1] Currently, more than 130 therapeutic proteins[1] and around 80 peptides[5] have been used clinically. Statistically, 55% of the marketed therapeutic proteins are indicated for hematology (29%) and oncology (26%),[6] while therapeutic peptides cover the applications in oncology, urology, pain, cardiovascular, respiratory, metabolic, and antimicrobial treatment.[3] According to a biobusiness brief published by Nature Reviews, 5 of the top 10 drugs by worldwide sales in 2022 were protein and peptide drugs.[7]

However, it remains quite challenging to promote clinical translation of proteins and peptides. Table 1 summarizes representative examples of innovative dose forms for therapeutic proteins and peptides. Oral administration is always the optimal route for patient compliance. However, only a few oral preparations such as capsules[8] and tablets[9] are clinically available due to poor absorption and easy degradation of peptides and proteins in the gastrointestinal tract. Recently, extended-release capsules (Mycapssa®) were developed with transient permeability enhancer (TPE®) technology by Chiasma in the USA and was approved by the FDA. Mycapssa® is applied for long-term maintenance treatment for

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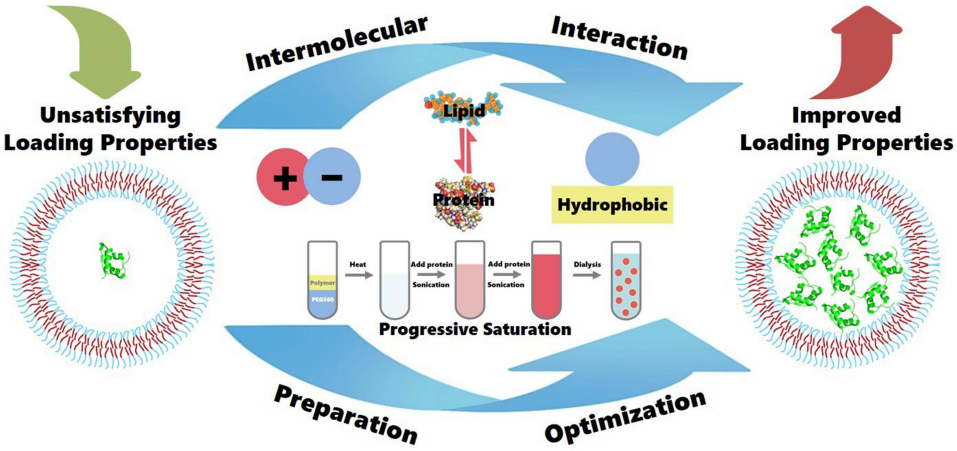
Graphical Abstract



patients with acromegaly who have tolerated lanreotide or octreotide initial treatment.[10] Another limitation of proteins and peptides is their short half-life. About 569 proteases are equipped in the body to degrade proteins and peptides, resulting in their fast elimination.[11] For example, the half-life of glucagon-like peptide-1 (GLP-1) is merely 1–2 min due to the degradation effect of dipeptidyl peptidase-4.[12] Microspheres present a successful solution to this issue. They enable controlled drug release by encapsulating drugs within polymers. As polymers degrade over time, the drug is released in a slow and continuous manner. This approach enhances drug stability, extends the drug's in vivo half-life, as well as reduces peak-valley fluctuations in plasma drug concentrations. To date, the FDA has approved 8 protein and peptide microsphere formulations for injection,[13] all using poly(lactic-co-glycolic acid) (PLGA) as biodegradable polymer carriers.[14] In addition, to address other challenges including difficulty in targeted delivery, low membrane permeability, and poor solubility, various nano-formulations such as liposomes, micelles, nanoparticles, and nanogels have been explored.[15] Currently, there are 3 liposomal formulations for protein and peptide delivery on the market, of which two act as vaccines[16] and one delivers peptide.[17] Epaxal® was the first liposomal vaccine developed in the 1980s. Hemagglutinin and neuraminidase, two important viral glycoproteins, are intercalated into the bilayer of the liposome. When liposomes attach to macrophages, the hemagglutinin protein is responsible for facilitating the delivery of Hepatitis A virus (HAV) antigen to immune cells, thereby triggering a stronger immune response. Besides, inactivated HAV virus particles are adsorbed on the surface, allowing the HAV virus to act as antigenic components and enhance the immune effect of the vaccine.[18] Mepact® is another liposomal formulation for osteosarcoma treatment and was marketed in 2009. The encapsulation of mifamurtide in liposomes can lead to better uptake by macrophages and prolong the drug retention time in the spleen, liver, and lung.[19] Micelles are self-assembled by amphiphilic molecules. The hydrophobic regions cluster together avoiding water, while the hydrophilic sections orient towards the aqueous solution.[20] Micelles could increase solubility of hydrophobic drugs through hydrophobic, π–π, or hydrogen bonding interactions between drugs and micellar hydrophobic core.[21] Guo et al studied the solubilization capacity of sodium cholate/lecithin-mixed micelles to deliver hydrophobic peptide Cyclosporine A. Solubility studies verified that this micellar formulation improves the solubility of Cyclosporine A to 5.42 mg/mL.[22] Other nanostructures, including solid nanoparticles[23] and nanogels,[24,25] could improve cellular uptake through enhanced permeability and retention (EPR) effect and active targeting.[26] For instance, the surface of nanoparticles can be modified by targeted moieties like folic acid to promote endocytosis via certain receptors on cell membranes.[27]

Compared to other dosage forms, polymersomes, also known as polymeric vesicles, offer a variety of advantages related to potential clinical applications. Firstly, polymersomes own hydrophobic membrane and hydrophilic lumen simultaneously, thus serving as effective carriers for both water-soluble and water-insoluble compounds.[36] The

Table 1 Representative Examples of Current Dosage Forms for Therapeutic Proteins and Peptides Delivery

Dosage Form	Material	Peptide/Protein	Indication or Objective	Product (Manufacturer)	Stage	Reference
Capsule	–	Peptide BBT-401-1S	Ulcerative colitis	- (Bridge Biopharmaceutics)	Phase I	[9]
	Gelatin, glycerol, polyoxyl 40 hydrogenated castor oil	Cyclosporin A	Graft-versus-host disease	Neoral®(Novartis)	On market	[8]
	-	Octreotide	Long-term maintenance treatment for patients with acromegaly	MYCAPSSA® (Chiasma)	On market	[10]
Tablet	Gelatin, mannitol, citric acid	Desmopressin acetate	Primary nocturnal enuresis	Minirin® (Ferring)	On market	[9]
Microsphere	PLGA	Triptorelin pamoate	Sustained release of 1–6 months to treat prostate cancer	Decapeptyl® (Ipsen)	On market	[13]
	PLGA	Exenatide	Sustained release of 1 week to treat diabetes	Bydureon® (AstraZeneca)	On market	[13]
	PLGA	Lanreotide	Sustained release of 2 weeks to treat acromegaly	Somatuline® (Ipsen)	On market	[13]
Liposome	DOPE and DOPC	Glycoprotein hemagglutinin, neuraminidase	Hepatitis A vaccine	Epaxal® (Crucell)	On market	[18]
	DOPE and DOPC	Glycoprotein hemagglutinin, neuraminidase	Influenza vaccine	Inflexal® (Crucell)	On market	[16]
	POPC and OOPS	Mifamurtide	Osteosarcoma	Mepact® (Takeda)	On market	[19]
Polymersome	Polyphosphazene	IFN-γ	Improve the anticancer effect of 5-fluorouracil as a cooperative attacker	-	Antitumor experiments in mice	[28]
	Polyphosphazene	Insulin	Oral delivery of insulin	-	Glucose regulation experiments in diabetic mice	[29]
	PMOXA-*b*-PDMS-*b*-PMOXA	NGF, CD109, BSA	Treatment of neurological diseases	-	Neurite outgrowth assay of mice	[30]
	PEG-*b*-PLA	L-Asparaginase	Treatment of acute lymphoblastic leukemia	-	In vitro experiments	[31]
	PEG-b-PLGA based polymers	Ovalbumin	Cancer immunotherapy	-	In vivo studies in mouse models	[32]
	PEG-SS-PPS	Stapled peptide ATSP-7041	Diffuse large B-cell lymphoma treatment	-	Xenograft experiments in mice	[33]
	PBD-b-PEO	Lipidated multi-epitope peptide vaccine	Dengue vaccine	-	Mice immunization study	[34]
	PBD-b-PEO	SARS-CoV-2 spike protein	Covid-19 vaccine	ACM-001 (ACM Biolabs)	Phase I clinical trial finished	[35]
Micelle	DSPE-PEG	Secretin	Modulating gastrointestinal and neuronal function	-	In vitro experiments	[20]
	Sodium cholate and lecithin	Cyclosporine A	Prevent rejection after transplantation	-	Pharmacokinetic experiments in rabbits	[22]

(*Continued*)

Table 1 (Continued).

Dosage Form	Material	Peptide/Protein	Indication or Objective	Product (Manufacturer)	Stage	Reference
Nanogel	Poly(vinyl alcohol) (PVA)	BSA	As a model protein drug	-	In vitro release experiments	[24]
	Poly acrylamide (PAM), PNIPAM, Poly vinylpyrrolidone (PVP)	Octreotide	Acromegaly	-	In vitro release experiments	[25]
Nanoparticle	Chitosan	Octreotide	Acromegaly	-	In vitro experiments	[23]
	PLGA and folic acid modified chitosan	Insulin	Treatment of diabetes	-	In vivo experiments in diabetic rats	[27]

combination of multiple drugs[37] provides additional options to address clinical challenges[38] like chemo resistance.[39] Secondly, the membrane of polymersomes could be easily decorated with diverse targeting ligands, from small molecules to antibodies. They could be designed to target inflammatory sites,[40] blood–brain barrier,[41] and cancer cells.[42] Such targeting strategy enhances therapeutic effects and minimizes off-target toxicity.[43] Moreover, various hydrophilic blocks, especially PEG, contribute to the stealth property of polymersomes and reduce their uptake by the reticuloendothelial system. Consequently, this property leads to an extended circulation half-life for polymersomes.[44] Thirdly, diverse polymeric blocks enable polymersomes to possess versatile stimuli-responsiveness, such as temperature, redox, glucose, enzyme, pH, and light.[45] These stimuli-responsive polymersomes are capable of releasing their payloads at the right time and right place, thus enhancing treatment accuracy.[46] Fourthly, the thick and stable membrane of polymersomes allows polymersomes to efficiently protect their encapsulated cargo and prolongs their circulation times.[47] Based on these advantages, polymersomes have been explored to encapsulate a large range of therapeutic proteins and peptides. Currently, biomolecules from model proteins like bovine serum albumin (BSA)[30] and ovalbumin (OVA)[48] to commercial therapeutic ones like insulin[29] and L-asparaginase[31] have been encapsulated.[28] In 2020, Shoaib et al reviewed the therapeutic applications of protein-loaded polymersomes.[49] Polymersomes have shown potential in cancer treatment, including prostate cancer,[50] lung cancer,[51] orthotopic glioblastoma tumor,[52] and multiple myeloma.[42] Besides, polymersomes have been investigated for vaccine delivery, such as influenza[53] and Bacillus Calmette-Guérin vaccine.[54] Other therapeutic applications, ranging from central nervous system diseases[30] to tissue regeneration,[55] have also been summarized in the above review. Recently, there have been several works on polymersomes loaded with proteins and peptides, with topics including cancer immunotherapy,[32] diffuse large B-cell lymphoma treatment,[33] and dengue vaccine.[34] As for ongoing clinical trial situations, in 2022, ACM Biolabs applied for a Phase I clinical trial of ACM-001, an adjuvanted SARS-CoV-2 spike protein (beta variant) vaccine. ACM-001 used poly(butadiene)-b-poly(ethylene glycol) (PBD-b-PEO) polymersomes to encapsulate the recombinant beta spike protein along with a synthetic CpG adjuvant.[35] In September 2023, positive topline results were reported for ACM-001 in the Phase I Trial.[56]

However, to the best of our knowledge, in 2023 there are still no polymersomes for therapeutic proteins or peptides commercially available or in advanced stages of clinical trials.[47] Several factors have hampered clinical translation of polymersomes including limited certified polymers, tough large-scale and reproducible production, and complicated in vivo metabolism characters.[57] Moreover, due to the large bulk size of biomolecules and the lack of intermolecular interactions between biomolecules and polymers, it is usually quite difficult to load proteins and peptides into polymersomes.[47] Adams et al calculated the encapsulation efficiency by assuming that the concentration of drugs in the formed polymersomes is equal to that in the continuous phase prior to self-assembly. It was found that the encapsulation efficiency is limited by the volume fraction of the vesicle inner aqueous cavity and would not exceed 10%.[58] This is a critical issue that many researchers tend to address, since loading capacity is an important item for pharmaceutical dosages.[59] A decent drug loading capacity not only helps to reduce production cost but also improves therapeutic effects and decreases safety risk of excipients.[60] Loading content and encapsulation efficiency are two frequently used indexes to characterize drug loading properties for certain formulations. Loading content is defined as the

weight of a loaded drug divided by the weight of drug-loaded polymersomes and reveals the weight proportion of the drug in the final product. Encapsulation efficiency is defined as the weight of a loaded drug divided by the weight of the drug in the feed and represents the use efficiency of the input drug during the fabrication process.

Currently, there have been a number of reviews summarizing the polymer types,[47] self-assembling process,[61] surface functionalization,[57] and therapeutic applications[49,62] for biomolecules-loaded polymersomes. However, to date, no reviews have been published discussing the loading property improvement. In this paper, focusing on the loading capability of polymersomes for peptides and proteins, we discussed novel approaches to achieve higher loading capability in detail and summarized encapsulation methods for biomolecules-loaded polymersomes.

Encapsulation Methods

The self-assembly of amphiphiles is governed by a balance between hydrophobic and hydrophilic interactions. The hydrophobic effect minimizes the interaction between water and the hydrophobic chains, while the hydration forces orient hydrophilic blocks towards the aqueous phase.[63] The hydrophilic block fraction is an important parameter that determines the geometry of the self-assemblies, ranging from micelles, worm-like micelles, polymersomes to lamellae. As a unifying rule, block copolymers with a hydrophilic block fraction of 35 ± 10% tend to yield polymersomes, while those with a fraction over 45% form micelles.[64] Currently, two types of methods have been developed for producing polymersomes loaded with proteins and peptides, namely the top-down method and the bottom-up method. In top-down strategies, vesicles are derived from bulk polymers, whereas in bottom-up approaches, vesicles are formed from individual polymer chains.[65] (Figure 1A).

Top-Down Approaches

During the top-down approach, the polymers would form vesicles from as-existed condensed matters, such as polymer films and polymer powders.[68] The film hydration method is the most widely used top-down method and is comprised of three steps (Figure 1B). Firstly, polymers are dissolved in organic solvent and formed into a thin film by the removal of solvent under reduced pressure. Secondly, an aqueous solution that contains hydrophilic cargos is added. Then, external energy, like sonication or mechanical stirring, is introduced to facilitate the hydration of the film.[43] As water diffuses into polymer layers, polymeric layers swell, amphiphilic membranes come into existence and finally detach to form polymersomes.[69] Alternatively, the hydration of the film could be achieved by applying an electric current, also known as electro-formation method. In this method, two platinum electrodes are deposited with block copolymers and then immersed in buffer solution with an alternating current electric field. Enhanced water diffusion through the polymer film is achieved by applying alternating current, providing control over the hydration rate.[70] Usually, the polymersomes prepared by the film hydration method have a quite broad size distribution. Therefore, further treatments like extrusion through membranes with certain pore sizes[70] or gel permeation chromatography purifying[41] to homogenize the size distribution would be needed. Discher's group first fabricated BSA-loaded polyethyleneoxide-*b*-polyethylethylene (PEO-*b*-PEE) polymersomes by film hydration method.[71] First of all, polymers of 4.0 mg/mL were dissolved in chloroform and coated uniformly on the inner wall of a glass vial. The chloroform was then evaporated under vacuum for 3 h. Subsequently, the addition of a sucrose solution resulted in the spontaneous formation of polymersomes detaching from the glass. After the work of Discher's group, more copolymer vesicle systems such as poly(ethylene oxide)-*b*-poly(butadiene) (PEO-*b*-PBD),[72–74] polyethyleneoxide-*b*-polylactic acid (PEO-*b*-PLA),[75] poly(ethylene glycol)-*b*-poly (ε-caprolactone)-*b*-poly(2-(diethylamino) ethyl methacrylate) (PEG-*b*-PCL-*b*-PDEA)[76] have been used to load proteins following the same process. Recently, Dinu et al coencapsulated enzymes with trehalose into polymethyloxazoline-*b*-polydimethysiloxane-*b*-polymethyloxazoline (PMOXA-*b*-PDMS-*b*-PMOXA) polymersomes using the film hydration method. It was indicated that trehalose could preserve and stabilize protein bioactivity under extreme states like overheating and dehydration.[77] To obtain vesicles with a homogeneous size distribution, the vesicle dispersions underwent 11 rounds of extrusion using a mini-extruder equipped with a polycarbonate membrane with 200 nm pore size.

For powder hydration method, the polymer powders are directly dissolved in the drug-containing solution to induce the self-assembly process. Usually, stirring or ultrasonication would be needed during this process. Wittemann et al directly dissolved triblock copolymer poly(ethylene oxide)-*b*-poly(caprolactone)-*b*-poly(acrylic acid) (PEO-*b*-PCL-*b*-PAA) in 10 mM MOPS buffer to form fluorescein isothiocyanate-BSA (FITC-BSA) loaded polymersomes. Due to

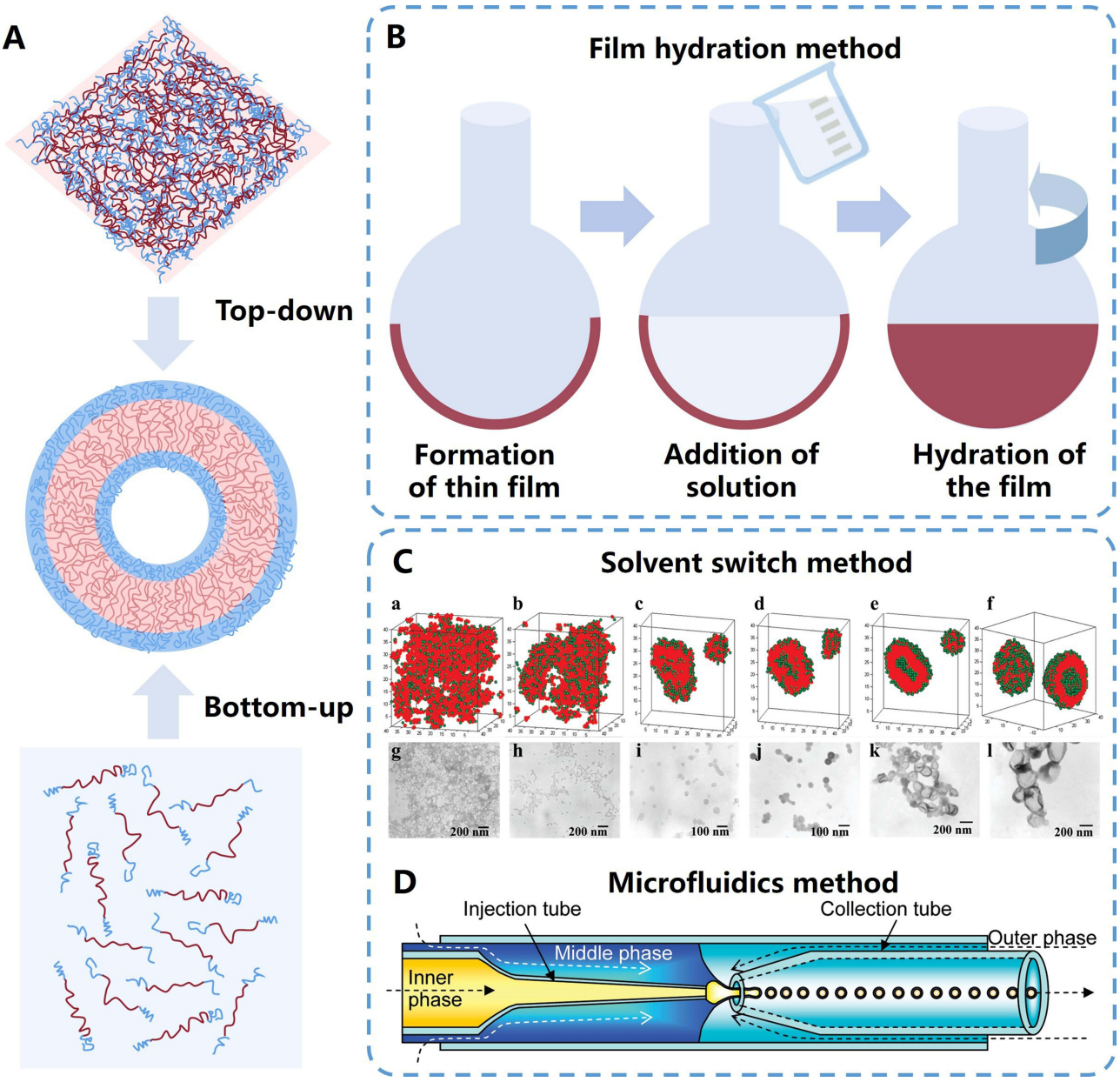


Figure 1 Encapsulation methods of biomolecules-loaded polymersomes. (**A**) A schematic expression of the top-down and bottom-up method to prepare polymersomes. (**B**) Schematic illustration of the film hydration process. (**C**) Monte Carlo simulation snapshots at a 8.3 × 10^4, b 9.0 × 10^4, c 1.1 × 10^5, d 1.2 × 10^5, e 2.0×10^5 and f 3.3×10^6 steps, illustrating the process of spontaneous vesicle formation by the amphiphilic triblock copolymer during slow addition (where green and red represent hydrophilic and hydrophobic molecules, respectively). Below are TEM images depicting the aggregates formed at various time points during the addition of deionized water at a rate of 0.2 wt% per day for g 15, h 18, i 20, j 25, k 28 and l 30 days. Reprinted with permission from Han Y, Yu H, Du H, Jiang W. Effect of selective solvent addition rate on the pathways for spontaneous vesicle formation of ABA amphiphilic triblock copolymers. *J Am Chem Soc*. 2010;132(3):1144–1150. Copyright 2010, American Chemical Society.[66] (**D**) Schematic diagrams of the microfluidic device for polymersome preparation. Reprinted with permission from Shum HC, Kim J-W, Weitz DA. Microfluidic fabrication of monodisperse biocompatible and biodegradable polymersomes with controlled permeability. *J Am Chem Soc*. 2008;130(29):9543–9549. Copyright 2008, American Chemical Society.[67]

the slow dissolution rate of PCL, solid polymers can still be observed in the solution after several hours of stirring. To ensure complete dissolution of the polymer, the samples were stirred vigorously for at least four days.[78] Similarly, using this method, Alibolandi M et al employed dextran (DEX) as the hydrophilic block to form Dextran-*b*-poly(lactide-co-glycolide) (DEX-*b*-PLGA) polymersomes containing insulin. DEX-*b*-PLGA (10 mg) was hydrated using 2 mL of insulin solution in PBS (pH 4–5) incorporating different amounts of insulin (0, 0.5, 1, 1.5, 2, 2.5, and 3 mg). The hydration process involved ultrasonication at 20–25°C for 1 h, followed by vigorous stirring (1250 rpm) for 24 h at room temperature.[79]

Bottom-Up Approaches

Unlike top-down approaches, the bottom-up ones involve the self-assembly of molecules to construct desired structures. In bottom-up approaches, polymers tend to form vesicles from a homogeneously dispersed initial state. Usually, for this process to take place, an external driving force must be introduced, which can either be a change in the type of solvent or a change in solvent conditions such as pH and temperature.

The solvent switch method is a widely used bottom-up approach.[80,81] In this method, the polymer is first dissolved in a feasible solvent for both hydrophobic and hydrophilic segments, and then the solvent is exchanged for a solvent which only dissolves the hydrophilic block.[69,82] The size and size distribution of the vesicles can be varied by selecting different organic solvents. The vesicle formation process could be visualized by a one-to-one correspondence between the Monte Carlo simulation results and transmission electron microscopy (TEM) images (Figure 1C).[66] The formation process could be divided into three stages. Initially, the amphiphilic triblock copolymer combines to form a large irregular aggregation. Subsequently, the large aggregation splits into large irregular spheres, with randomly distributed hydrophilic molecules. Finally, some hydrophilic molecules diffuse towards the surface, while others move towards the center, resulting in the formation of small vesicles. Using the solvent switch method, insulin-loaded poly(lactic acid)-*b*-pluronic-*b*-poly(lactic acid) (PLA-*b*-F127-*b*-PLA) polymersomes were prepared. Firstly, a solution of 60 mg PLA-*b*-F127-*b*-PLA in 3 mL THF was prepared. The polymer solution was then added dropwise to the insulin solution at room temperature, with gentle stirring. Subsequently, the solution was dialyzed for 4 h to remove the organic solvent. Finally, the solution was lyophilized to obtain insulin-loaded polymersomes.[83]

For stimuli-responsive polymers, the temperature or pH switch method is a commonly used bottom-up approach.[84,85] In this method, the polymersomes are formed by increasing the hydrophobicity of the polymer with the change of the pH or temperature. Du et al reported pH-sensitive PMPC-*b*-PDPA vesicles formed by pH switch method. Due to the protonation of tertiary amine groups on PDPA blocks (pKa around 6.3), PMPC-*b*-PDPA is easily dissolved in water below pH 6 and exists as a weakly cationic polyelectrolyte. However, above a pH value of approximately 7, the deprotonation of tertiary amine groups occurs. Such an increase in hydrophobicity causes the formation of polymersomes.[86] Zhang et al developed pH-sensitive poly (ethylene glycol)-SS-poly(2-(diethyl amino)ethyl methacrylate) (PEG-SS-PDEA) diblock copolymers with pH-sensitivity and proteins were loaded into the polymersomes by adjusting an acidic solution to pH 7.4. To achieve desirable size distributions, the solution pH was incrementally increased from pH 5.5 to pH 6.5, followed by pH 7.0, and ultimately pH 7.4, and each pH level was allowed 30 min equilibrium.[87,88] The temperature switch method takes advantage of the temperature responsiveness of thermosensitive polymers. Poly(N-Isopropylacrylamide) (PNIPAM) is a commonly used thermosensitive polymer that exhibits a rapid and reversible phase separation from the solvent upon reaching temperatures above its lower critical solution temperature (LCST) of 32°C.[89] Cheng et al formed protein loaded polymersomes by increasing the temperature of thermosensitive poly(ethylene glycol)-poly(acrylic acid)-poly(*N*-Isopropylacrylamide) (PEG-*b*-PAA-*b*-PNIPAM) triblock copolymer from 4°C to 40°C.[90]

Microfluidics is a preparation method that produces vesicles by precise manipulation of microscale fluid. Usually, multiple mobile phases are involved in microfluidics (Figure 1D). Typically, the inner phase is an aqueous solution containing hydrophilic cargoes such as proteins and the middle phase is an organic solvent dissolving polymer. At the tip of the injection tube, the inner aqueous phase is sheared into single emulsified droplets by the middle polymer oil phase. Simultaneously, the middle oil phase is sheared into W/O/W double emulsified droplets by the outer water phase. After the formation of double emulsion within the collection tube, the organic solvent departs from the structure by means like evaporation or diffusion. This leads to the ultimate formation of a polymeric bilayer.[43] Since all the flow parameters such as drug concentration, polymer concentration, polymer-to-drug ratio, solvent type, and solvent-to-water ratio could be manipulated, enhanced control over polymersomes self-assembly is possible.[91] Weitz's group has reported the microfluidic fabrication of monodisperse giant polymersomes since 2005.[67,92] In 2010, Brown et al used a flow focusing microfluidic device to induce self-assembly of PMPC-*b*-PDPA block copolymer by changing the pH of the flows within the microchannels. In this method, since self-assembly was induced using pH changes instead of solvent exchange, the middle oil phase was eliminated and both the inner and outer phases were aqueous. Polymersomes with hydrodynamic diameters in the range of 75–275 nm were obtained. BSA was loaded into vesicles and the encapsulation efficiency was

even slightly higher than that of the traditional pH switch method.[93] Using the microfluidics technique, Chiara et al achieved the expression of actin-like structural proteins within polymersomes. The inner aqueous phase consisted of the E. coli ribosomal extract and the MreB DNA plasmid, both necessary for protein expression. The middle phase contained a mixture of hexane and chloroform (volume ratio of 62:38), 5 mg/mL poly(ethylene glycol)-*b*-polylactic acid (PEG-*b*-PLA) (MW 5 000 or 10,000), and 2.5 mg/mL PLA homopolymer (MW 15 000). PEG-*b*-PLA acted as the amphiphile, while the homopolymer PLA was introduced to improve the stability of the resulting vesicles. The polymersomes provided a stable compartment for protein expression, and their semipermeability facilitated the release of expressed proteins through a negative osmotic shock.[94]

Novel Approaches to Improve Loading Capability

How many proteins and peptides are encapsulated into polymersomes? This is a basic and pivotal question in determining drug action in vivo to a great extent. Accordingly, loading capability is one of the basic evaluation criteria of drug delivery systems. The characterization procedure of protein and peptide loading properties could be summarized in three steps, namely separation, quantification, and calculation. After preparation, the hybrid system contains both encapsulated and unencapsulated proteins and peptides. To quantify the encapsulation efficiency, the first step is to separate using methods such as centrifugation, dialysis, chromatography, etc.[76] Upon separation, quantification against either the encapsulated part or the un-encapsulated part could be carried out. Finally, the loading properties, as either encapsulation efficiency or loading content, could be calculated. Many efforts, mainly focused on innovations of materials and preparation methods, have been made to achieve higher loading capability.

Introduction of Intermolecular Interactions

If there exist no or weak intermolecular interactions between the self-assembled polymers and proteins, the loading content would generally be low.[47,95–98] This is because during the self-assembly process, the drug is loaded into vesicles in a volume-based manner.[99] Table 2 lists some comparisons of loading properties with and without intermolecular interactions. Clearly, polymers that possess specific intermolecular interactions with biomolecules typically exhibit enhanced encapsulation efficiency compared to those lacking such interactions. The most widely used strategy is to design polyelectrolytes bearing charges, positive charges in most cases, to adsorb proteins or peptides more tightly via electrostatic interaction. Besides, inspired by natural biomembranes, lipid–protein interactions are taken to achieve better loading properties.[100,101] Genetically modified proteins could also help to introduce hydrophobic interactions.

Table 2 Comparisons of Encapsulation Efficiency with and without Intermolecular Interactions

Protein	Materials	Interaction	EE	Preparation Method	Reference
OVA	PEG-*b*-PPS	None	45%	Solvent exchange	[95]
	PEG-*b*-PCL-*b*-PDEA	Electrostatic interaction	84.7%	Film hydration	[76]
	PEG-*b*-PAA-*b*-PNIPAM	Electrostatic interaction	88.3%	Temperature switch	[90]
	PEDP	Electrostatic interaction	84%	Solvent exchange	[100]
	PEOP	Lipid-protein interaction	70%	Solvent exchange	[101]
BSA	PMOXA-*b*-PDMS-*b*-PMOXA	None	48.6%	Film hydration	[30]
	PEG-*b*-PTMC(AC)	None	47.6%	Powder hydration	[96]
	PEG-*b*-PCL-*b*-PDEA	Electrostatic interaction	78.5%	Film hydration	[76]
	PEO-*b*-PCL-*b*-PAA	Electrostatic interaction	83%	Powder hydration	[78]
	PEG-*b*-PTMC(COOH)	Electrostatic interaction	87.1%	Powder hydration	[96]
	PEG-*b*-PTMC(NH_2)	Electrostatic interaction	95.9%	Powder hydration	[96]
Insulin	PEG-*b*-P(Ser-Ketal)	None	0.7%	Solvent exchange	[97]
	PEG-*b*-P(Ser-S-NI)	None	3.2%	Solvent exchange	[98]
	PEOP	Lipid-protein interaction	84.7%	Solvent exchange	[29]

Polyelectrolytes–Protein Interaction

Polyelectrolytes are defined as a series of macromolecules with charged units, while proteins are polyampholytes possessing both positive and negative charges on their surface. Electrostatic interaction between proteins and polymeric materials plays a major role in the protein encapsulation process.[102,103] Generally, charged amphiphilic copolymers own higher protein loading capacity than their uncharged counterparts after self-assembly into polymersomes, as illustrated in Table 2. As for OVA, while using the same encapsulation method, the encapsulation efficiency of poly(ethylene glycol)-b-poly(ε-caprolactone)-*b*-poly(2-(diethylamino) ethyl methacrylate) (PEG-*b*-PCL-*b*-PDEA),[76] where the hydrophobic block PDEA exhibits positive charges due to diethylamino groups, was 84.7%, higher than that of uncharged PEG-b-PPS (EE 45%).[104] The same trend could be observed for BSA, with the encapsulation efficiency of positively charged PEG-*b*-PCL-*b*-PDEA (78.5%)[76] higher than that of uncharged PMOXA-*b*-PDMS-*b*-PMOXA (48.6%).[30]

Since most proteins have isoelectric points less than 7 and are negatively charged under physiological conditions,[105] a common strategy in constructing proteinophilic polyelectrolytes is to incorporate positively charged blocks. Table 3 lists various charged units of polyelectrolyte blocks in amphiphilic copolymers to form biomolecules-loaded polymersomes. A frequently used polyelectrolyte block is polyethylenimine (PEI). PEI has become the gold standard cationic polymer for gene delivery. It has a high charge density ranging from 20 to 25 microequivalents per gram.[106] Yang et al took advantage of PEI to construct copolymer PEG-*b*-P(TMC-DTC)-*b*-PEI.[107] As a result, the loading content of FITC-CC reached 17.8% when the theoretical loading content was 20%. Granzyme B-loaded PEG-*b*-P(TMC-DTC)-*b*-PEI polymersomes exhibited strong cytotoxicity against A549 lung cancer cells, demonstrating the half-maximal inhibitory concentration (IC_{50}) of 20.7 nM. In vivo, the functionalization with a cell-selective penetrating peptide resulted in enhanced tumor accumulation compared to nonfunctionalized counterparts, while maintaining a blood circulation time of 6.6 h. Moreover, it exhibited complete growth inhibition of orthotopic A549 tumor xenografts in nude mice within a 20-day period.[102]

Qiu's group constructed a polymersome system to deliver protein antigen OVA based on amphiphilic graft polyphosphazene derivative PEDP containing pH-sensitive N,N-diisopropylethylenediamine (DPA) groups (Figure 2A). The pK_b of the polymer was estimated to be 6.2 through potentiometric measurement.[103] The loading content of OVA was up to 7.19% under a theoretical loading content of 10%. Compared with blank polymersomes (Figure 2B), OVA-loaded polymersomes (Figure 2C) exhibited the similar outline but the center hydrophilic cavity became darker due to the considerable OVA loading. Different from the instant and short-term release behavior of polymersomes formed by common pH-responsive copolymers, the OVA diffusion of PEDP nanovesicles lasted for about a week at pH 5.5. This phenomenon might be attributed to the structure feature of graft copolymer that the hydrophobic polyphosphazene backbone chain can maintain vesicles stable to some extent in acidic solution despite the protonation of DPA side groups. The BALB/c mice exhibited sustained elevated antibody titers for 8 weeks in response to the prime-boost vaccine. The subcutaneous administration of OVA polymersomes biased the immune response toward a type 1 T helper (Th1) response. Results from animal experiments revealed that

Table 3 Polymers Containing Polyelectrolyte Blocks to Form Biomolecules-Loaded Polymersomes

Charged Unit of Polyelectrolyte Block	Polymer	pK value	Reference
Ethylenimine (EI)	PEG-*b*-P(TMC-DTC)-*b*-PEI	pK_a=7.8–9.9	[102]
N,N-diisopropylethylenediamine (DPA)	PEDP	pK_b=6.2	[100]
2-(diethyl amino)ethyl methacrylate (DEA)	PEG-*b*-PCL-*b*-PDEA PEG-SS-PDEA PEG-*b*-PAA(SH)-*b*-PDEA	- pK_a=7.2 -	[76] [87] [88]
Acrylic acid (AA)	PEO-*b*-PCL-*b*-PAA PEG-*b*-PAA-*b*-PNIPAM	pK_a=4.5 -	[78] [90]
Trimethylene carbonate (TMC) derivatives	PEG-*b*-PTMC($COOH/NH_2$)	-	[96]
Succinic acid carbonate (SAC)	PEG-*b*-PTMBPEC-*b*-PSAC	-	[103]

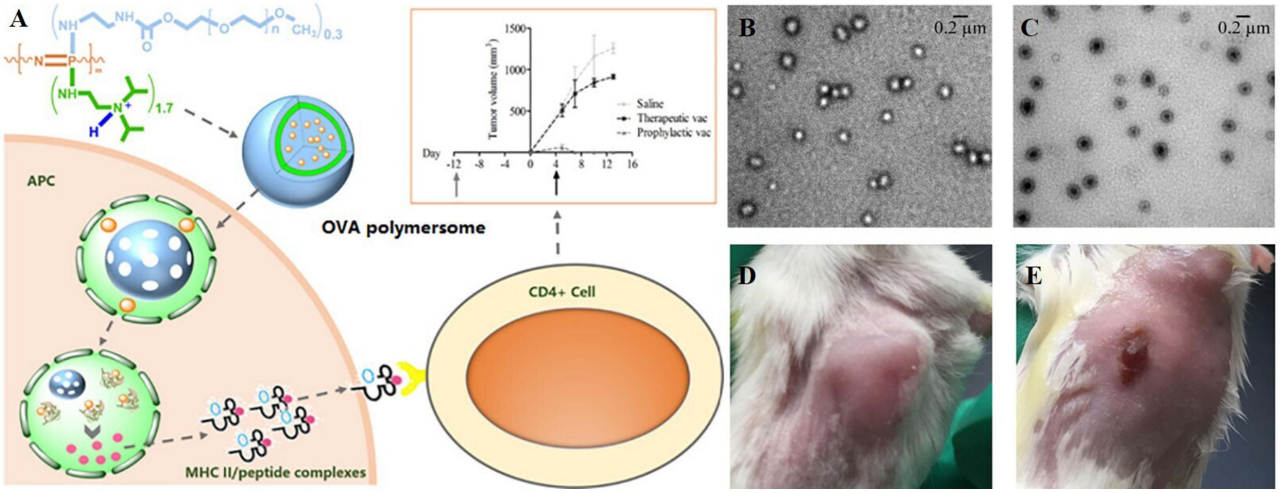


Figure 2 Polymersome system to deliver protein antigen OVA based on amphiphilic graft polyphosphazene derivative PEDP containing pH-sensitive N, N-diisopropylethylenediamine (DPA) groups. (**A**)Schematic of OVA-loaded pH-responsive PEDP vesicles inducing enhanced Th1 immune response. (**B**) Blank PEDP polymersomes. (**C**) OVA polymersomes with the theory protein loading content of 5%. Photographies of the location of cell inoculation on (**D**) therapeutic group and (**E**) prophylactic vaccine group mice at day 10. Reprinted from Gao M, Peng Y, Jiang L, Qiu L. Effective intracellular delivery and Th1 immune response induced by ovalbumin loaded in pH-responsive polyphosphazene polymersomes. *Nanomedicine*. 2018;14(5):1609–1618. Copyright (2018), with permission from Elsevier.[100]

prophylactic vaccination using PEDP polymersomes significantly accelerated and improved the suppression of tumor growth compared to therapeutic vaccination (Figure 2D and E). These findings suggested that PEDP-based polymersomes hold great promise for the controlled cytosolic protein antigens delivery and could enhance Th1-specific immune responses.[100]

PDEA is an extensively studied weak polybase that exhibits a pK_a of approximately 7.3 in water.[108] Liu et al prepared asymmetric polymersomes using PEG-*b*-PCL-*b*-PDEA copolymers to deliver proteins into cells. Nuclear magnetic resonance (NMR) results proved that PDEA blocks were shielded inside of polymersomes, indicating the asymmetric structure of polymersomes. The PDEA blocks protonate at acidic pH, which gives rise to a strong interaction between the cationic blocks and proteins during the polymersome formation at pH 5.3. Five species of FITC-labeled proteins were all loaded with rather high encapsulation efficiency. At a theoretical loading content of 25%, the encapsulation efficiency values of FITC-BSA, FITC-CC, FITC-Lys, FITC-OVA, FITC-IgG were 78.5%, 89.1%, 84.3%, 84.7%, and 89.6%, respectively.[76] Similarly, PEG-SS-PDEA polymersomes were prepared based on the PDEA blocks with high protein encapsulation efficiency, nearly 100% for FITC-CC under a theoretical loading content of 10%. Confocal laser scanning microscopy (CLSM) observations demonstrated the efficient delivery and release of proteins into MCF-7 cells by FITC-CC-loaded PEG-SS-PDEA (9.2 k) polymersomes after 6 h of incubation. Notably, flow cytometry assays revealed that these polymersomes induced significantly increased apoptosis of MCF-7 cells.[87] Afterwards, by modifying the PAA blocks with cysteamine, the thiol-containing PAA(SH) was yielded and the reduction and pH dual-responsive PEG-*b*-PAA(SH)-*b*-PDEA polymersomes were prepared. The encapsulation efficiency values of FITC-BSA and FITC-CC both reached nearly 100% under a theoretical loading content of 5%. The PEG-*b*-PAA(SH)-*b*-PDEA copolymer could be readily dissolved in water at physiological pH or below but would aggregate into polymersomes upon increasing solution pH over 7.4. The pendant thiol groups could be facilely crosslinked under exposure to the air and could be reversibly decrosslinked in response to 10 mM glutathione (GSH) (Figure 3A). TEM micrograph verified the vesicular structure of these nanoparticles (Figure 3B). Moreover, confocal laser scanning microscopy (CLSM) observation of giant crosslinked polymersomes co-loaded with Nile red (hydrophobic) and FITC (hydrophilic) clearly showed that Nile red was distributed uniformly in the membrane while FITC was distributed in the aqueous lumen, in accordance with the above mentioned vesicular structure (Figure 3C). At the cellular level, MTT assays showed that cytochrome C-loaded PEG-b-PAA(SH)-b-PDEA polymersomes induced potent MCF-7 and HeLa cells apoptosis after 48 h of incubation, proving the efficient intracellular delivery and release of cytochrome C.[88]

Though against common sense, proteins could interact with polyelectrolytes even on the "wrong side" of isoelectric point, which means they are like-charged.[109] For example, under physiological conditions, both the PAA block and BSA

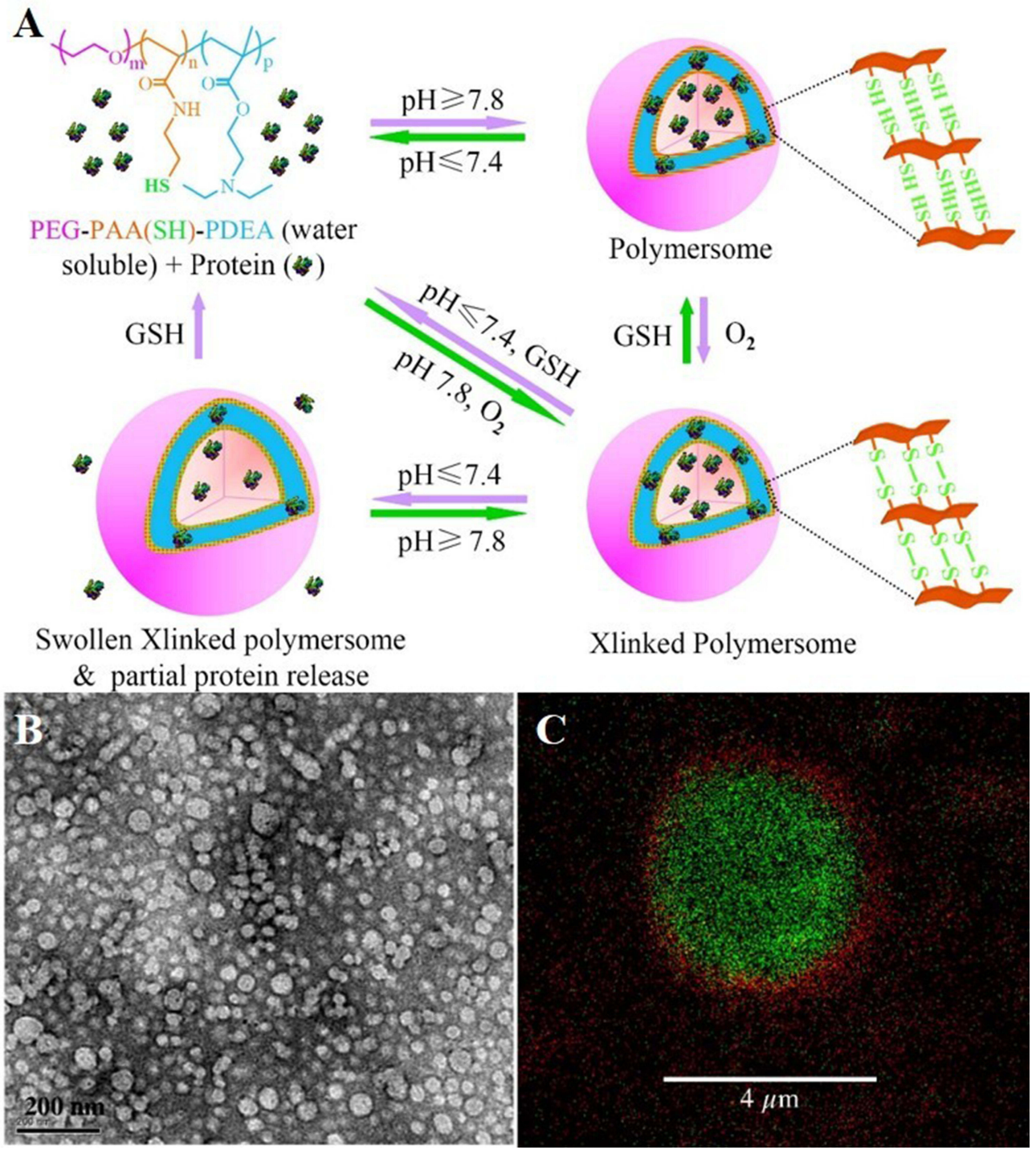


Figure 3 Reduction- and pH-responsive disulfide-crosslinked polymersomes based on PEG-PAA(SH)-PDEA triblock copolymer. (**A**) Scheme of efficient encapsulation and controlled release of proteins. (**B**) TEM image of disulfide-crosslinked polymersomes. (**C**) CLSM image of giant polymersome co-loaded with Nile red and FITC. Reprinted from Sun H, Meng F, Cheng R, Deng C, Zhong Z. Reduction and pH dual-bioresponsive crosslinked polymersomes for efficient intracellular delivery of proteins and potent induction of cancer cell apoptosis. *Acta Biomater.* 2014;10(5):2159–2168. Copyright (2014), with permission from Elsevier.[88]

exhibit overall negative charges,[110] yet PEO-*b*-PCL-*b*-PAA polymersome could load FITC-BSA with high encapsulation efficiency of over 80%. Dubin et al attributed this phenomenon to the presence of positive "patches" on the protein surface persisting beyond the isoelectric point. Thus, the protein–polyelectrolyte interaction can be rationalized by a balance between the attraction among these positive patches and polyelectrolytes and the repulsion caused by the

overall negative charge of the protein.[111] The Delphi images of electrostatic potentials for different proteins at pH 7 verified the above mentioned charge "patches" model for proteins (Figure 4A). As proteins interact with polyanions, counterions previously attached to the surface of proteins and polyanions would be released, thus increasing the total entropy and making the whole process spontaneous (Figure 4B).[105] Wittemann et al synthesized triblock copolymer PEO-*b*-PCL-*b*-PAA to form polymersomes and studied their protein encapsulation and adsorption behavior. UV/vis spectroscopy results confirmed high encapsulation efficiency for FITC-BSA of over 80%. Zeta potential measurements showed that the PAA blocks are preferentially segregated to the outer interface, while the PEO blocks prefer the inner interface. Transmission electron microscopy (TEM) and laser scanning confocal microscopy images indicated a strong enrichment of proteins onto the vesicle walls.[78] Using a temperature switch method, Cheng et al prepared polymersomes by triblock copolymer PEO-*b*-PAA-b-PNIPAM since PNIPAM block displays phase transfer at the lowest critical solution temperature (LCST). Several kinds of proteins, including BSA, lysozyme, and cytochrome C (CC), were loaded into the polymersomes with high encapsulation efficiency of 60–100% at theoretical loading content of 10–50%. After the formation of PEO-*b*-PAA-*b*-PNIPAM polymersomes, crosslinking the PAA segment with cystamine (Cys) viacarbodiimide chemistry could yield reduction-responsive polymersomes that could rapidly dissociate with the reducing agent. Flow cytometry analysis revealed that Cys-crosslinked polymersomes loaded with CC significantly enhanced apoptosis in MCF-7 cells compared to both reduction-insensitive and free CC controls.[90] In 2015, copolymer poly(ethylene glycol)-*b*-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate)-*b*-poly(succinic acid carbonate) (PEG-*b*-PTMBPEC-*b*-PSAC) containing carboxylic acids with negative charges was prepared. The polymersomes derivated from this copolymer showed high encapsulation efficiency for both FITC-BSA (95.2%) and FITC-CC (90.2%) under a theoretical loading content of 10%. After 2-[3-[5-amino-1-carboxypentyl]-ureido] pentanedioic acid (Acupa) decoration, Acupa-PEG-*b*-PTMBPEC-*b*-PSAC could form polymersomes that target PSMA positive LNCaP prostate cancer cells. MTT experiments showed that Granzyme B-loaded Acupa decorated polymersomes induced potent apoptosis in LNCaP cells and exhibited a low IC_{50} of 1.6 nM. In nude mice, the Acupa decorated polymersomes demonstrated a long elimination half-life of 3.3 h. The long circulation time suggested their promising potential for targeted in vivo protein delivery.[50] In 2018, triblock copolymers poly(ethylene glycol)-b-poly(α-aminopalmitic acid)-b-poly(L-aspartic acid) (PEG-b-PAPA-b-PAsp) were prepared. Negatively charged PAsp blocks contributed to the good protein loading ability. The polymersomes prepared by this copolymer showed high encapsulation efficiency over 90% for FITC-labeled cytochrome C. After intravenous injection into mice via a tail vein, polymersomes exhibited an extended half-life of 3.5 h. The good circulation of polymersomes facilitated elevated protein accumulation in orthotopic A549 lung tumor xenografts. Effective inhibition of tumor growth was observed in saporin-loaded polymersomes. Mice treated with this formulation experienced a significant extension in median survival time (50 d) compared to PBS (28 d). Therefore, this polymersome acted as a robust platform for good loading and intracellular delivery of therapeutic proteins in vivo.[112]

The interaction between proteins and polyelectrolytes is influenced by several factors, including the system ionic strength, polyelectrolyte structure, and protein charge anisotropy.[115] Firstly, the ionic strength in the system could largely influence protein–polyelectrolyte interactions. Dubin et al studied the binding between BSA and polyanions, hydrophobically modified poly(acrylic acid) (HMPAA) and found that the binding was the strongest at the medium ionic strength (Figure 4C). This is because the binding of polyanions to protein positive "patches" is actually a combination of short-range attractions and long-range repulsions (Figure 4D). When the ionic strength is medium, its charge shielding effect could screen long-range repulsions while still maintaining short-range attractions, thus leading to the best binding effect. The charge shielding effect of ionic strength on charged units could be quantified by Debye length $\kappa^{-1}=0.3/\sqrt{I}$(nm). Thus, the critical I corresponding to the strongest binding would be reached when $\kappa^{-1}=R_{pro}$. Considering R_{pro} is usually 2–4 nm, the critical I would be in the range of 6–20 mM.[113]

Secondly, charge density of polyelectrolyte is another influencing factor. Dubin et al studied the interactions between polyanions with various charge densities and BSA. It was found that polyanions with lower charge density bound with BSA more strongly than those with higher charge density. This is because BSA has a global negative charge under neutral conditions. Thus, the most favorable linear arrangements of polyelectrolyte charges are those maximizing the attraction with the positive protein "patches" while minimizing the repulsion with the adjacent negative domain.[116]

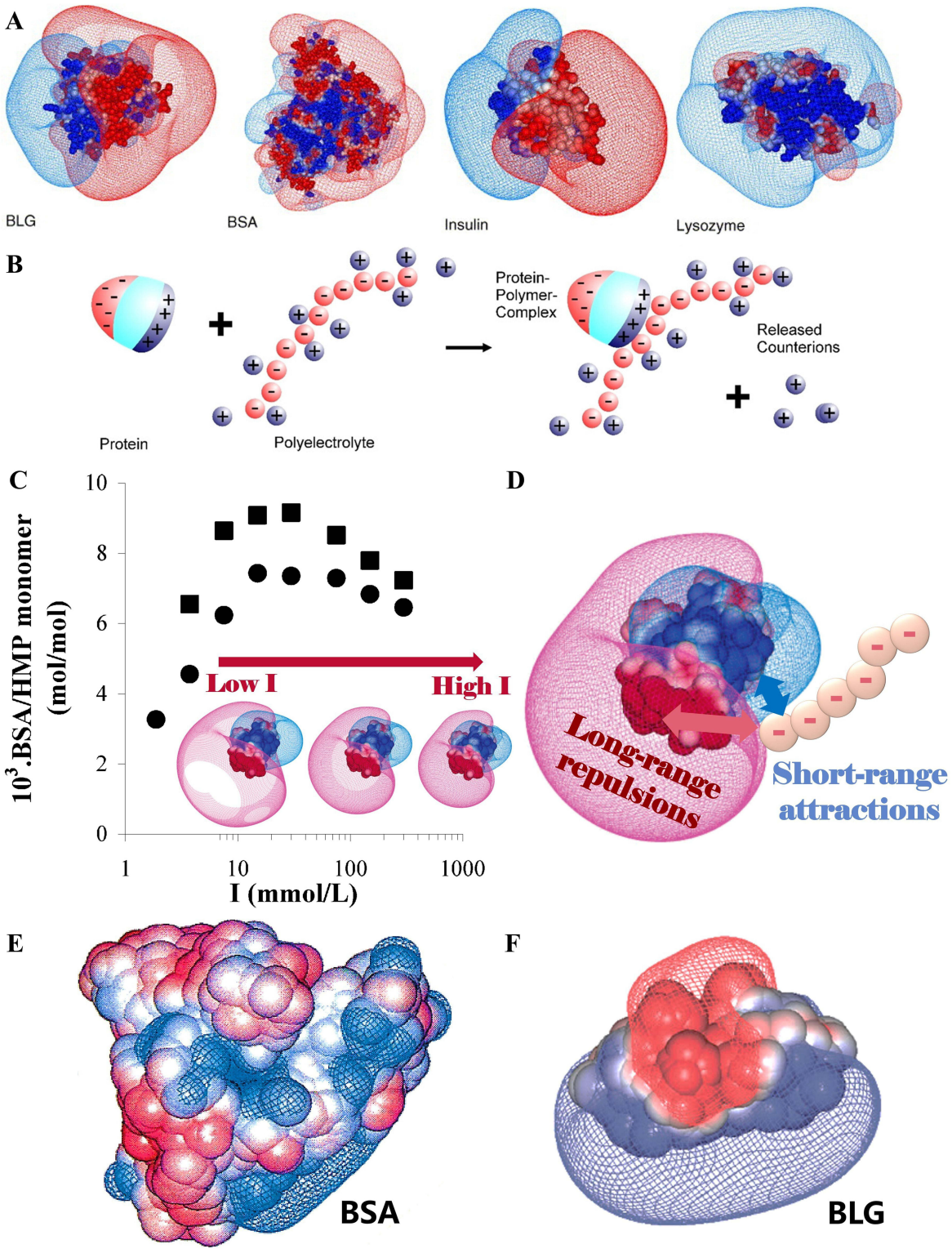


Figure 4 Interactions between proteins and polyelectrolytes. (**A**) Delphi images of electrostatic potentials for different proteins at pH 7. Adapted from Cooper CL, Dubin PL, Kayitmazer AB, Turksen S. Polyelectrolyte–protein complexes. *Curr Opin Colloid Interface Sci*. 2005;10(1):52–78. Copyright (2005), with permission from Elsevier.[111] (**B**) The combination process of polyanions and proteins with negative total charge. Adapted from Achazi K, Haag R, Ballauff M et al. Understanding the Interaction of Polyelectrolyte Architectures with Proteins and Biosystems. *Angew Chem Int Ed*. 2021;60(8):3882–3904. Creative Commons.[105] (**C**) Binding capacity of BSA on polyanions HMPAA and Delphi images of BSA from low I to high I; (**D**) Schematic presentation of long-range repulsions and short-range attractions between polyanion unit and BSA. Potential surfaces are colored red (negative charges) and blue (positive charges) Adapted with permission from Seyrek E, Dubin PL, Tribet C, Gamble EA. Ionic strength dependence of protein–polyelectrolyte interactions. *Biomacromolecules*. 2003;4(2):273–282. Copyright 2003, American Chemical Society.[113] Electrostatic potential contours (+0.5 (blue) and −0.5 (red) kT/e) around (**E**) BSA and (**F**) BLG at I=5 mM. Adapted with permission from Xu Y, Mazzawi M, Chen K, Sun L, Dubin PL. Protein Purification by Polyelectrolyte Coacervation: Influence of Protein Charge Anisotropy on Selectivity. *Biomacromolecules*. 2011;12(5):1512–1522. Copyright 2020, American Chemical Society.[114]

Thirdly, the protein charge anisotropy would impact the interactions between proteins and polyelectrolytes. Dubin et al studied the binding affinity of cationic polyelectrolyte, poly(diallyldimethylammonium chloride) (PDADMAC), for BSA and β-lactoglobulin (BLG).[114] The binding constant K was obtained by the isothermal titration calorimetry (ITC) method, and it was found that the binding constant for BLG (1900 ± 30) is around 3 times larger than that for BSA (740 ± 340). The DelPhi images showed the electrostatic potential contours around BSA and BLG at I = 5 mM (Figure 4E and F). The negative patches (red) are rather dispersed and attached to the surface of BSA tightly, while the negative domain of BLG (red) is intact and more extensive. Therefore, cationic polyelectrolytes would preferentially bind to BLG under the same condition.

Lipid–Protein Interactions

Cellular membranes are mainly composed of diversified lipids and membrane proteins. The lipid–protein interaction performs a crucial role in cellular biological functions.[117] For instance, lipids could interact with amyloid-beta (Aβ) peptides and lead to variation in Aβ aggregation, a major cause for Alzheimer's disease.[118] Inspired by such lipid–protein interaction, Qiu's group designed a polyphosphazene material poly[methoxy-poly(ethyleneglycol)/octadecylphospho-ethanolamine]phosphazene (PEOP) with hydrophobic branch octadecylphosphoethanolamine (OPA) to construct nano-vesicles mimicking bacteria membrane and explored its usage in oral cancer immunotherapy (Figure 5). Aqueous OVA solution was added to polymer DMF solution under stirring, and OVA-loaded polymersomes were obtained after 5 h dialysis. The FT-IR results indicated that there exist interactions between hydroxyl groups in OVA and amino groups in OPA, which contributed to the high OVA encapsulation efficiency of 70%. RGD peptide-conjugated cholesterol (RGD-Chol) was also synthesized and inserted into polymersome hydrophobic membrane to mimic the function of bacteria. The

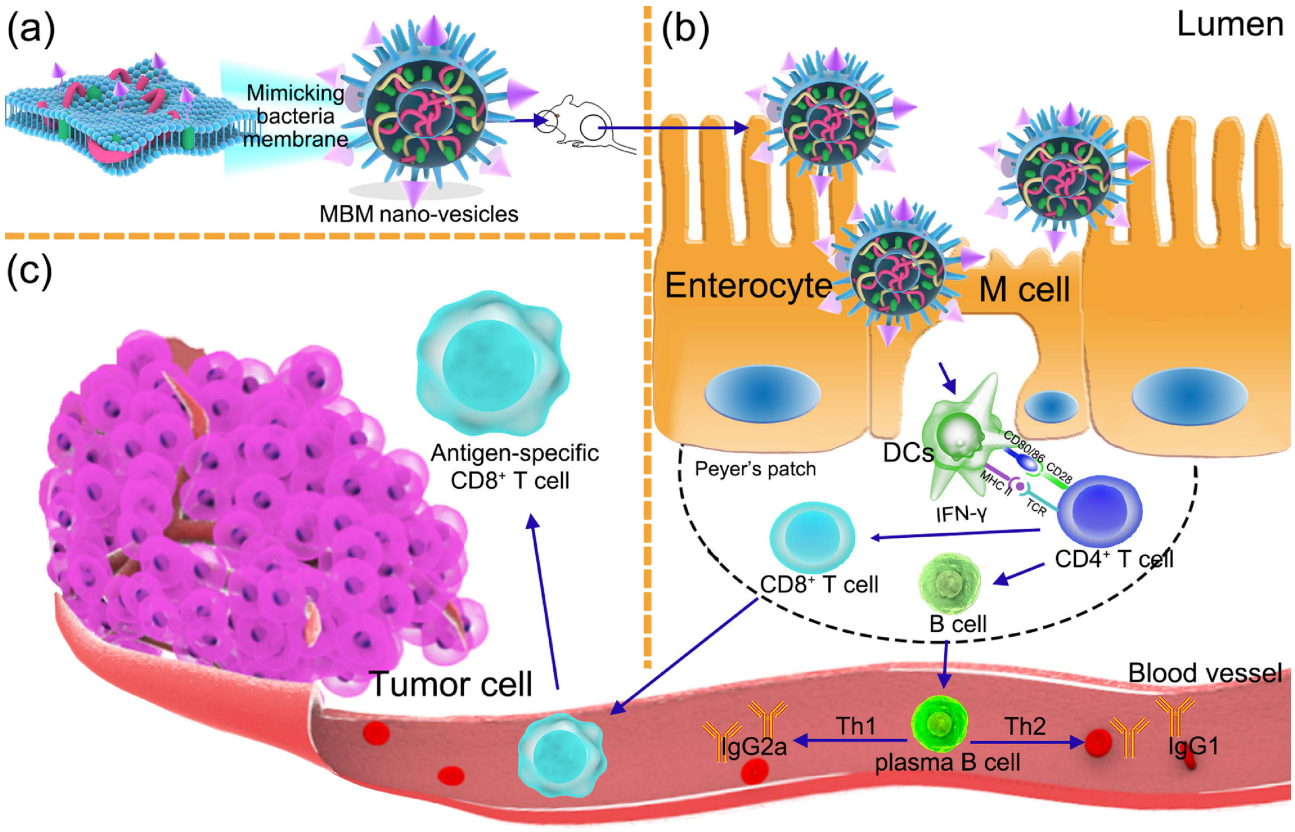


Figure 5 Schematic picture of the process that bacteria-mimicking vesicles activate immunization through oral administration. (**A**) The design of OVA/RGD-PEOP nano-vesicles. (**B**) The process of targeting to M cells, evoking the OVA-specific immune response in gastro-intestine tract. (**C**) The activated antigen-specific CD8+ T cells kill tumor cells. Reprinted from Shen Y, Hu Y, Qiu L. Nano-vesicles based on phospholipid-like amphiphilic polyphosphazenes to orally deliver ovalbumin antigen for evoking anti-tumor immune response. *Acta Biomater.* 2020;106:267–277. Copyright (2020), with permission from Elsevier.[101]

systemic immune response to OVA loaded RGD-modified polymersomes (OVA/RGD-PEOP) in vivo was investigated by detecting serum IgG antibody titers. The anti-OVA IgG1 and IgG2a antibody titers for OVA/RGD-PEOP were 5.8-fold and 6.0-fold higher than those for the free OVA group. In the in vivo antitumor test, the OVA/RGD-PEOP group achieved the most substantial tumor inhibition (69%) against E.G7-OVA cells compared to all other oral immune groups. Hence, the PEOP polymersomes represent a highly promising approach for oral antitumor immunotherapy.[101]

In another work by Qiu's group, insulin was encapsulated into the above polyphosphazene vesicles with a high encapsulation efficiency of 84.7% (Figure 6). Insulin aqueous solution at a concentration of 1 mg/mL was added into 10 mg/mL PEOP copolymer DMF solution, and the mixture was stirred for 20 min. The TEM images showed that the blank PEOP vesicles displayed a typical vesicle structure with a hollow core (Figure 6B), while the INS-PEOP vesicles appeared like solid nanoparticles with a shell (Figure 6C). After 12 h dialysis against distilled water, insulin-loaded vesicles were obtained. The strong molecule interactions, mainly hydrogen bonding between the lipid-like OPA and protein insulin, were verified by 2D ^{1}H NMR NOESY and FT-IR measurements. Due to the unique structure of polymersomes, insulin could be encapsulated both in the aqueous cavity and into the hydrophobic membrane. Such synergetic effect explains the high insulin loading properties of this polymersome. The in vivo hypoglycemic experiment demonstrated a gradual reduction in blood glucose levels upon oral administration of insulin-loaded PEOP vesicles, with a maximum decrease (up to 40%) observed at 6 h. The outcome proved that orally administered polymersomes could effectively protect the bioactivity of insulin (Figure 6D). According to the plasma INS concentration curves, the oral bioavailability of insulin encapsulated in PEOP polymersomes was 47.32 times higher compared to that of free insulin solution (Figure 6E). Further lymphatic transport fluorescence examination verified the insulin-loaded polymersomes could accumulate in the mesenteric lymph nodes and further release insulin into the systemic circulation through lymphatic transport.[29]

Based on these studies, they also encapsulated interferon-γ (IFN-γ) into the similar polymersome with an encapsulation efficiency of 85.1% (Figure 7). IFN-γ-loaded polymersomes (IFN-γ-EDP) were prepared by the solvent switch method. In vitro cell assays indicated obvious cell growth inhibition, strong cellular apoptosis and serious retardation of cell cycle. In vivo antitumor experiments were carried out in CT-26 cancer-bearing mice. The tumor inhibition rate of intravenous injection of IFN-γ-EDP followed by intraperitoneal injection of 5-FU (IFN-γ-EDP /5-FU) was 56.9%, compared to that of IFN-γ/5-FU (35.2%). This result suggested that polymersomes effectively protected IFN-γ from degradation during systemic circulation. Besides, CD4+ and CD8+ T cell analysis and cytokine qualification experiments

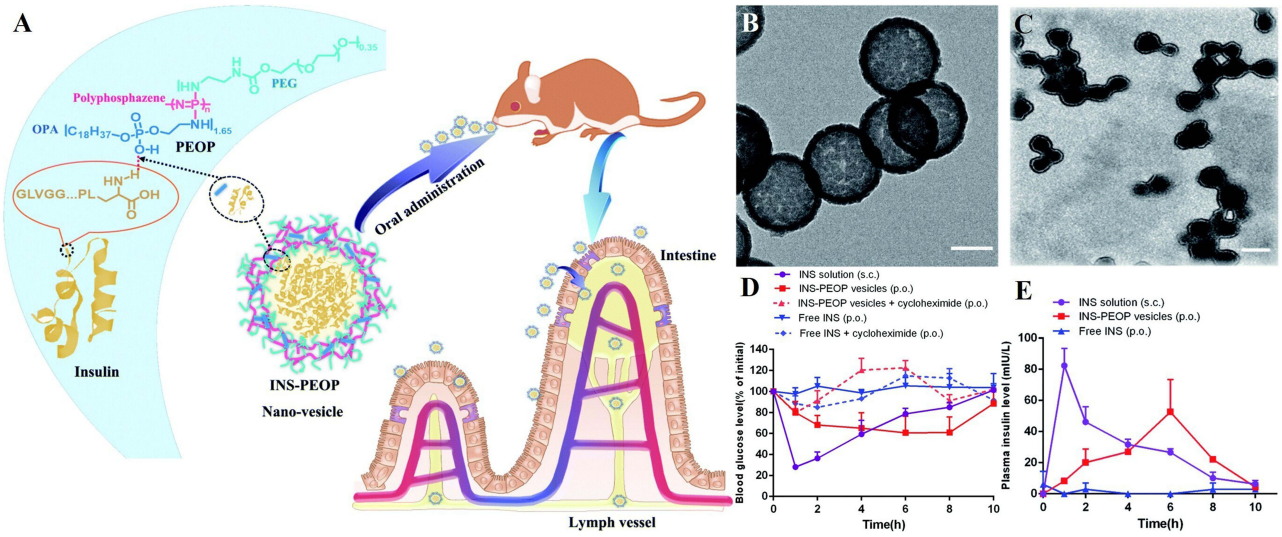


Figure 6 Polymersomes via intermolecular action to load and orally deliver insulin. (**A**) The formation of the INS-PEOP vesicles and applied for oral cure of diabetes mellitus. (**B**) TEM images of blank PEOP vesicles (scale bar 100 nm). (**C**) TEM images of INS-PEOP vesicles (scale bar 250 nm). (**D**) Variation of blood glucose levels of diabetic rats (nontreated and cycloheximide-treated rats) after orally administering INS-PEOP vesicles or insulin solution at dose of 100 IU kg-1, subcutaneous injection with insulin solution at 3 IU kg-1. (**E**) Variation of plasma insulin concentration of diabetic rats after orally administering INS-PEOP vesicles or insulin solution at dose of 100 IU kg-1, or subcutaneous injection with insulin solution at 3 IU kg-1. Reprinted from Hu Y, Wang J, Qiu L. Polymeric nano-vesicles via intermolecular action to load and orally deliver insulin with enhanced hypoglycemic effect. *RSC Adv.* 2020;10(13):7887–7897. Creative Commons.[29]

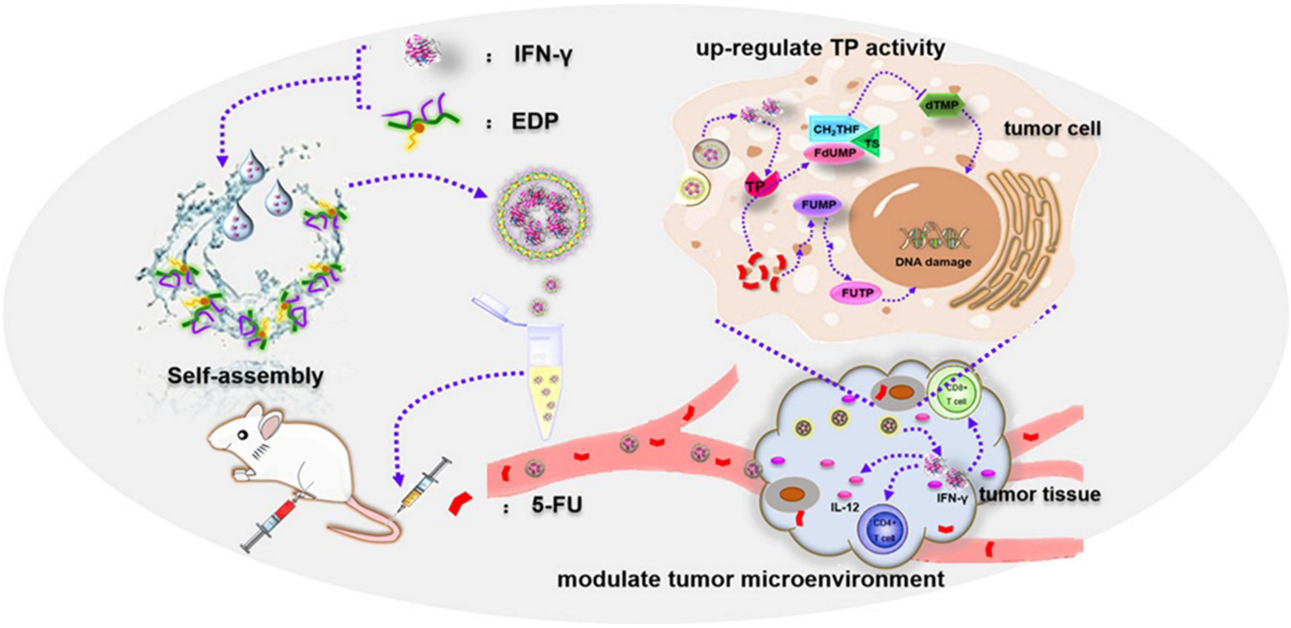


Figure 7 Scheme of improved anticancer effect of 5-FU when cooperatively used with IFN-γ. Reprinted from Peng Y, Hu Y, Qiu L. Vesicular IFN-γ as a cooperative attacker to enhance anti-cancer effect of 5-fluorouracil via thymidine phosphorylase upregulation and tumor microenvironment normalization. *Nanomed Nanotechnol Biol Med*. 2022;40:102,501. Copyright (2022), with permission from Elsevier.[28]

proved that IFN-γ-EDP effectively normalized the tumor microenvironment. This was achieved through increased CD4+ and CD8+ T cell populations, elevated IL-12 secretion, and suppressed IL-10 secretion within the tumor.[28]

Hydrophobic Interactions Introduced by Modified Proteins

Apart from innovations of polymers, an alternative approach is modifying proteins to arouse molecular interactions. In a recent study, enhanced green fluorescent proteins (eGFP) were genetically modified with hydrophobic peptides. 0.6 mL PMOXA-*b*-PDMS-*b*-PMOXA ethanol solution was injected into a 11.4 mL protein buffer to obtain polymersomes. Due to the hydrophobic interactions, the encapsulation efficiency could reach 25.7% for modified proteins compared to 0.18% for original eGFPs. Figure 8 indicates the presence of hydrophobic peptide-modified eGFPs on both the inner and outer vesicle surfaces.[119]

Innovations of Preparation Methods

Vesicles undergo different morphologies during their formation process. The formation mechanism will significantly affect the loading properties of protein and peptide drugs. If the internal aqueous chamber of the polymersomes is not easily accessible to the cargo, the final concentration of the drugs inside the polymersomes will be significantly lower than the external loading phase.[47] Therefore, several novel approaches have been explored to address this problem.

Improvements of Film Hydration Method

Due to the microscopic mechanism of the hydration process, the encapsulation efficiency of traditional film hydration methods tends to be low.[120] For instance, using the film hydration method, encapsulation efficiency of myoglobin by PEO-*b*-PBD was just 3.5%[120] while that of BSA by PEO-*b*-PEE was merely 5%.[71] To increase the loading limit of the film hydration method, Neil et al proposed an improved hydration method to encapsulate FITC-OVA into poly(ethylene glycol)-*b*-poly(propylene sulfide) (PEG-*b*-PPS) and named it the "direct hydration method" (Figure 9A). In this method, the hydrophilic polymer PEG500 was employed as a predispersed solvent for PEG-*b*-PPS. Initially, PEG-*b*-PPS was melted and mixed with PEG500 at 95°C. After the sample was cooled down, protein solutions were added, which caused PEG500 matrix to be rapidly hydrated. Such hydration generated an interconnected sponge phase that is quite essential for the improvement of encapsulation efficiency. The proteins are trapped throughout the hydrated polymer/PEG matrix

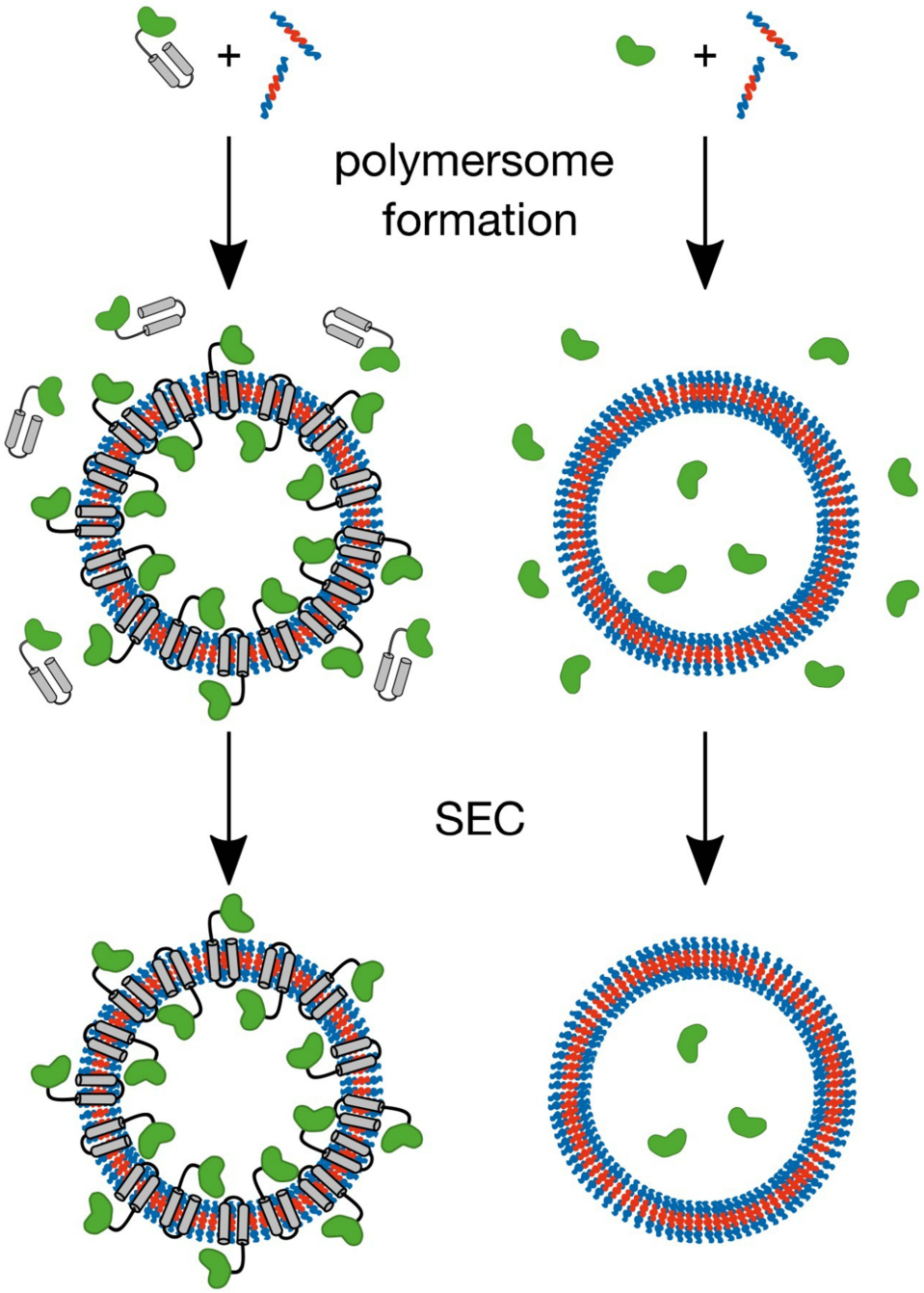


Figure 8 Comparison of protein distribution of genetically modified eGFPs and original eGFPs. Reprinted from Mertz M, Castiglione K. Increased Protein Encapsulation in Polymersomes with Hydrophobic Membrane Anchoring Peptides in a Scalable Process. *Int J Mol Sci.* 2021;22(13):7134. Creative Commons.[119]

prior to polymersome formation during the "direct hydration method", while encapsulation occurs only at the surface during the thin-film hydration method. An improved encapsulation efficiency of 37% for OVA was provided by "direct hydration method", compared to the 9% observed for the conventional thin-film hydration method.[104] However, there are two drawbacks of this method. Firstly, the loading content was quite low, which was only 0.24%. Secondly, the yield of this method is limited (less than 1 mL) and thus it is only suitable for small-batch production.

Based on the above "direct hydration method", Yewle et al proposed a "progressive saturation" method that could produce scalable protein loaded polymersomes (Figure 9B). There were two pivotal innovations for the "progressive saturation" method. Firstly, the protein solutions were added in a stepwise manner, ensuring complete encapsulation and

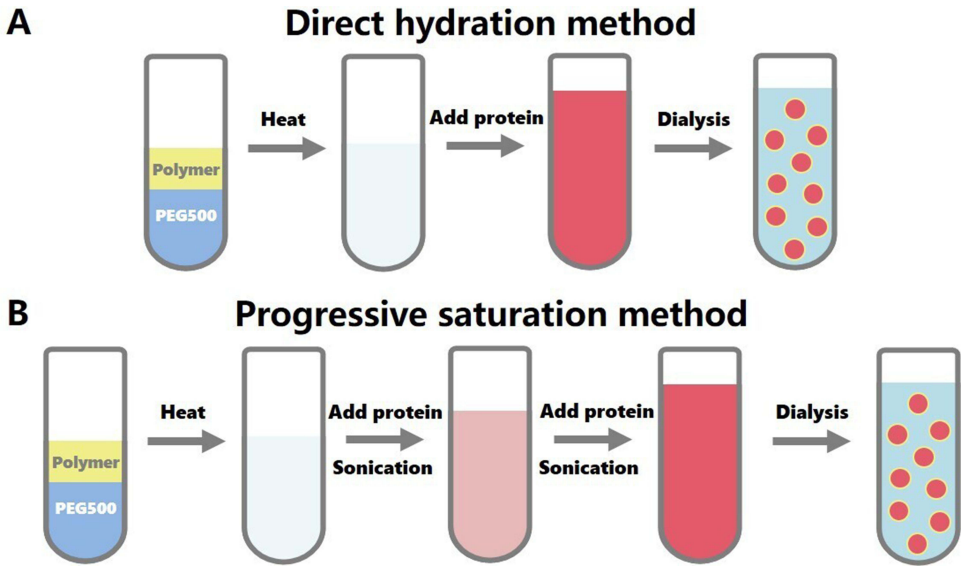


Figure 9 Schematic illustration of the process route of (**A**) direct hydration method and (**B**) progressive saturation method.

prompt thermodynamic equilibrium. Secondly, a sonication step was introduced and optimized after the addition of protein solutions, which facilitated the dispersion of the protein throughout the mixture. This method increased the final loading content of myoglobin to 4%. Several larger proteins, including hemoglobin, BSA, immunoglobulin G, catalase, fibrinogen, and apoferritin, were also tested and exhibited improved loading properties.[120]

Phase-Guided Assembly Method

For most encapsulation methods, drugs are usually loaded into polymersomes in a volume-based manner and these methods usually lead to low encapsulation efficiency. To address this issue, Zhang et al developed an encapsulation method where proteins were loaded by the thermodynamic driving force (Figure 10). The highlight of this method is that

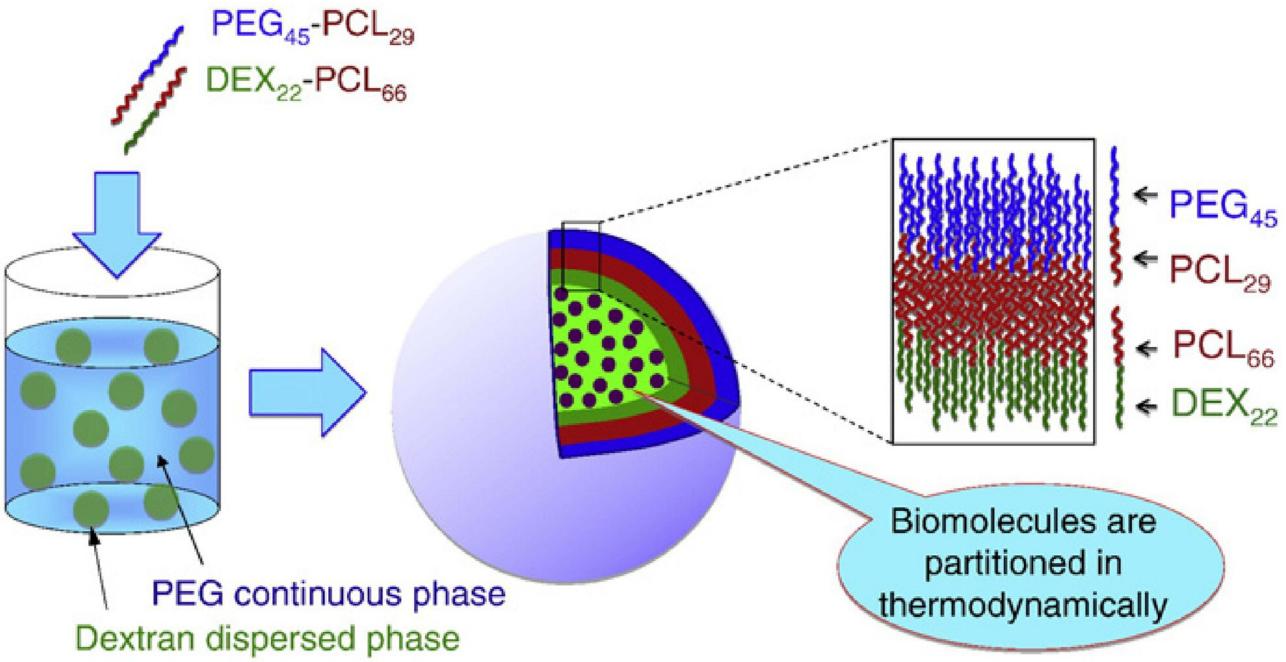


Figure 10 Schematic description of the preparation procedure and structure of polymersomes of asymmetrical bilayer membrane. Reprinted from Zhang Y, Wu F, Yuan W, Jin T. Polymersomes of asymmetric bilayer membrane formed by phase-guided assembly. *J Control Release*. 2010;147(3):413–419. Copyright (2010), with permission from Elsevier.[99]

it achieved high encapsulation efficiency through the partition coefficient difference for proteins in two phases. Two diblock copolymers, poly(ethylene glycol)-block-poly(ε-caprolactone) (PEG-b-PCL) and dextran-block-poly(ε-caprolactone) (DEX-b-PCL), were added to a dextran-in-PEG aqueous two-phase system. DEX-b-PCL formed the inner leaflet around the dispersed dextran phase, while PEG-b-PCL formed the outer leaflet, with the PEG block facing the PEG continuous phase. When the protein erythropoietin was introduced into the dextran phase, it was encapsulated with a pretty high loading efficiency (89%), which could be explained by its higher partition coefficient in the dextran phase than the PEG phase. Several experiments were carried out to confirm that the PEG block and the dextran block of the two amphiphilic diblock copolymers were aligned facing the two sides of the dextran/PEG interface, respectively. However, it should be noted that polymersomes formed by such phase-guided assembly method have a rather large diameter over 2 μm, and thus this method needs to be modified to prepare nano-sized polymersomes for intravenous administration of protein and peptide drugs.[99]

Conclusions and Perspectives

Loading proteins or peptides into polymersomes is the first but most important step to develop high-quality formulation products. Both the introduction of intermolecular interactions and the innovation of preparation methods offer viable means to improve the loading properties of polymersomes. To produce electrostatic interactions between polymers and proteins, various charged blocks have been used to effectively promote the loading capacities of polymersomes. The interaction between proteins and polyelectrolytes is influenced by several factors. To ensure a robust interaction, it is recommended to maintain the system ionic strength between 6 and 20 mM. Factors such as polymer structure, polyelectrolyte charge density, and protein surface charge distribution should also be taken into account. Besides, taking advantage of lipid–protein interaction, OVA, insulin, and IFN-γ could be loaded into polyphosphazene vesicles at high encapsulation efficiency. From the perspective of protein modification, hydrophobic interaction was established by utilizing genetically modified green fluorescent proteins as a typical case. Modifying encapsulation methods is another option to perfect loading properties. The difficulty lies in how to promote protein enrichment to the aqueous interior or hydrophobic membrane while maintaining the fine structure of polymersomes. This requires precise adjustments along the original thermodynamic path, as in the direct hydration method, progressive saturation method, and phase-guided assembly method.

It should be noted that the loading capacity of proteins and peptides is only an initial indicator for evaluating formulations, as conventional drug loading tests, like bicinchoninic acid assay (BCA),[121] fluorescence detection,[122] high-performance liquid-based chromatography (HPLC),[123] and the UV–vis[124] method, do not distinguish between active and inactive biomolecules. It is necessary to examine how much of the encapsulated biomolecules retain their biological activity, as only this portion can ultimately generate therapeutic effects. When choosing the encapsulation methods for biomolecules-loaded polymersomes, careful attention should be paid to the protection of payload structural integrity and biological activity. Relevant variables such as types of organic solvents, pH, and temperature need to be tuned cautiously because biomolecules are prone to unfold and destabilize when exposed to organic solvents, pH variations, and temperature fluctuations. It was reported that lipase, pig-liver esterase, and α-chymotrypsin lost approximately 96%, 75%, and 40% of their initial activity after 4 days of incubation in octane.[125] From this perspective, the film hydration method, in contrast to solvent switch, could protect the structural integrity of proteins and peptides more effectively, as it is free of organic solvent.

Compared with other nano-sized carriers, such as liposome, nanoparticle, and micelle, polymersome has a more complex and higher-order structure. Thus, delicate control over preparation parameters, like so-solvent composition and mixing rate,[126] is essential to achieve thermodynamic steadyness of a closed bilayer structure. Currently, there remain several manufacturing challenges that must be tackled for the commercialization of polymersomes. As for powder hydration and film hydration methods, prolonged exposure to intensive turbulence is needed for thorough hydration. This would lead to greater energy consumption and increased cost.[61] As for the solvent switch method, polymers dissolved in the first solvent are mixed and diluted by the second solvent. Therefore, extra concentration steps are required and production cost might be uneconomically increased. Microfluidics is a rapid and continuous polymersome preparation method. It could achieve homogeneous size distribution along with high throughput production via parallel

arrangement.[43] The overall cost of microfluidics is reduced as a result of the lowered energy consumption and diminished post-processing steps. Besides, the separation of free proteins and peptides following the encapsulation process is difficult and costly.[127] Such expenditure could be decreased by improving the loading capability of polymersomes.

Overall, polymersome is one of the most attractive supramolecular structures for therapeutic protein and peptide delivery. More detailed work needs to be done to realize therapeutic application of biomolecules-loaded polymersomes, including accurate assessment of loading properties and further increase in protein and peptide loading content. With more deep-going research and performance improvements, we believe polymersome-based protein and peptide formulations will exert predominant and unique effects for clinical use in the near future.

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Disclosure

The authors declare no competing interest in this work.

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