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Strategies and Research Progress of Gelation Methods in Maintaining the Stability of Protein Drugs

Hongyan Fan*, Pengfei Cui

School of Pharmacy, Changzhou University, Changzhou 213164, P. R. China

*Correspondence: fanhongyan1008@126.com.

Abstract

Proteins are promising biotherapeutics that offer advantages such as higher specificity, greater activity, and lower toxicity compared to traditional small-molecule drugs. However, due to their complex structures, proteins are prone to degradation and denaturation, which affects the safety and efficacy of drugs. Over the past few decades, lyophilized formulations, liposomes, and polymer nanoparticles have been commonly used for drug delivery, but they have significant limitations. Thus, there is an urgent need for novel carriers to address these issues. This review summarizes the unique advantages of hydrogels in improving protein stability, as they effectively reduce protein aggregation, oxidation, and enzymatic degradation through mechanisms such as confinement effect, hydration regulation, and microenvironment optimization. Studies have shown that gel systems constructed based on natural/synthetic polymers have achieved key applications in vaccine sustained-release carriers, long-acting protein drugs, and tumor-targeted therapy.

Keywords

Hydrogel; Protein; Stability; Gelation.

1. Introduction

Proteins play an irreplaceable role in modern medicine as therapeutic agents, and their unique biological activity and targeting have demonstrated invaluable therapeutic value in the modern disease treatment system. Protein-based therapies account for a large proportion of the current market. Since the launch of the first protein drug in 1982, various macromolecular drugs such as enzymes, hormones, coagulation factors, antibodies, and cytokines have been developed[1]. These drugs not only overcome the "undruggable" protein-protein interactions that are difficult to achieve with traditional small-molecule drugs but also break through multiple limitations in the treatment of complex diseases by virtue of their ability to specifically recognize disease targets, making them important therapeutic tools in fields such as tumors, metabolic diseases, and rare diseases[2]. However, protein drugs themselves have fragile structures, with poor physicochemical stability and high immunogenicity risks. Especially for oral administration, there are barriers such as gastrointestinal proteases, epithelial barriers, and efflux pumps, resulting in a bioavailability usually less than 1%[3]. Therefore, the vast majority of protein drugs have to rely on complex delivery systems or invasive administration routes (such as subcutaneous injection and intravenous infusion) to maintain effective therapeutic concentrations[4]. These technologies and processes not only significantly increase production costs and regulatory complexity but also limit patients' access to drugs and compliance, becoming a bottleneck that needs to be addressed for the next generation of protein drugs.

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In the past few decades, significant progress has been made in the development of protein delivery systems, but there are still some problems to be solved. The design and formulation of protein drug delivery systems should maximize the bioavailability of drugs, maintain their stability to avoid degradation, denaturation, or aggregation of protein molecules (which may lead to immunogenicity and loss of pharmacological activity after administration), and at the same time achieve targeted or on-demand precise release to enhance therapeutic effects[5, 6]. In view of this, delivery strategies such as liposomes, microspheres, polymer microneedles, and solid nanoparticles have emerged one after another. Compared with these delivery systems, hydrogels, with their highly hydrophilic environment, can significantly reduce hydrophobic interactions, thereby avoiding protein conformational changes that may be induced by liposome bilayers[7]. The combination of the physical barrier effect of their cross-linked network and the chemical bond-controlled release mechanism can effectively overcome the burst release phenomenon caused by residual organic solvents in polymer microspheres[8]. Meanwhile, their excellent biocompatibility can reduce the risk of immune reactions caused by some lipids in lipid nanoparticles[9]. In addition, hydrogels can precisely regulate the spatiotemporal release and localization of drugs, and their hydrolyzable or enzymatically degradable backbones can be gradually degraded into harmless small molecules (such as PEG, oligosaccharides, and amino acids) under physiological conditions, which are excreted from the body through the kidneys or hepatobiliary system, avoiding the long-term accumulation of carrier materials that may exist in other delivery systems[10]. These characteristics together lay the unique advantage of hydrogels in protein delivery systems.

This review provides an overview of several common protein delivery systems, as well as the unique stabilization strategies and advantages of hydrogels as delivery systems.

2. Factors Contributing to the Instability of Protein Drugs

The development of protein drugs is highly dependent on their stability. Instability may lead to reduced efficacy, increased safety risks, and pressures on production costs and storage challenges. The degradation mechanisms of protein drugs are complex and diverse, involving the influence of multiple factors such as physical, chemical, biological factors, and abnormal post-translational modifications.

2.1 Physical Factors

Most proteins cannot maintain their natural structures at high temperatures. High temperatures accelerate the thermal movement of protein molecules, destroy hydrogen bonds and hydrophobic interactions, leading to protein denaturation[11]. Many enzymes lose their activity at high temperatures. For example, the secondary structures such as α-helices and β-sheets in the three-dimensional structure of trypsin will change under high-temperature conditions, making it unable to bind to substrates normally and thus losing activity[12]. Although low temperatures are usually conducive to protein preservation, extreme low temperatures (such as freezing) may also cause protein instability. During freezing, water in the protein solution will freeze to form ice crystals. The growth of ice crystals may squeeze protein molecules, causing them to aggregate or denature[13]. Moreover, when proteins are thawed from the frozen state, the rapid temperature change may also cause changes in protein structure, so some protein drugs are prohibited from being re-thawed. Ultraviolet light can directly act on aromatic amino acids such as tryptophan and tyrosine in protein molecules, triggering photochemical reactions and generating reactive substances such as free radicals[14]. These reactive substances will attack other parts of the protein molecule, leading to protein oxidation, cross-linking, or fragmentation[15]. For example, some protein drugs containing tryptophan will be oxidized when exposed to ultraviolet light for a long time, and tryptophan residues may undergo deamination reactions, damaging the structure and function of the protein. The aggregation level of certain monoclonal antibodies increases significantly after exposure to ultraviolet light, resulting in decreased biological activity[16].

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Protein aggregation is one of the most common and serious stability problems of protein drugs. Protein molecules bind together through non-covalent interactions or partial covalent bonds to form oligomers, polymers, visible or insoluble particles. Aggregation mainly occurs in the form of amyloid fibrils. The aggregation of amyloid-β (Aβ), tau, or α-synuclein in the brain can lead to neurodegenerative diseases[17]. The aggregation of amyloid-β (Aβ) in the brain to form amyloid plaques is one of the main pathological features of AD. These plaques will interfere with the normal function of nerve cells, leading to neuroinflammation and nerve cell death[18]. The aggregation of α-synuclein in nerve cells not only forms Lewy bodies but also causes synaptic dysfunction, thereby inducing PD[19]. The abnormal phosphorylation of tau protein leads to conformational changes, resulting in the loss of its ability to polymerize with tubulin and impaired function. The free tau protein in the cytoplasm interacts to form insoluble straight filaments and paired helical filaments, eventually forming neurofibrillary tangles (NFTs), which in turn leads to damage to the structure and function of nerve cells[20]. This aggregation will cause damage and dysfunction of nerve cells. The aggregation-prone regions (APRs) on the protein structure interact through β-strands, and the interaction between the same or homologous APRs is the most common structural mechanism driving protein aggregation.

2.2 Chemical Factors

The structure and function of proteins are closely related to the pH environment in which they are located. Changes in pH will not only change the dissociation state of many dissociable groups in protein molecules, such as carboxyl groups (-COOH) and amino groups (-NH_2) of amino acid residues, affecting the charge distribution and spatial structure of protein molecules but also may change the structure of protein active sites, thereby affecting their biological activity[21]. For example, pepsin has optimal activity in an acidic environment (pH about 1.5-2.5), while its structure will change and lose activity in a neutral or alkaline environment[22].

Protein oxidation refers to the reaction of proteins with reactive oxygen species (ROS) or other oxidants, mainly affecting protein side-chain residues. Reactive oxygen free radicals such as superoxide anions, hydroxyl radicals, and hydrogen peroxide can directly attack amino acid residues of proteins, such as cysteine and methionine, triggering chain reactions, leading to protein folding, aggregation, or fragmentation[23]. This will affect their functions as receptors, enzymes, carriers, or structural proteins. For example, α_1-antitrypsin is an important serine protease inhibitor. The active center of α_1AT contains a key methionine residue (Met358), which plays a crucial role in inhibiting proteases. Oxidation can oxidize Met358 to methionine sulfoxide (MetSO), making it unable to correctly embed into the active site of elastase, further reducing its inhibitory activity[24]. At the same time, protein drugs may undergo chemical modifications under oxidative conditions to form new epitopes. These oxidatively modified epitopes may be recognized by the immune system as new antigens, thereby causing immune reactions[25]. In some cases, oxidatively modified drugs can break immune tolerance and induce the production of ADA[26]. In the development process of protein drugs, it is necessary to consider the impact of oxidative modification on immunogenicity, reduce the production of ADA, and improve the safety and efficacy of drugs.

PTMs refer to the chemical modifications carried out on the amino acid side chains or terminals of proteins after synthesis to regulate their structure, function, and interactions. It mainly involves abnormal phosphorylation caused by the addition of phosphate groups to specific amino acid residues, methylation, acetylation, and dysregulation of protein degradation mechanisms caused by ubiquitin molecules marking proteins for degradation[27]. Abnormal post-translational modifications can lead to the occurrence of neurodegenerative diseases.

2.3 Biological Factors

Protein drugs may encounter proteases in the body or during storage. Protein enzymatic hydrolysis is the process of decomposing proteins into peptides or amino acids through trypsin, pepsin, and chymotrypsin. Insulin, which we use daily, is generally administered by injection. Oral administration will be degraded by extreme pH values and pepsin in the gastrointestinal tract, resulting in a

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bioavailability of less than 1%[28]. Therefore, its stability in the body is poor. During the storage of protein drugs, the growth and metabolic activities of microorganisms will have a negative impact on the stability of protein drugs. If microbial contamination exists, proteases secreted by microorganisms may also degrade protein drugs. During the production process of protein drugs, it is generally carried out in a sterile environment. If sterile control fails, proteases or metabolites secreted by microorganisms will destroy the structure of proteins, posing a serious threat to product quality and safety.

Protein instability is the result of the synergistic effect of multiple mechanisms. In the process of drug research and development, it is necessary to formulate reasonable strategies to address these instability pathways on the basis of understanding these mechanisms to improve the safety of drugs.

3. Common Protein Delivery Systems

3.1 Polymer Microneedles

Due to the poor oral bioavailability of protein drugs, transdermal drug delivery systems using microneedles have attracted wide attention. Polymer microneedles are a new type of transdermal drug delivery technology (Figure 1). By encapsulating drugs in polymer microneedles and penetrating the stratum corneum of the skin through tiny needle-like structures to form micron-scale channels, protein drugs are directly delivered to the deep skin or blood circulation to achieve therapeutic effects[29]. This administration method combines the efficient delivery capacity of injection and the painlessness and convenience of transdermal patches[30].

Different materials used to prepare microneedles determine their different applications and characteristics, including solid microneedles, coated microneedles, dissolvable microneedles, and hollow microneedles[31]. Due to the rich variety of polymer materials and low cost, polymer materials have good biocompatibility and biodegradability, so polymer microneedles have become a key research object in the field of medical microneedles[32]. Harit et al. prepared four-point star-shaped microneedles using photolithography[33]. These microneedles are composed of non-degradable hydrogels, forming a hydrogel network by combining sulfobetaine (SPB) monomers with dextran-glycidyl methacrylate/acrylic acid. Zwitterionic polysulfobetaine (poly-SPB) was incorporated into the microneedles, which can effectively inhibit protein aggregation. Experiments have shown that the microneedles have high drug-loading capacity and efficient drug release rate, and exhibit sufficient mechanical strength. Even under external pressure, the proteins in the microneedles can remain stable, be released in their natural state, and have no loss of activity. This technology can be used to effectively deliver sensitive therapeutic drugs or vaccines, and is suitable for untrained people with better compliance. After the drug is injected into the body, in order to maintain a stable blood drug concentration and achieve continuous administration, the drug release rate must be controlled. The microneedles mentioned in the above article are prepared based on non-degradable hydrogels. Although they have sufficient mechanical strength, the dissolution rate of this material on the skin may need more precise regulation. If the dissolution rate is too fast, it may lead to premature drug release, affecting the controlled release effect of the drug; if the dissolution rate is too slow, it may affect the user experience of the microneedles and the bioavailability of the drug. Concetta Di Natale[34] et al. developed a dual-chamber microneedle system containing two independent chambers. One chamber is used to load collagenase, and the other chamber is used to load auxiliary components (stabilizers or buffers). Microneedles are prepared using biodegradable polymer materials (polylactic-co-glycolic acid copolymer, PLGA), which have good biocompatibility and degradability and can be gradually decomposed in the body. Experiments have shown that the dual-chamber microneedles can effectively protect collagenase from degradation, and the activity retention time of collagenase is significantly prolonged during storage. By adjusting the composition of the polymer and the structure of the microneedles, the continuous release of collagenase at a stable rate within a certain period of time is achieved. It also shows good skin care effects, which can effectively improve skin elasticity and reduce wrinkles and scars.

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Microneedle technology provides a new and effective solution for the transdermal delivery of protein drugs, which is expected to improve patients' treatment experience and compliance. By designing microneedles of different shapes and sizes to optimize penetration capacity and drug-loading capacity[35], but high-dose drugs require increasing the density of microneedle arrays, which affects skin tolerance, and cannot deliver drugs that require high systemic concentration distribution[36], mainly suitable for local or moderate low-concentration administration; for proteins administered in milligram levels, the size of microneedle patches may be too large to be practical[37]. In future research, it is necessary to further study the stability of microneedles during long-term storage, especially under different environmental conditions, as well as the problems of puncture efficiency and consistency of drug release affected by individual differences and local inflammatory reactions caused by long-term use.

3.2 Nanoparticle Delivery Systems

Nanoparticle delivery systems usually refer to a delivery technology that uses nanoparticles as carrier materials to deliver therapeutic drugs such as organic small molecules and biomacromolecules to specific parts of the body. Lipids, polymers, and inorganic nanoparticles are used as carrier materials for drug delivery (Table 1). Proteins are loaded on the carrier materials by strategies such as embedding proteins inside nanoparticles or adsorbing them on the surface of nanoparticles through electrostatic or hydrophobic interactions[38,39]. Moreover, the size, surface charge, and composition of nanoparticles can be controlled to adjust the drug-loading capacity and release rate. PLGA nanoparticles are prepared from polylactic-co-glycolic acid copolymer, which has biodegradability, sustained release, and low toxicity, but may have problems such as burst release effect and low cell uptake efficiency caused by surface hydrophobicity[40]. Lu[41] et al. modified the surface of PLGA with chitosan through electrostatic adsorption or chemical cross-linking to form a core-shell structure, which improved the drug encapsulation efficiency. Moreover, chitosan modification significantly reduced the initial burst release effect, achieving a more stable and sustained drug release. The cationic surface charge promotes the interaction with negatively charged cell membranes, improving the efficiency of cell uptake. In vitro cytotoxicity experiments also show low toxicity, which is suitable for long-term administration. As a peptide drug, insulin faces problems such as gastric acid degradation and first-pass effect when administered orally. Zou[42] et al. developed a new type of oral insulin delivery system, using folate-modified metal-organic framework nanoparticles (Folate-MOF NPs) to achieve efficient delivery of insulin, improving the bioavailability and therapeutic effect of insulin. As a targeting ligand, folic acid can enhance the targeting of nanoparticles to intestinal cells. Insulin is loaded into the pores of MOF nanoparticles through physical adsorption. The combination of FA and MOF (PCN-777) nanoparticles not only selectively enhances the intestinal transport efficiency of diabetic animals by upregulating the endocytosis mediated by intestinal FA transporters but also regulates the decomposition of PCN-777 in the phosphate-rich blood environment to maintain long-acting basal insulin release kinetics within a narrow therapeutic range. In diabetic animal models, after a single oral administration of folate-MOF-insulin, the blood glucose decreased significantly within 2-4 hours and maintained for more than 12 hours, with a relative bioavailability of 35%. This represents a potential long-acting oral formulation that can reduce the risk of hypoglycemia.

Traditional carriers such as liposomes may have unstable phenomena such as lipid oxidation, hydrolysis, leakage, aggregate formation, and even liposome fusion during preparation, storage, and further administration[43], leading to premature recognition and degradation of carriers in the blood, premature drug release or inactivation. Several strategies for developing protein-loaded nano-carriers often enhance drug encapsulation to improve stability, which may limit the protein release rate or require strong external stimulation to release[44]. Secondly, the complex interaction between nano-carriers and the biological environment makes it difficult to achieve precise tissue or organ targeting, resulting in drug distribution in non-target tissues and side effects[45].

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3.3 Microsphere Delivery Systems

Microspheres are spherical particles made of natural or synthetic polymer materials with a particle size usually of 1-1000 μm. They load proteins through physical encapsulation or chemical binding to achieve controlled release[46]. Microspheres can protect protein drugs from degradation by gastrointestinal enzymes, improve their oral bioavailability. After surface modification with targeting ligands or antibodies, they can achieve targeted delivery to specific tissues or cells, increase the drug concentration at the target site, and reduce side effects on normal tissues[47]. PLGA is a polymer with self-healing properties, which can be used to prepare porous microspheres widely used in drug delivery and sustained release of biomacromolecules[48]. Yuyoung Kim[49] et al. prepared similar sponge-structured PLAG microspheres by ammonolysis single emulsification microencapsulation. These microspheres have high porosity and uniform interconnected pores. Different from traditional solid or closed-pore PLAG microspheres, pre-formed porous microspheres are mixed with an aqueous solution containing lysozyme, and the pores of the microspheres are closed through a self-healing process to encapsulate lysozyme. Studies have shown that the porous structure of sponge microspheres provides a large specific surface area for protein adsorption, which can achieve high drug-loading capacity, and the through channels allow uniform distribution of proteins, avoiding surface enrichment, and the burst release rate is also lower. Since the protein is directly adsorbed on the pore walls, the contact between water and oil interfaces is reduced, and the mild low-temperature process reduces thermal/chemical damage. The activity of lysozyme released from sponge microspheres is retained by 95%. Compared with traditional microspheres, the activity retention rate has increased by more than 65%. In addition to improving their performance by optimizing the composition and structure of microspheres, modifying the surface of microspheres can maintain the activity, biocompatibility, and safety of proteins during preparation. Huang et al.[50] proposed an innovative drug-loaded core-shell microsphere (RPG@FN) prepared by electrospray technology, which encapsulates resveratrol (Res) with a shell layer of poly(lactic-co-glycolic acid) (PLGA) and a core composed of polycaprolactone-polyethylene glycol (PCL-PEG) micelles, and is surface-modified with fibronectin (FN). The results show that RPG@FN microspheres can achieve the sustained release of fibronectin and resveratrol. In vitro experiments have proved that they can promote the proliferation and migration of alveolar epithelial cells, and at the same time inhibit the secretion of inflammatory factors by macrophages, showing good cytocompatibility and biological activity.

Polymer microspheres are widely used for the encapsulation and sustained release of protein drugs. However, the degradation of PLGA in the body will produce an acidic microenvironment, which may have an adverse effect on the encapsulated protein drugs, especially for some environment-sensitive proteins, such as bovine serum albumin (BSA). This acidic microenvironment may lead to protein degradation and inactivation[51]. By adjusting the composition ratio of PLGA or modifying its surface, it may be possible to enhance the stability and release efficiency of microspheres[52].

3.4 Lipid Nanoparticles

Lipid nanoparticles are a class of nano-scale drug delivery systems composed of physiologically compatible and biodegradable lipid materials, with good drug-loading capacity. They are mainly divided into solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). The particle size is usually between 10-1000 nanometers, allowing the encapsulation of hydrophilic or hydrophobic drugs with higher encapsulation efficiency than liposomes.

Traditional LNPs are mostly designed for nucleic acid delivery, and the formulation needs to be re-optimized to adapt to the encapsulation of macromolecular protein complexes. The clinical application of gene editing tools such as CRISPR-Cas9 is limited by delivery efficiency, safety, and immunogenicity. Therefore, Wang et al. developed an efficient gene editing protein delivery system based on bioreducible lipid nanoparticles[53]. The lipid molecules of bioreducible lipid nanoparticles (bLNPs) contain disulfide bonds, which can be degraded in the reductive environment inside cells to release the loaded Cas9 protein or ribonucleoprotein complex (RNP), realizing the direct delivery of

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proteins rather than DNA/RNA, avoiding the risk of gene integration. The degraded products are non-toxic, reducing the long-term accumulation risk of traditional liposomes. By optimizing the LNP formulation, the efficiency and specificity of gene editing can be improved, while reducing potential side effects. Brown et al. designed a series of new nanoparticle formulations, optimized the physicochemical properties of LNPs by adjusting the ratio of cationic lipids, helper lipids, and PEGylated lipids, mixed RNP with the optimized LNPs to form stable nanocomplexes for in vivo delivery[54]. The optimized LNP formulation achieved up to 40% base editing efficiency in mouse livers, with off-target efficiency lower than that of traditional Cas9-RNP. Compared with mRNA-LNP, RNP delivery did not activate the TLR or RIG pathway, significantly reducing the level of inflammatory factors. The degradable lipid design promotes rapid intracellular release, reducing protein degradation caused by lysosomal retention.

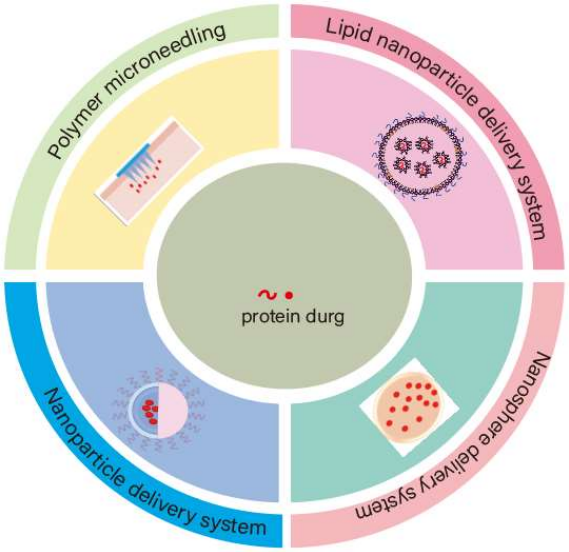

Figure 1. Schematic Diagrams of Different Delivery Systems

Table 1. Protein Drugs Loaded by Different Delivery Systems

Protein	Method	Changes in Major Indicators	References
Insulin	Nanoparticles	Folate-modified metal-organic framework nanoparticles enhance the targeting of nanoparticles to intestinal cells.	[13]
Collagenase	Polymer microneedles	Dual-chamber microneedles can effectively protect collagenase from degradation and prolong the activity time.	[10]
Cas9 protein	Lipid nanoparticles	By adjusting the ratio of cationic lipids, helper lipids, and PEGylated lipids, up to 40% base editing efficiency is achieved, with off-target efficiency lower than that of traditional Cas9-RNP.	[21]
GLP-1 analogs, vaccine antigens	Microspheres	The high porosity and uniform interconnected pores of sponge microspheres achieve high drug-loading capacity and low burst release.	[18]

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At present, the limitation of nano-carriers lies in the complex interaction with the biological environment, resulting in efficient editing still limited to target organs such as the liver and spleen. Delivery to tissues such as the brain and muscles requires local injection or higher doses. For example, Bio-LNPs may rely on passive targeting and lack the ability to actively target tumors or specific cell types, limiting their application efficiency in non-hepatic tissues. The disulfide bonds in bioreducible lipids may be prematurely degraded by redox substances in plasma during blood circulation, leading to problems such as drug leakage or nanoparticle disintegration, which need to be solved.

4. Stabilization Strategies of Gel Systems

Hydrogels are polymer materials with a three-dimensional cross-linked network structure. The three-dimensional network structure can fix a large amount of water and at the same time endow the material with a certain elasticity and mechanical strength[55]. The cross-linked nature of hydrogels is conducive to protein transport because it can not only prevent large foreign molecules from interacting with encapsulated proteins but also its high water content environment can simulate the extracellular matrix environment, reducing hydrophobic aggregation of proteins. In hydrogels simulating the ECM environment, the aggregation rate of monoclonal antibodies can be controlled below 5%, while in liposomes, it may exceed 15%[56]. This helps maintain the active form of proteins and reduce their vulnerability to chemical degradation[57], showing higher stability (Table 2).

Protein drugs are encapsulated in hydrogels through in-situ encapsulation technology, which can avoid damage to protein activity by organic solvents and high temperatures[56]. For example, when using photo-crosslinked hyaluronic acid hydrogels to load lysozyme, the activity retention rate can exceed 95%, avoiding damage to proteins by traditional methods. Moreover, by precisely controlling and adjusting the photo-crosslinking conditions and the concentration of hyaluronic acid, the mechanical properties and biodegradability of hydrogels can be regulated, which not only improves the stability of protein drugs but also provides more precise control for drug delivery and release[58].

4.1 Modification of Hydrogels

Common synthetic gels are formed by chemical cross-linking of synthetic polymer materials, including polylactide, polyacrylate, polyacrylamide, etc. They are synthesized by introducing covalent bonds using chemical cross-linking agents and initiators. The gels have good mechanical strength and stability but may cause additional cytotoxicity[59]. Natural hydrogels are cross-linked by natural polymer materials such as hyaluronic acid, chitosan, alginate, collagen, and nucleic acids, which have good biocompatibility, biodegradability, and non-immunogenicity (Table 3) [60,61]. The stability of hydrogels can be enhanced and their applications in the biomedical field can be expanded by modifying their structure through various physical or chemical methods or selecting appropriate cross-linking strategies and cross-linking agents[62]. Chen et al. synthesized poly(lactic-co-glycolic acid)-b-polyethylene glycol-b-poly(lactic-co-glycolic acid) (PLGA-PEG-PLGA) triblock copolymer, and then prepared an injectable, thermosensitive hydrogel system loaded with Herceptin to reduce the risk of local recurrence of breast tumors after breast-conserving surgery and minimize systemic side effects[63]. By adjusting the PLGA/PEG ratio, zero-order release of trastuzumab was achieved within 80 days, which is the longest reported sustained release cycle of HER2 antibodies. Yang et al. mixed dopamine-modified silk fibroin with classic thermosensitive hydrogels, chitosan, and β-glycerophosphate sodium, and then encapsulated zeolitic imidazolate framework-8 (ZIF-8@QCT) to form an injectable composite hydrogel (SFD/CS/ZIF-8@QCT). The gel shows good antibacterial, immunomodulatory, pro-osteogenic/angiogenic, and pro-recruitment properties, providing a new therapeutic strategy for the repair of periodontitis-induced bone defects[64].

Designing and developing double-network gels. Double-network gels have significant advantages: the first network provides rigidity, and the second network enhances toughness, significantly improving the strength and toughness of the gel[65]. By introducing dynamic cross-linking points and reversible chemical bonds, double-network gels can achieve self-healing after mechanical damage[66]. Professor Sun Wei's team[67] innovatively used the ALG/PEGDA network to improve

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the mechanical properties and network density of the hydrogel, and added the positively charged monomer 2-(acryloyloxy)ethyltrimethylammonium chloride (AETAC) to generate electrostatic interactions with the electronegative anti-human papillomavirus (HPV) protein (3-hydroxyphthalic anhydride-modified bovine β-lactoglobulin) to achieve the effect of delayed release. The ALG/PEGDA-AETAC DN hydrogel uses the principle of affinity-controlled release and the dense network structure of the double-network material, which can not only achieve long-term and stable release of antiviral proteins but also has high stability, good mechanical strength, and flexibility.

4.2 Structural Engineering Strategies of Hydrogels

Precisely design the structure of gels through 3D printing or microfluidic technology to design hierarchical porous structures, optimizing their ability to protect protein stability, control release kinetics, and adapt to complex biological environments[68]. 3D printing technology can prepare personalized hydrogel scaffolds and hydrogels with complex and highly geometric structures by designing models and adjusting printing parameters. Specific structures can promote cell adhesion, proliferation, and differentiation[69]. Dong et al. prepared 4-arm polyethylene glycol (4armPEG)-OPA/gelatin hydrogels loaded with bone morphogenetic protein 2 (BMP2), and incorporated the hydrogels into 3D-printed PLAG scaffolds. The hydrogels form a porous network at low concentrations, which is used for local and sustained release of BMP2 to repair large segmental bone defects[70]. The sustained release effect lasts for several weeks, inducing osteogenic differentiation of bone marrow mesenchymal stem cells and promoting bone repair, providing a new therapeutic method for bone defects.

4.3 Design of Multifunctional Hydrogels

Smart responsive hydrogels can quickly sense and respond to changes in the internal and external environment, thereby producing changes such as high hydrophobicity, ionization, and conformational changes, intelligently releasing drugs, and improving the controllability and targeting of drug release[71]. The team of Cai Zhongyu from Beihang University[72] designed a stimulus-responsive hydrogel based on proteins/peptides. Protein/peptide sequences with specific responsiveness were designed through genetic engineering or chemical modification, and functional groups such as sulfhydryl groups and photosensitive groups were introduced to enhance responsiveness. These hydrogels can respond to various stimuli and real-time feedback the degradation of the gel and the release of proteins through optical or electrical signals.

In addition to releasing drugs through intelligent response systems, dynamic microenvironment regulation can also be achieved by changing the microenvironment inside the gel, such as real-time adjusting the pH, ionic strength, or redox state of the gel, which will play a key role in optimizing protein function and activity and achieving targeted delivery[73-75]. Zhang et al. constructed a dynamic Schiff base cross-linked hydrogel platform with "dynamic response + dual-drug regulation" and phase-adaptive regulation functions for efficient repair of chronic wounds infected with drug-resistant bacteria and significant inhibition of scar formation[76]. The hydrogel can release different active ingredients according to the wound healing stage, realizing programmed regulation of chronic infected wounds. By releasing ε-polylysine and nano-cerium enzymes, it effectively inhibits the growth of multidrug-resistant bacteria and the formation of biofilms, promotes the formation of a regenerative environment, and solves the problem of difficult healing of chronic wounds. Excessive oxidative substances in cells can cause oxidative stress. Free radicals and other active oxidants in cells will damage biomolecules such as proteins, lipids, and DNA, causing cell damage, tissue aging, and the development of various diseases[77].

Hydrogels can inhibit inflammatory reactions and reduce the production of ROS by integrating antioxidants or reactive oxygen scavenging materials, thereby alleviating symptoms and preventing the progression of oxidative stress-related diseases[78]. For example, diabetic wounds are difficult to heal due to hyperglycemia, easy infection, hypoxia, accumulation of reactive oxygen species, and chronic inflammation, and traditional therapies are difficult to solve multiple pathological factors at the same time. Tu et al. prepared an antibacterial hydrogel with ROS scavenging, O2 generation, and

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NO production by cross-linking PEGMA-GMA-AAm copolymer containing epoxy groups with HBPL attached to MnO2 nanosheets, in which pravastatin sodium was loaded. The hydrogel can kill bacteria in MRSA-infected diabetic skin wounds and alleviate inflammation in vivo, and significantly promote wound healing without obvious scars[79].

4.4 Multifunctional Synergistic Therapy

4.4.1 Drug-Cell Co-Delivery

Cardiac cell therapy, as an emerging therapy, aims to repair damaged myocardial tissue through cell transplantation. However, the low survival rate and retention rate of transplanted cells severely limit its efficacy[80]. Injectable hydrogels have become a key strategy for optimizing cell delivery because they can simulate the biophysical and biochemical properties of the extracellular matrix (ECM). By providing a three-dimensional supportive microenvironment, they significantly promote cell adhesion, proliferation, and differentiation. The team of Cecilie Hoeeg[81] encapsulated mesenchymal stem cells (MSCs) in functionalized gelatin hydrogels containing arginine-glycine-aspartic acid (RGD) peptides. This design not only significantly improves the survival rate and myocardial retention rate of MSCs but also enhances their paracrine ability of vascular endothelial growth factor (VEGF) by activating the integrin signaling pathway, thereby synergistically promoting angiogenesis and myocardial repair. In the myocardial infarction model, the treatment with MSCs encapsulated in this RGD-gelatin hydrogel showed significant efficacy, and the infarct area was significantly reduced by 35%. This result confirms that the RGD-modified gelatin hydrogel not only provides a bionic microenvironment for MSCs to maintain their activity and function but also directly drives angiogenesis and tissue regeneration by enhancing VEGF secretion.

4.4.2 Immune Cell Recruitment

Table 2. Core Characteristics and Advantages of Protein Drugs Loaded by Hydrogels

Strategy	Hydrogel System	Example	References
Stability protection	High water content environment (>90%) maintains the natural conformation of proteins	The aggregation rate of monoclonal antibodies can be controlled below 5%.	[42]
Release precision	pH/enzyme/light multiple responses	Stimulus-responsive hydrogels based on proteins/peptides can respond to various stimuli and real-time feedback the degradation of the gel and the release of proteins through optical or electrical signals.	[53]
Local retention	In-situ gelation forms a drug reservoir to achieve long-term stable release	The ALG/PEGDA-AETAC DN hydrogel uses the principle of affinity-controlled release and the dense network structure of the double-network material, which can achieve long-term and stable release of antiviral proteins.	[50]
Biocompatibility	Degradation products are sugars/amino acids (non-toxic) with low immunogenicity	The inflammation score of silk fibroin hydrogels in the subcutaneous implantation model is significantly lower than that of traditional synthetic materials.	[10], [46]
Synergistic therapy potential	Hydrogels loaded with multiple antigens and immunomodulators promote in situ recruitment of immune cells and improve the efficacy of cancer vaccines	The hydrogel loaded with aPD-L1 and DOX (Gel/aPD-L1+DOX) inhibits tumor growth more effectively.	[62]

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The high encapsulation efficiency of hydrogels enables them to load multiple antigens and immunomodulators simultaneously. They can accurately recruit and in situ activate key immune cells such as dendritic cells and T cells through controlled release of chemokines, physical capture of microstructures, surface chemical modification, etc., and can further couple immune checkpoint regulation, thereby significantly improving the efficacy and breadth of the immune profile of cancer vaccines[82]. Liu et al. constructed an injectable supramolecular hydrogel for local sustained release of immune checkpoint inhibitors (DPPA-1 peptide, blocking PD-1/PD-L1) and chemotherapeutic drugs (doxorubicin, DOX) in the same scaffold to produce a synergistic chemo-immunotherapy effect and reduce systemic toxicity[83]. The immune-favorable microenvironment created by DOX enhances the efficacy of aPD-L1; at the same time, aPD-L1 protects activated T cells, making the chemotherapy-induced anti-tumor immune response more effective and durable. The two synergistically activate a strong anti-tumor immunity. In the primary tumor model, the hydrogel loaded with aPD-L1 and DOX (Gel/aPD-L1+DOX) inhibited tumor growth more effectively than single drugs, free drug combinations, or hydrogels loaded with a single drug. This strategy can not only control local tumors but also induce a systemic immune response, effectively inhibiting the growth of untreated contralateral tumors (abscopal effect) and tumor metastasis.

5. Application Scenarios and Typical Cases

Like other proteins, insulin has a high tendency to aggregate and is sensitive to temperature, light, and vibration, resulting in loss of biological activity. Insulin injection solutions usually require low-temperature storage and careful handling to avoid shaking. Therefore, a simple storage method is needed to eliminate the need for temperature control, and it should be cheap, easy to prepare, meet safety requirements, and ensure its efficacy. Bianco et al. proposed an innovative and simple strategy to store and deliver proteins in gels to protect insulin solutions from heat and mechanical stress[84]. After high-speed stirring at 600 revolutions per minute for six hours, when pushed through a filter with a syringe, the molecular network of the gel disintegrates under the applied pressure. The drug solution recovered from the gel is detected to be a pure, basically non-aggregated insulin solution. Potential applications are not limited to insulin storage, but also can be used for messenger RNA vaccines against COVID-19 and vaccines against measles, mumps, and rubella, both of which require cold chain management. Such gels also promise to safely store and transport hazardous chemicals, including liquids (such as fuels), for which traditional storage and transportation methods pose safety risks.

Tumor immunotherapy mainly achieves tumor treatment by intervening in the body's immune response, including activating and enhancing the immune response, and improving the ability to recognize and kill tumor cells[85]. The tumor immune escape mechanism is affected by PD-1/PD-L1. Immune checkpoint inhibitor therapy uses immune checkpoint inhibitors to block the interaction between PD-1 and PD-L1, thereby enhancing the immune response, activating patients' T cells, and achieving continuous killing of tumor cells[86]. Hu et al. developed a local sustained-release hydrogel for delivering anti-PD-1 antibodies and small-molecule drug Pexidartinib (PLX). The hydrogel achieves local sustained release of drugs by encapsulating PLX nanoparticles and platelets modified with anti-PD-1 antibodies. In the postoperative inflammatory environment, platelets are activated and release anti-PD-1 antibodies to block immune checkpoints, while PLX clears TAMs, promotes T cell infiltration, and enhances anti-tumor effects. In various tumor recurrence models, this strategy significantly inhibits postoperative tumor recurrence and metastasis[87]. Other anti-tumor immunotherapies whose efficacy is hindered by the tumor immunosuppressive microenvironment, such as adoptive T cell therapy and cancer vaccines, may also benefit from this strategy.

The formation of glial scars after spinal cord injury is a complex pathophysiological process. It severely hinders the repair and functional recovery of spinal cord injury by forming physical and chemical barriers, exacerbating inflammatory reactions, affecting nerve function recovery, and leading to tissue structure remodeling[88]. Therefore, inhibiting the formation of glial scars or regulating their composition and function has become an important research direction in the treatment

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of spinal cord injury. Liu[89] et al. developed a nano-antagonist hydrogel (Nano-antGel) containing N-cadherin nano-antagonists and polyphenol hydrogels. The hydrogel exhibits significant anti-inflammatory properties, specific calcium ion adsorption capacity, and antagonistic effect on N-cadherin, effectively preventing the formation and aggregation of astrocytes that form scars. In the SCI model, Nano-antGel effectively inhibited the formation of glial scars, promoted axon regeneration, and significantly improved the motor function of SCI mice. In addition, the researchers also designed an N-Cadherin-functionalized aligned fibrin nanofiber hydrogel (AFGN), which provides a superior microenvironment for the delivery and regulation of exogenous neural stem cells (NSCs), including biophysical and biochemical cues. The combined use of AFGN hydrogel and NSC can regulate the local neuroinflammatory microenvironment, reduce inflammation-induced secondary damage by converting activated macrophages into M2 subtypes. These research results provide new perspectives for the design of future neural tissue engineering.

Table 3. Disease Application Scenarios and Cases

Disease Field	Hydrogel Scheme	Advantage	References
Diabetes	Reversible supramolecular hydrogels are used to achieve controlled release of homogeneous proteins	Under the conditions of no chemical stimulation, no solvent, and no high temperature, the controlled release of homogeneous proteins can be achieved only through mechanical force, thereby avoiding damage to protein activity by traditional methods.	[61]
Tumor immunotherapy	Hydrogels encapsulating PLX nanoparticles and platelets modified with anti-PD-1 antibodies to achieve local sustained release of drugs	Block immune checkpoints, while PLX clears TAMs, promotes T cell infiltration, and enhances anti-tumor effects.	[62]
Glial scar	Nano-antagonist hydrogels including N-cadherin nano-antagonists and polyphenol hydrogels	Significant anti-inflammatory properties, specific calcium ion adsorption capacity, and antagonistic effect on N-cadherin, effectively preventing the formation and aggregation of astrocytes that form scars.	[64]

6. Conclusion

The rise of hydrogel delivery systems marks a paradigm shift of protein drugs from "passive transport" to "intelligent organisms". Compared with the limitations of transdermal efficiency of polymer microneedles, burst release effect and immunogenicity risk of lipid nanoparticles, metabolic accumulation concerns of inorganic nanoparticles, and organic solvent damage and acidic degradation dilemmas that are difficult to overcome by microsphere systems, hydrogels have opened up a new path as guardians of bionic microenvironments. Their high water content network (>90%) builds a natural barrier against denaturation for proteins, and the dynamic cross-linked network has achieved evolution from "sustained-release warehouses" to "on-demand pharmacies". When liposomes cause antibody aggregation due to bilayer disturbance, and when PLGA microspheres etch drug activity during acidic degradation, hydrogels precisely release functional proteins through pH/enzyme/light multiple response mechanisms under the tumor microacidic environment or specific enzymatic hydrolysis signals.

This intelligence is reconstructing treatment scenarios: in diabetes management, glucose-responsive hydrogels release insulin in a closed loop, which is significantly superior to the fixed drug release mode of microneedle patches; in postoperative tumor cavities, in-situ gelation forms drug reservoirs

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that can synergistically sustain the release of antibodies and cytokines, overcoming the systemic exposure risk of nanoparticles; in the field of gene editing, the efficiency of TAT peptide-modified hydrogels carrying CRISPR-Cas9 to penetrate cell barriers is far higher than the random encapsulation strategy of lipid nanoparticles. More profoundly, the compatibility of hydrogels with living cells makes them the cornerstone of regenerative medicine. RGD gels loaded with mesenchymal stem cells simultaneously deliver VEGF and extracellular matrix in myocardial repair, achieving the "drug-cell-scaffold" trinity therapy that microspheres or liposomes cannot match.

However, hydrogels are not "disruptors" that replace other systems, but "catalysts" for collaborative evolution. Cutting-edge research has revealed integration paths: nanoparticles embedded in hydrogels to construct hierarchical controlled release systems (such as liposome@gels to achieve pulsed vaccine release), microneedle arrays combined with hydrogels to form transdermal intelligent patches (gels respond to local microenvironments after breaking through the stratum corneum), and 3D printing technology to precisely arrange microspheres in hydrogel scaffolds to achieve spatiotemporal sequential release of growth factors. This multi-system hybrid strategy is eliminating traditional boundaries—when polymer microneedles solve penetration problems, lipid nanoparticles optimize intracellular delivery, and hydrogels provide microenvironment regulation, protein drugs finally cross the "last mile" from the laboratory to the lesion.

Looking forward to the future, the evolution of hydrogel delivery systems will develop in depth along three axes: intelligence, vitalization, and personalization. When these breakthroughs are deeply integrated with the penetration of microneedles, the membrane fusion of liposomes, and the long-acting nature of microspheres, protein delivery will truly enter the era of "tailor-made medicine"—no longer drugs adapting to carriers, but carriers customized for life.

Looking forward to the future, although hydrogels are simpler to prepare and lower in cost than microspheres, nanoparticles, and other formulations, there are still problems in clinical transformation. In the future, we will continue to conduct in-depth research on their clinical transformation in promoting the intelligent development of hydrogels.

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