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Strategies for overcoming protein and peptide instability in biodegradable drug delivery systems

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ABSTRACT

The global pharmaceutical market has recently shifted its focus from small molecule drugs to peptide, protein, and nucleic acid drugs, which now comprise a majority of the top-selling pharmaceutical products on the market. Although these biologics often offer improved drug specificity, new mechanisms of action, and/or enhanced efficacy, they also present new challenges, including an increased potential for degradation and a need for frequent administration via more invasive administration routes, which can limit patient access, patient adherence, and ultimately the clinical impact of these drugs. Controlled-release systems have the potential to mitigate these challenges by offering superior control over *in vivo* drug levels, localizing these drugs to tissues of interest (e.g., tumors), and reducing administration frequency. Unfortunately, adapting controlled-release devices to release biologics has proven difficult due to the poor stability of biologics. In this review, we summarize the current state of controlled-release peptides and proteins, discuss existing techniques used to stabilize these drugs through encapsulation, storage, and *in vivo* release, and provide perspective on the most promising opportunities for the clinical translation of controlled-release peptides and proteins.

Contents

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1. Introduction

Pharmaceutical drugs have had an enormous impact on human wellness and longevity and will continue to do so for the foreseeable future. The vast majority of drugs on the market today are small molecules; however, small-molecule drugs are typically limited in some respects. For example, their limited complexity can lead to off-target activity, potentially resulting in toxicity or other side effects. Furthermore, many bacterial and cancer cells have developed resistance to small molecule drugs, and certain diseases remained undruggable.

Recent progress in pharmaceutical technologies and recombinant protein production methods has resulted in the identification and commercial availability of biological drugs with distinct therapeutic activity. These "biologics" encompass a variety of products, including peptides, recombinant proteins, and nucleic acids. Their main advantages over conventional small-molecule drugs lie in their high complexity relative to small molecules, enabling them to act more potently and with greater specificity, thereby limiting off-target effects. A summary of the currently licensed biologics for use in the US can be viewed in Table 1.

Protein and peptide drugs have been in the clinic the longest of any biologics, first as vaccines against infectious diseases. In contrast, nucleic acid therapeutics have just arrived in the clinic with the approval of Patisiran in 2018. Proteins and peptides can also be direct biological substitutes in response to disease with full functionality that may not be achievable with a small molecule. Protein and peptide therapeutics are also attractive from a commercial perspective since they are linked to faster clinical development and approval time as well as better patent protection than small molecule drugs [1]. The global protein therapeutics market size reached $298 billion in 2021 and is expected to reach $490.2 billion by 2028 [2].

Most of these peptide and protein therapeutics are administered by repeated injections either in solution or suspension dosage forms because they are not orally bioavailable. This approach is inconvenient, often requires medical personnel or hospitalization, and is expensive. Like many protein drugs, monoclonal antibodies (mAbs), a key sub-class of therapeutic proteins, are typically delivered intravenously (IV) or subcutaneously (SC) in multiple doses administered over an extended period of time, underscoring the potential benefit of formulating these drugs in controlled-release formats that greatly reduce dosing frequency.

Unfortunately, the extended delivery of proteins is complicated by their generally poor stability relative to small-molecule drugs [3]. mAbs, for example, have a delicate structure that makes them more susceptible to conformational changes during formulation and storage, which could render them biologically (i.e., functionally) inactive [4]. Changes to the drug microenvironment, such as temperature, hydration status, organic solvent exposure, pH, or the presence of enzymes, can structurally alter peptides and proteins, potentially rendering them irreversibly inactivated. The unique environmental conditions that a biologic experiences while being formulated in, or released from, a drug delivery system can

Table 1
Peptide and protein drugs currently licensed for human use in the US.

Drug	Therapeutic area	Examples (number of drugs*)
Monoclonal antibodies	Oncology	Herceptin, Rituxan, Keytruda, Opdivo, Avastin (n = 58)
	Immunology	Humira, Stelara, Cosentyx, Remicade, Xolair (n = 46)
	Infectious diseases	RabiShield, Ronapreve, Trogarzo,Inmazeb, Ebanga (n = 17)
	Neurological disorders	Leqembi, Donanemab, ADUHELM, Emgality, Ajovy (n = 7)
	Hematological disorders	Soliris, Hemlibra, Adakveo, Reopro, Praxbind (n = 8)
	Genetic diseases	Takhzyro, Evkeeza, Ilaris, Crysvita, Concizumab (n = 5)
	Musculoskeletal disorders	Evenity, Narlumosbart, Prolia (n = 3)
	Ophthalmology	Lucentis, Tepezza, Vabysmo, Beovu (n = 4)
	Others	Tafolecimab, Repatha, Praluent (n = 3)
Antibody-drug conjugates	Oncology	Kadcyla, Mylotarg, BESPONSA, Adcetris, Polivy (n = 14)
Other proteins and peptides	Oncology	Leuprolide, Thyrotropin Alfa, Sipuleucel-T, Pegaspargase, Aflibercept (n = 16)
	Immunology	Etanercept, Interferon alfa-n3, Anakinra, Filgrastim, Aprotinin (n = 31)
	Infectious diseases	Drotrecogin alfa, OspA[1] lipoprotein, Teicoplanin, Enfuvirtide, Gramicidin D (n = 14)
	Neurological disorders	Glatiramer Acetate, Peginterferon beta-1a, Botulinum Toxin Type A, Botulinum Toxin Type B (n = 4)
	Hematological disorders	Darbepoetin alfa, Epoetin alfa, Collagenase, Desirudin, Susoctocog alfa (n = 34)
	Genetic diseases	Insulin Lispro, Sacrosidase, Conestat alfa, Lucinactant, Rasburicase (n = 18)
	Hormonal disorders	Secretin, Vasopressin, Thyroglobulin, Liraglutide, Sermorelin (n = 42)
	Metabolic disorders	Dornase alfa, Imiglucerase, Pegademase, Laronidase, Aliskiren (n = 43)
	Others	Teriparatide, Nesiritide, C1 Esterase Inhibitor, Beractant, Pegaptanib (n = 13)
Subunit vaccines	Infectious diseases	MenB[2] vaccine, Pneumococcal Vaccine, Hepatitis B Vaccine, Anthrax Vaccine, Hib[3] Conjugate (n = 25)
	COVID-19 vaccine	Novavaxovid (EUA) (n = 1)
Viral vaccines	Infectious diseases	Measles, Mumps and Rubella Vaccine, Hepatitis A Vaccine, DTaP[4], Dengue Tetravalent Vaccine, BCG[5] (n = 65)
	COVID-19 vaccine	Janssen COVID-19 Vaccine (EUA) (n = 1)

*Note: Current as of 02/03/2023.
[1] OspA: Outer surface protein A.
[2] MenB: Meningococcal B.
[3] Hib: Haemophilus influenzae type b.
[4] DTaP: Diphtheria, Tetanus toxoids & Acellular Pertussis vaccine.
[5] BCG: The Bacille Calmette-Guérin vaccine.

present a substantial problem for retaining bioactivity through *in vivo* residence and release. Therefore, understanding the mechanisms of peptide and protein degradation, environmental stressors encountered during controlled-release device preparation, storage, and release, and methods that can be used to mitigate drug degradation are critical for developing successful extended-release peptide and protein formulations.

For the sake of conciseness, proteins and peptides will be collectively referred to as "proteins," except in cases where the principle discussed does not apply to both. Additionally, protein PEGylation, amino acid substitution, and other structural modifications to the drug molecule itself are meaningfully different from the sustained release of native proteins in both approach and outcomes. These approaches are well-covered by existing reviews and are not discussed here [5–8].

2. Types of peptide and protein drugs and their applications

2.1. Peptides

Peptide therapeutics are polypeptides—oligomers up to 40 amino acids in length—which are used for the treatment or prevention of diseases. Peptides can be further classified into three categories based on their relationship to endogenous peptide molecules. The first category, native peptides, have the same sequence as a naturally occurring peptide, are well-tolerated *in vivo*, and have minimal adverse side effects; however, they also typically have short half-lives in circulation. Most native peptides are currently produced synthetically or through recombinant expression depending on their length and production scale—methods that have largely supplanted isolation from animal tissues. The next category, peptide analogs, are modified or substituted versions of native peptides that enhance the properties of the drug, achieved through modifying the amino acid sequence or conjugating the peptide with other molecules. The benefit of these peptide analogs generally includes an extension of the half-life in circulation, improved resistance to digestive enzymes, and an increased specificity for the intended target. Given these advantages, peptide analogs have become the dominant category used in the majority of peptides in clinical trials [9]. Finally, the third category of peptides is heterologous peptides, which have no relation to native sequences. Instead, these peptides are short amino acid sequences discovered through library screening of naturally produced compounds of other species, rational and computational design, or phage display. Without a native analog, heterologous peptides may have longer half-lives in circulation and/or exhibit novel functionality; however, they are also more likely to have unexpected side effects [10].

To date, more than 80 peptide drugs have been approved by the United States Food & Drug Administration (FDA), more than 170 are in active clinical development, and an additional 260 have been tested in human clinical trials [9,10]. Most of these peptides are used or being evaluated for use in metabolic disease, oncology, and cardiovascular disease. One major use of peptide therapeutics is for "replacement therapy," in which the drug adds or supplements peptide hormones in patients whose endogenous levels are inadequate (e.g., due to mutation). Perhaps the most well-known example of peptides as replacement therapeutics is the use of insulin in diabetic patients who do not produce enough of the hormone naturally. Another popular application of peptides is as cell-penetrating peptides (CPPs), which was identified in the early 1990s from the transactivator of transcription protein of human immunodeficiency virus (HIV) [11]. CPPs can facilitate the transport of different molecules, including peptides, DNAs, siRNA, and drugs, into cells [12]. Peptides can also function as antimicrobial and antiviral agents. Antimicrobial peptides (AMPs) function by acting on multiple targets on the plasma membrane and intracellular targets, including interacting with nucleic acids, inhibiting protein synthesis and enzyme activity, and perturbing cell wall

synthesis [13]. As of March 2023, a total of 3,594 AMPs have been reported in the antimicrobial peptide database (APD3), underscoring the interest in these peptides. Antiviral peptides (AVPs) have also demonstrated clinical utility by interfering with the viral replication cycle through a variety of mechanisms [14]. Enfuvirtide is the first FDA-approved AVP, which is a 36-amino acid peptide that blocks HIV infection [15].

The stability of each peptide therapeutic is unique and is dependent on its sequence. The shelf-life of peptides in solution is very limited and much shorter than lyophilized peptides. Most lyophilized peptides are stable for several years if stored at −20 °C protected from light; however, certain amino acid residues in peptide sequences can undermine their long-term stability. Peptides containing cysteine (Cys), methionine (Met), or tryptophan (Trp) residues are prone to oxidation, which is accelerated by freeze–thaw cycles and a high pH [16]. Glutamic acid (Glu) and aspartic acid (Asp) are prone to deamidation, especially when dried under acidic conditions. Certain positions are more susceptible to deamidation, including the N-terminus and the amino acid N-terminal to glycine. Aspartic acid is sensitive to hydrolysis, and amino acids containing aromatic rings are susceptible to photochemical degradation, such as phenylalanine (Phe) and Trp [17].

2.2. Antibodies

Immunoglobulin G (IgG) and fragments of IgG present the most abundant protein class of pharmaceutical antibodies. IgG consists of two Fab (fragment antigen binding) regions and a single Fc (fragment crystallizable) region. These are located on two heavy chains (~50 kDa each) and two light chains (~25 kDa each). The amino acid sequence of the complementary determining regions (CDR) confers the antibody's specificity and affinity for antigens. IgG-derived fragments, such as Fab or single chain variable fragments (scFv), can replace full IgGs without losing molecule functionality. However, their smaller size leads to a shorter half-life *in vivo*, and the lack of Fc-dependent activation of immune cells may reduce the efficiency of immunotherapy when a robust inflammatory response is required [18].

2.2.1. Monoclonal antibodies

Monoclonal antibodies (mAbs) are uniform populations of immunoglobulins that bind to an antigen with high specificity. The native immune response to an antigen triggers the polyclonal production of antibodies by B cells. These antibodies can each bind to a different epitope of the antigen—some of which are functionally useful for a neutralizing response and others that may not be. A single (i.e., clonal) population of useful antibodies can then be identified and used to treat disease. The hybridoma technique, developed in 1975, allows for the production of cell lines that stably produce mAbs, making it possible to produce these proteins in large quantities at a relatively low price. Globally, at least 1,200 therapeutic mAbs have been studied in clinical trials, and 177 therapeutic mAbs have been approved or are currently under regulatory review [19,20].

mAbs are used to treat a wide range of diseases, though oncology, immunology, and hematology remain their most prevalent medical applications despite their high specificity, which might otherwise seem to be application-limiting. Many mAbs have multiple disease indications—often at least one that is cancer-related (lymphoma, myeloma, melanoma, glioblastoma, neuroblastoma, sarcoma, colorectal, lung, breast, ovarian, head and neck cancers)—because they act on proteins that are dysregulated across multiple conditions [21]. mAbs have recently found a major use in stimulating or inhibiting protein function in the immune checkpoint signaling pathway with the implementation of numerous antibody therapeutics targeting programmed cell death protein 1 (PD-1, cemiplimab, nivolumab, pembrolizumab), its ligand programmed death-ligand 1 (PD-L1, durvalumab, avelumab, atezolizumab), and cytotoxic T-lymphocyte-associate antigen 4 (CTLA-4, ipilimumab), which have received regulatory approval [22].

There are also mAbs in use and under development for the treatment of infectious diseases. Currently, there are 17 therapeutic antibodies that have been approved by FDA, and more than 200 manuscripts about neutralizing mAbs against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Products that have at one point received emergency use authorization (EUA) from the FDA include bamlanivimab/etesevimab, casirivimab/imdevimab (brand name REGEN-COV), sotrovimab, and bebtelovimab. However, because Omicron sub-variants with sufficiently mutated spike proteins (the target of these mAbs) have become dominant in the United States, the EUAs for these drugs have been revoked as of January 2023, and there is no monoclonal antibody currently authorized for the treatment of COVID-19. This underscores one limitation of mAbs, which is that their function is fully dependent on the existence of one particular motif, and thus evasion can occur far more readily than it would for a polyclonal antibody cocktail.

mAbs can also be used to enhance the targeting of other drugs. A prominent example of the first generation of antibody-drug conjugates (ADCs) is presented by BR96-doxorubicin, which consists of a chimeric mAb directed against the Lewis tetra-saccharide commonly expressed on human carcinomas, modified with eight molecules of doxorubicin—a small-molecule cancer drug—conjugated to hinge cysteines [23]. However, those early products didn't achieve widespread clinical success owing to a combination of technological, targeting, and potency issues that did not allow the integrity of the antibody to be maintained. Refinements in linker technology combined with greater knowledge of targets have led to the emergence of second-generation ADCs, such as the FDA-approved brentuximab vedotin (Adcetris®), a CD-30 specific ADC, and a cytotoxin-conjugated biobetter of trastuzumab, ado-trastuzumab emtansine (Kadcyla®).

In comparison to sensitive proteins such as enzymes, most antibodies are quite robust and typically retain the ability to bind to their targets for up to 12 months if stored at 2–8 °C. However, they are sensitive to repeated freeze/thaw cycles and will lose stability at excessive dilute concentrations. One major problem encountered in antibody-based therapies is their tendency to aggregate under high-concentration formulations required for disease treatment. All levels of a protein's structure have an impact on its stability. The amino acid sequence (primary structure) is a critical determinant of a protein's susceptibility to aggregation. For example, a low isoelectric point (pI) of CDR is likely to form soluble aggregates due to increased electrostatic interactions between mAbs, while a high pI leads preferentially to insoluble aggregates, especially when in contact with negatively charged surfaces [24]. Minor variations in mAb sequence and structure can also have a significant impact on their stability when exposed to stress conditions. The aggregation potential of different subclasses of IgG in pH 4–7 is IgG1 < IgG2 < IgG4, which is attributed to the different flexibility of the hinge region [25]. Many stability studies about commercially available mAbs have been published independently or requested by the manufacturers, which have been well summarized by Le Basle and colleagues [26].

2.2.2. Fc fusion proteins

Fc fusion proteins are composed of an immunoglobin Fc domain directly linked to another peptide. The fused partner can be any other proteinaceous molecule of interest but is typically selected for its therapeutic potential. The presence of the Fc domain markedly increases the plasma half-life beyond the fused partner's typical half-life, which prolongs therapeutic activity [27,28]. The

attached Fc domain also enables these molecules to interact with Fc-receptors (FcRs) on immune cells, which can be leveraged for cancer therapy and vaccination. Most Fc-fusion proteins target receptor-ligand interactions, working either as antagonists to block receptor binding (e.g., etanercept, aflibercept, rilonacept, belatacept, abatacept), as agonists to directly stimulate a receptor to reduce (e.g., alefacept) or increase immune activity (e.g., romiplostim) [29]. Besides improving the therapeutic potential of the biological partner, the Fc-domain also greatly affects the stability and solubility of its partner. The Fc fragment of Ig is glycosylated and highly charged at neutral pH, which helps increase the solubility of otherwise hydrophobic proteins. The aggregation of Fc-fusion protein is affected by intrinsic factors (e.g., free thiols in the Fc-fusion protein peptide moiety) and extrinsic factors (pH, protein and salt concentration, state of matter) [30]. In comparison with native IgG proteins, wherein interdomain interactions presumably have evolved to provide mutual stabilization, fusion proteins, such as abatacept, often lack such stabilizing interdomain stabilization and show overall lower colloidal stability than naturally occurring multidomain proteins, such as antibodies [31]. The linker technology used is also important for drug stability and the efficacy and toxicity of the conjugates. Specific details on the various linker options can be found elsewhere [32].

2.2.3. Nanobodies

Nanobodies refer to the variable domain of a heavy-chain-only antibody (HcAbs), which are only 15 kDa in molecular weight—approximately 10% of the molecular weight of a complete IgG antibody. The antigen-binding capacity of nanobodies remains similar to that of conventional antibodies; however, they typically offer superior structural stability and solubility. Nanobodies are commonly used as targeting ligands to specifically direct chemotherapy drugs, radionuclides, or toxins toward areas of therapeutic interest [33]. More sophisticated bivalent or bispecific nanobodies have also been constructed with higher binding affinity, avidity, improved specificity, and consequently better therapeutic efficacy than their monovalent counterparts [34]. Caplacizumab, a drug for acquired thrombotic thrombocytopenic purpura (aTTP), was the first bivalent nanobody to be approved by the FDA in 2019, representing a landmark event in the clinical advancement of nanobodies [35]. Owing to their efficient refolding capacity after chemical or thermal denaturation, nanobodies are resistant to irreversible functional losses at elevated temperatures (T_m normally between 60 and 80 °C) [36]. They retain their full binding capacity after one week at 37 °C [37], and their thermal unfolding behavior has been described as completely reversible after a 2-hour heat shock at 90 °C [38]. The exposure to stress conditions (pH ranging from 3.0 to 9.0; pressure at 500–750 MPa) and chemical denaturants (2–3 M guanidinium chloride, 6–8 M urea) also reduce their antigen-binding capacity by very little [39]. In addition, the folding of the CDR3 loop and the hydrophilic content of the framework-2 region makes them highly water-soluble and helps to prevent aggregation [40].

2.3. Other therapeutic proteins

Like peptide therapeutics, only a small number of protein therapeutics are purified from their native source (e.g., pancreatic enzymes from hog and pig pancreas, α-1-proteinase inhibitor from pooled human plasma). Instead, most are now produced recombinantly by inserting an exogenous gene into a production host, such as bacteria, yeast, insect cells, mammalian cells, or using transgenic animals or plants, and then purifying out the desired protein. According to their mechanisms of action and applications, therapeutic proteins can be classified by function into the following categories:

1) Replacing a protein that is deficient or abnormal. These protein drugs are widely used in endocrine and metabolic disorders with defined molecular etiologies. For example, providing lactase in patients lacking the gastrointestinal enzyme [41] and replacing vital blood-clotting factors such as factor VIII and factor IX in patients with hemophilia [42,43]. The bioactivity of most clotting factors remains high over the 24 h after phlebotomy (FP24) when stored at 4 °C, except factor VIII, which loses 20–30% of its activity over that time period [44].
2) Augmenting an existing pathway to enhance the magnitude or timing of a particular normal protein activity. This class of protein therapeutics has been successfully used in treating hematopoietic defects, including the use of recombinant erythropoietin to increase erythrocyte production in anemic patients [45,46]; interleukin 11 (IL-11) to increase platelet production in thrombocytopenic patients [47], and various interferons to improve immunoregulation [48,49]. The stability concerns of therapeutic cytokines mainly relate to their hydrophobicity, which can lead to aggregation and adsorption, as well as the chemical instability that arises from oxidation. Many of the cytokines in the market are stable in solution when kept at a low temperature in an appropriate buffer system since pH can deleteriously affect conformational stability. Cytokine structural stability has been summarized in multiple papers [50,51].
3) Supplying exogenous proteins with novel functions or endogenous proteins that act at a novel time or location in the body. In these use cases, a protein can ameliorate a problem even though it is not normally present in that situation. For example, Papain can be used to degrade proteinaceous debris in wounds [52], collagenase can be used to digest collagen in the necrotic base of wounds [53], and recombinant human deoxyribonuclease 1 (DNase 1) can be used to degrade the DNA left over from dying neutrophils in the respiratory tract of patients with cystic fibrosis [54]. Two major challenges facing the use of therapeutic enzymes are their instability and half-life in circulation. The temperature, pH, and salinity of the body are often not optimal for microbial enzymes, potentially leading to protein denaturation [55]. Most enzymes readily degrade when stored at room temperature, and degradation is further accelerated at a body temperature of 37 °C. The effect of native proteolytic enzymes and rejection by the immune system are also important factors affecting the long-term stability and efficacy of exogenous proteins.

2.4. Vaccines

In addition to disease-responsive therapeutics, recombinant proteins can also serve as prophylactics. The most common use of this strategy is for the generation of vaccines against infectious diseases. Vaccination is the most economical method to prevent many infectious diseases that cause morbidity or mortality; however, the administration schedule can be cumbersome—especially in low- and middle-income countries—and patient motivation for prophylactic with a potentially deferred benefit may be lower, making vaccines an attractive candidate for drug delivery systems. At present, vaccines can prevent more than 20 life-threatening diseases [56].

2.4.1. Live attenuated vaccines

Live attenuated vaccines use a weakened (or attenuated) form of the pathogen, which leads to the desired protective immune response without causing disease in healthy individuals. Live vaccines tend to create a strong and lasting immune response, which

may obviate the need for require additional doses or booster shots later in life. This class of vaccines includes some of the most effective vaccines, such as the measles, mumps, rubella (MMR) vaccine and varicella (chickenpox) vaccine. However, live vaccines may not be suitable for people who are immunocompromised, either due to drug treatment or underlying illness [57], and are associated with some inherent risk, albeit very little relative to the potential benefits of vaccination [58].

Live attenuated vaccines typically do not require adjuvants to boost the immune responses but are more sensitive to potency loss during storage and distribution, especially at elevated temperatures. Live attenuated bacterial (LAB) vaccines are regarded as unstable when exposed to high residual moisture, high temperatures, extreme pH, or ultraviolet radiation. As a result, only a few LAB vaccines are commercially available. The infectivity and viability of live attenuated virus (LAV) vaccines are often directly linked to their replication competency; therefore, their protein structure must typically be maintained to remain replicative and effective. Unfortunately, LAV vaccines are highly unstable under aqueous conditions due to chemical and physical reactions. Hydrolysis, deamidation, oxidation, and alteration of disulfide bonds are the major chemical instability of LAV vaccines. The unfolding of proteins increases the otherwise-unfavorable thermodynamic interactions, resulting in the aggregation, precipitation, or adsorption of LAVs. It is estimated that about 50% of vaccines formulated by lyophilization are discarded annually due to the poor thermostability of LAV vaccines [59]. One advantage to live vaccines, however, is that since they are dependent on *in vivo* expansion after administration, so a reduction in the initial dose of an attenuated pathogen due to inactivation during processing or storage will sometimes only delay the onset of an adaptive immune response as a diminished "seed" dose with fewer viable pathogens takes slightly longer to expand.

2.4.2. Inactivated vaccines

Inactivated vaccines contain whole bacteria or viruses which have been killed or altered—often by treating them with formalin or β-propiolactone [60]. They have been developed for many diseases, such as influenza, hepatitis A, rabies, polio, and encephalitis. Because inactivated vaccines do not contain live pathogens, they cannot cause the infectious disease that they protect against, which may be less efficacious.

Inactivated vaccines are typically developed as liquid formulations stored in glass vials or prefilled syringes, potentially due to the crosslinking imparted by inactivation. However, these vaccines remain sensitive to heat and sometimes freezing damage, making them susceptible to potency loss during storage and distribution. Commercially available inactivated poliovirus vaccine (IPV) can maintain their stability and potency in the cold chain (2–8 °C) for up to 3 years if properly maintained, yet rapidly lose potency quickly when exposed to temperatures above 25 °C [61]. Many stabilizers, such as disaccharides and sodium citrate, have been shown to enhance the thermal stability of trivalent IPV. D_2O can also protect poliovirus from swelling or aggregating, making IPV more stable; however, formulations employing D_2O have not been widely used for geopolitical reasons [62].

2.4.3. Subunit vaccines

Subunit vaccines are immunogenic fragments of pathogenic proteins that evoke an immune response with the goal of conferring immunity without exposing the patient to the risks associated with the native pathogen. This can be achieved by administering a pathogen-derived protein or polysaccharide that is key for pathogen function but otherwise innocuous (e.g., the spike protein in Novavax's COVID-19 vaccine) or chemically inactivate a protein fragment to prevent cellular toxicity (e.g., tetanus toxoid and diphtheria toxoid). One successful example is the hepatitis B vaccine, created by producing recombinant hepatitis B surface antigen (HBsAg) protein [63]. Vaccines to prevent human papillomavirus (HPV) infection also are based on recombinant protein antigens.

Subunit vaccines are generally more stable than multi-protein pathogens, whether they are inactivated or live. This is likely intuitive as well since the ability to use a subunit antigen in the first place means that it remains effective even after significant modification (e.g., cross-linked with formalin or recombinantly produced as a fragment). Studies have shown that the subunit tetanus toxoid vaccine is sensitive to freezing temperatures but not highly sensitive to heat. It was shown that the tetanus toxoid vaccine could remain immunogenic and safe in a CTC (controlled temperature chain) at 40 °C for up to 30 days before reconstitution [64]. However, like other protein drugs, the tetanus toxoid vaccine begins aggregating when it absorbs moisture, which reduces its potency. The recombinant protective antigen (rPA) of anthrax vaccines can elicit an effective immunologic response with fewer doses than anthrax vaccine adsorbed (AVA) [65]. However, rPA is more sensitive to elevated temperatures than AVA, and it has been reported that rPA loses its bioactivity when stored at 37 °C for 48 h [66].

2.4.4. Conjugate vaccines

Conjugate vaccines are similar to recombinant vaccines, except that they are composed of more than one component to enhance the immune response and are most commonly used for antigens that are otherwise poorly immunogenic. For example, the pathogen-associated polysaccharide attached to diphtheria or tetanus toxoid proteins helps to generate a stronger immune response against that polysaccharide [67]. Clinical examples of vaccines that employ this strategy include Haemophilus influenzae type B (Hib) vaccines and children's pneumococcal vaccines (PCV), and meningococcal vaccines.

Conjugated polysaccharide vaccines are polysaccharide haptens covalently attached to protein carriers. DNA bases are linked to a deoxyribose backbone or carbohydrate monomers through glycosidic bonds. The stability of these bonds is affected by pH, with the greatest stability within the range of pH 6 and 8. Ionic strength also has a significant effect on the stability of these vaccines. One particularly successful thermostable lyophilized vaccine from this family is the meningococcal A vaccine MenAfriVac, which is stable at 40 °C for up to 4 days and is the first vaccine to be approved for transport outside of the cold chain.

2.4.5. Combination vaccines

Combination vaccines, which contain antigens from multiple pathogens, have been used to consolidate vaccination schedules for multiple diseases. The first combination vaccine licensed to prevent multiple diseases combined with diphtheria, tetanus, and whole-cell pertussis (DTwP) antigens to vaccinate infants and children. To improve its reactogenicity profile, the whole cell pertussis was replaced with acellular pertussis. This advancement helped enable the addition of other vaccines, such as the *H. influenzae* vaccine (Hib), the IPV, and the hepatitis B vaccine (HepB), to the existing diphtheria, tetanus, and acellular pertussis (DTaP) vaccine.

In contrast to monovalent and single-pathogen vaccines, the stability challenges facing combination vaccines are exacerbated due to the complex interactions between antigens, adjuvants, and excipients. Vaccine components must remain stable after they are combined, and the World Health Organization (WHO) states that the effective shelf life of a combination vaccine should be determined by the antigen with the shortest shelf life [68]. In addition, multiple antigens can compete for the same binding sites on an adjuvant. For example, this occurs with pertussis antigens, diphtheria toxoid, and the hepatitis B antigen, often reducing the immunogenicity of the diphtheria component as the other antigens

displace it. Immunological, physical, and/or chemical interactions between combination vaccine components have the potential to alter the immunity conferred by the constituent antigens. The most widely reported example of immune interference in DTaP-based combination vaccine is the reduction in antibody titers to the Hib component of the polyribosylribitol phosphate antigen [69,70], which has also been reported in the hexavalent vaccine DTaP-HBV-IPV/Hib [71,72]. Therefore, at present, there is some limitation to what vaccine antigens can be used together—a problem that could potentially be overcome through asynchronous release from a protein delivery system.

3. Benefits of controlled-release biologics

Achieving better control over drug release has the potential to improve the effectiveness of therapeutics and prophylactics across a number of disease applications. Controlled delivery could potentially allow a drug to be administered once yet mimic the benefits of administering the drug repeatedly over an extended period of time by maintaining drug concentrations within the therapeutic window for days, months, or even years. The potential advantages of controlled-release systems are both physiological and logistical. These physiological benefits could include a potential reduction in adverse side effects, improved efficacy, and reduced chronic toxicity on metabolizing organs owing to a reduction in the cumulative amount of drug processed by avoiding the peaks and troughs associated with periodic dosing. On the logistical side, controlled-release systems can reduce the dosing frequency to lessen the inconvenience of administration, reduce cumulative administration pain (or frequency thereof), increase patient adherence, and reduce the cost of the treatment via reduced administration by healthcare professionals and/or dose sparing.

3.1. Safety enhancement

As with any biomedical device, safety is of paramount importance, though the acceptable safety profile for each drug could be different depending on the severity and urgency of administration (e.g., only minimal safety risks are acceptable for a prophylactic vaccine compared to higher acceptable risks for drugs that treat advanced-stage cancer). Safety considerations for drugs used to treat chronic diseases should factor in the cumulative effects of repeated dosage over months and years. Controlled drug delivery systems can help to dramatically reduce drug toxicity through the sustained release of the therapeutics at rates that achieve systemic concentrations at the low end of the therapeutic window or provide selective delivery to the intended site of action through local administration, which creates a favorable drug gradient between the target and non-target tissues.

3.2. Effectiveness enhancement

To maximize the benefits of a drug, it is crucial that drugs are present at the correct levels for the correct duration, which is something that can be achieved using controlled-release systems. Various clinical circumstances call for differential drug release rates with variable patterns for ideal treatment, which may depend on a variety of factors, including patient weight, age, gender, duration of need, and other factors. The most successful personalized disease management is the closed-loop insulin pump for diabetes, which has been shown to improve the duration spent within the desired glucose range (3.9–10.0 mmol/L) by 9.6% while also reducing the risk of hypoglycemia [73]. Unfortunately, bulky external devices are not desirable for most conditions. Responsive injectable and implantable systems offer the potential to internalize or reduce the fingerprint of these devices; however, in most cases, the levels of drug required in the future are not dynamic but rather can be known or approximated at the time of administration; such as for chronic diseases. In these cases, biodegradable polymers with tunable physicochemical properties offer the ability to improve drug pharmacokinetics and/or biodistribution without altering drug chemistry.

Novel ocular drug delivery technologies including nanoformulations, implants, and other ocular devices have been developed to greatly extend the duration over which drug remains at effective levels within a target tissue after dosing with a goal of reducing intravitreal injection frequency [74]. The ability of these injections to cross the blood-ocular barrier provides further benefits for peptides and proteins, which would otherwise not typically cross this barrier. Those techniques are actively used to sustain and extend medication delivery to treat back-of-the-eye illnesses, such as diabetic retinopathy (DR), age-related macular degradation (AMD), retinal detachment, posterior uveitis, and retinal vein occlusion [74].

Vaccine delivery systems also show great promise for improving global health. The release platform can also serve as an adjuvant since particulate antigens have been shown to be more efficient than soluble antigens for the induction of immune responses [75]. Antigen uptake by dendritic cells (DCs) is enhanced by the association of antigens with nanoparticles, which plays a crucial role in initiating the T-cell-mediated immunity [76]. In addition, most vaccines are delivered intramuscularly, which results in relatively muted immunogenicity compared to injection sites with greater concentrations of antigen-presenting cells, such as the dermis and lymph nodes. Microneedle-based dermal immunization showed more dose effectiveness than conventional intramuscular and subcutaneous immunization, but typically requires multiple doses for immunization [77]. However, microneedles can be used to deliver controlled-release microparticles [78], potentially enabling a single administration to confer immunity.

3.3. Dosing advantages

Drug delivery systems also offer dosing benefits that can increase the probability that the treatment performs as intended. Since the oral bioavailability of peptides and proteins is extremely low due to their poor absorption through the gastrointestinal (GI) tract, patient acceptance and adherence can be quite low for these drugs. As a result, reducing the invasiveness of protein drug administration or the dosing frequency could help to increase patient acceptability and compliance. SC-delivered controlled-release systems could increase the bioavailability of proteins and peptides up to 50–80% [79]. Further, rather than depend on the pharmacokinetics (i.e., plasma half-life) intrinsic to each protein, drug delivery systems can continue to release drugs sequestered within the device to counteract drug metabolism and elimination, such that the decrease in systemic drug concentration is greatly prolonged. The potential reduction of doses offered by controlled-release products can improve patient compliance and help to avoid missed doses.

4. Materials and techniques for preparing controlled-release systems for peptide/protein delivery

4.1. Particulate delivery systems

Particulate delivery systems are clinically attractive due to their ability to be injected through standard needles. This route of administration fits readily into existing clinical workflows, is minimally invasive, and is accessible to many patients worldwide—

especially for intramuscular administration. By avoiding more invasive delivery methods (e.g., implantation), the number of potentially viable applications for which controlled-release technologies could be appropriate is greatly expanded. Herein, we discuss the polymeric particles, inorganic particles, and their associated preparation methods (Fig. 1).

Classical mechanisms of drug release from polymeric systems include: 1) desorption of drug bound to the particle surface; 2) diffusion of drug through water-filled pores; 3) degradation and clearance of an encapsulating material; and 4) a combination of these mechanisms [80]. In general, the rate of protein drug release from a system depends on both the protein's intrinsic properties (e.g., hydrophobicity, charge, globular diameter) as well as the solubility, porosity, pore size, and biodegradation rate of the encapsulating matrix, which is a consequence of both the material and preparation method used. In many cases, the drug release rate can be further modified by changing drug loading and/or particle size. Larger particles generally exhibit a proportionally smaller initial burst release than smaller particles due to a smaller proportion of drug at the device's surface. Therefore, controlling the material and particle properties that determine release kinetics is key to achieving the desired release kinetics.

Biodegradable polymeric microparticles (MPs) and nanoparticles (NPs) show great promise as drug delivery systems due to their ability to prolong drug release and be entirely cleared from the body. Additionally, if appropriate materials are selected, these particles also exhibit favorable biocompatibility with tissue and are compatible with various drugs, including proteins and peptides. Depending on the payload and the desired duration of release, polymeric MPs and NPs have commonly been synthesized using emulsion-solvent evaporation, spray drying, nanoprecipitation, and self-assembly processes.

The material(s) chosen to encapsulate biologics are just as important as the methods used to form the materials into particles. The organic solvent solubility, degradation rate, degradation products, hydrophobicity, biocompatibility, and immunogenicity of the encapsulating biomaterial should all be key considerations when creating MPs and NPs. Although many materials have been used or proposed for the encapsulation of biologics, poly(lactic-co-glycolic acid) (PLGA), polylactic acid (PLA), polycaprolactone (PCL), polyanhydride, chitosan and cyclodextrin are among the most common. Of these, PLGA is the most common material used to encapsulate peptides or proteins because it has ample precedence in FDA-approved formulations, is generally considered to be biocompatible, and degrades into monomers that are found endogenously (i.e., lactic acid and glycolic acid) and readily eliminated from the body.

4.1.1. Polymeric particle composition

PLGA has been widely used in commercial drug formulations over the past 30 years to extend the release of numerous drugs, including luteinizing hormone-releasing hormone (LHRH) analogs for treating advanced prostate cancer and endometriosis [81], glucagon-like peptides (GLPs) for treating type 2 diabetes [82] and human growth hormone (hGH) for treating pituitary dwarfism [83]. Surface modification of PLGA, drug encapsulation method, particle size, additives added during formulation, the molecular weight of the drug and polymer, the ratio of lactide to glycolide moieties, and the polymer end group all exert a strong influence on the release of drugs from PLGA particles. Unfortunately, as PLGA

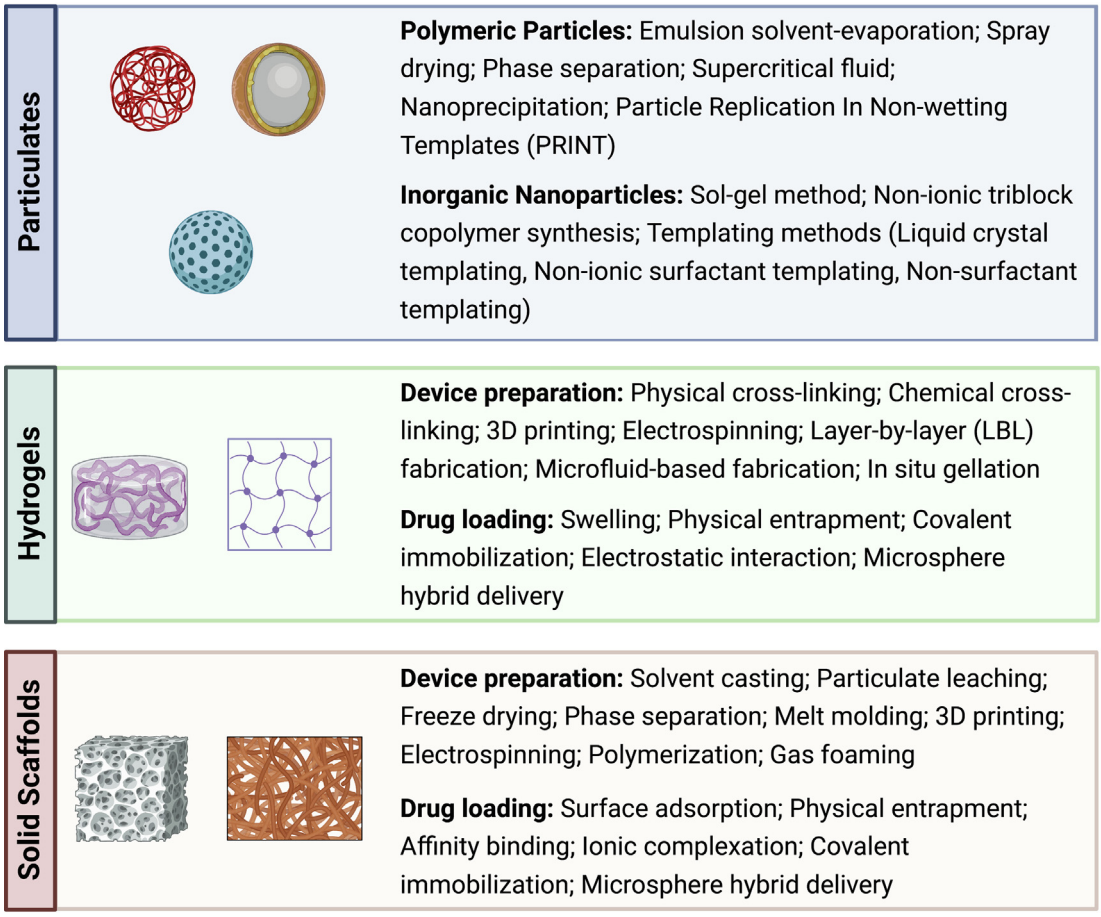

Fig. 1. Current controlled-release systems and their preparation methods.

degrades via hydrolysis, it generates acidic degradation products, which are non-ideal for biologics since these products can lower local pH, potentially causing biologics to assume alternative, non-native (and inactive) conformations. However, several approaches have been developed to overcome this challenge. For example, blending PLGA with other materials, such as alginate [84–86], chitosan [87], pectin [88], poly(propylene fumarate) [89], polyvinylalcohol [90,91], and poly(orthoester) [92], has been shown to improve the bioactivity of released biologics.

PLA, a homopolymer that comprises one of the two monomers in PLGA, shares many similarities to PLGA, including undergoing hydrolysis and generating lactic acid. Unlike PLGA, however, PLA is crystalline rather than amorphous, which confers both a high melting point, elevated hydrophobicity, and slower hydrolytic degradation. Like PLGA and PLA, PCL is degraded by the hydrolysis of its ester linkages and has therefore received a great deal of attention for use in drug delivery. It is especially interesting for the preparation of long-term implantable devices, owing to its slow degradation rate, which is often on the order of years. Like PLA, PCL is crystalline; however, its melting point is much lower (around 60 °C), offering some potential advantages in MP and NP preparation methods that require heat to flow the material when encapsulating biologics.

Chitosan is a modified natural carbohydrate polymer prepared by the partial N-deacetylation of chitin—a natural biopolymer chitin often harvested from crustaceans whose degree of deacetylation determines its crystallinity and degradation rate. Chitosan is broken down in the body by lysozyme, and the degradation rate can be accelerated by disrupting the network of hydrogen bonding through the inclusion of bulky side groups. Chitosan has been previously used in wound dressing and healing due to its antibacterial properties [93], gene delivery due to its positive charge [94], and oral and pulmonary drug delivery due to its mucoadhesive properties [95,96].

4.1.2. Polymeric particle preparation methods

A number of methods have been employed to encapsulate biologics within biodegradable particles, typically with a focus on extending the release of peptides and proteins. Although there are too many methods to cover in this review, we highlight a few of the most popular methods of encapsulation since proteins were first released from polymers by Langer and Folkman in 1976 [97]. These methods include double emulsion-solvent evaporation, spray drying, phase separation, nanoprecipitation, and Particle Replication In Non-wetting Templates (PRINT).

The most commonly reported method of encapsulating proteins and peptides in biodegradable microparticles is the solvent emulsion-evaporation technique [98]. There are two versions of this technique—single and double emulsion—which are preferentially used based on the hydrophobicity of the drug. Single emulsion-solvent evaporation is not an efficient method for the entrapment of hydrophilic molecules like peptides and proteins, a double emulsion approach is typically employed [99]. In double emulsion-solvent evaporation, an aqueous solution containing the protein and any relevant excipients is poured into a relatively larger solution of an organic solvent containing a dissolved polymer. Next, those immiscible solutions are emulsified, poured into a larger volume of water containing a surfactant, and emulsified again—typically at lower energy than the first emulsion. The resulting suspension is then stirred continuously until the organic solvent evaporates, leaving it to drug encapsulated within solidified polymeric particles. Protein concentration, choice of organic solvent, polymer concentration, volumes of the water and organic solvent phases, emulsification speeds, and surfactant concentration all play key roles in determining particle structure and release rate.

Spray drying is another method commonly used to encapsulate peptides and proteins in polymeric MPs and NPs. In this process, water-in-oil emulsions containing protein in the inner aqueous phase and polymer in the outer organic phase are passed through a nozzle and atomized in a stream of hot air, resulting in rapid solvent evaporation and polymer solidification before landing on a surface. Several processing parameters that should be considered in this approach are very similar to those when using a double emulsion, except that nozzle temperature, flow rate, and chamber temperature replace second emulsion factors.

Phase separation can also be used to fabricate protein-loaded polymeric MPs. In this approach, hydrophilic drugs (i.e., proteins and peptides) are dispersed into polymers dissolved in an organic solvent, then a non-solvent agent (e.g., silicone oil) that decreases the solubility of the polymer in the solvent is gradually added to the emulsion while stirring, extracting the polymer's solvent and initiating polymer phase separation by forming a soft coacervate of drug-loaded droplets. Then, the protein is encapsulated in a polymer-rich liquid phase (coacervate), and newly formed microspheres are immersed in heptane to quench and solidify the microdroplet [100]. The polymeric particles are harvested by washing, sieving, filtering, centrifugation or freeze-drying. A wide range of processing parameters, including polymer concentration, quenching temperature, quenching time and solvent composition, could be controlled to alter the morphology and size of resultant particles [98].

Nanoprecipitation is another relatively simple technique commonly used for encapsulating peptides and proteins [101]. Polymer is dissolved in a water-miscible organic solvent and then mixed with an aqueous solution containing protein, an emulsifier such as Pluronic F683, and other excipients (if desired). The resulting solution is then diffused into an aqueous phase. As the organic solvent diffuses into water, the polymer concentration exceeds its solubility in a mixed water-organic solvent solution, causing it to solidify and create protein-loaded polymeric NPs [102]. Parameters affecting the properties of nanoparticles produced using this method include the selection of solvents and antisolvents and their volume ratio, the choice of polymers and drugs and their concentrations, and the mixing method (slow mixing, turbulent mixing or laminar mixing) [98]. The main advantage of this procedure is that it minimizes stress on the encapsulated protein [103].

PRINT is a continuous, roll-to-roll, high-resolution molding technology providing monodisperse particles ranging from NPs to MPs. In PRINT, an elastomeric mold containing wells or cavities of predefined shape and size is used to define the structure of the particles [104,105]. The encapsulating material is either loaded as a pre-polymer and photocured inside the mold or deposited under heat or solvation into the mold. Proteins or peptides can be incorporated in PRINT particles by mixing with the encapsulating material or post-fabrication via adsorption. For example, PRINT particles encapsulating pneumococcal polysaccharide type 14 (PnPs14) antigen have been tested for their ability to evoke an anti-polysaccharide immune response [106], and toll-like receptor (TLR) 7/8 ligand has been encapsulated in PLGA particles as an adjuvant, with circumsporozoite surface protein (CSP) absorbed on the surface to enhance T cell-mediated immunity [107]. Hemagglutinin (HA) antigens in three commercial trivalent influenza vaccines (TIV) were electrostatically bound to the surface of cylindrical cationic PLGA-based NPs prepared by PRINT technology [108].

4.1.3. Inorganic nanoparticles

In addition to biodegradable polymers, inorganic nanoparticles have also been explored for the controlled release of protein. In these systems, proteins/peptides can be carried on the nanoparticle surface or within the structure.

Mesoporous silica nanoparticles (MSNs) are one common example of inorganic NPs that can serve as protein carriers due to their biocompatibility and biodegradability. The synthesis of MSNs occurs due to the self-assembly of pore template (frequently ionic, such as cetyl trimethyl ammonium bromide, or nonionic surfactants, such as poloxamers) into micelles during the preparation of mesoporous silica materials, creating voids in the silica matrix. The ordered structures with closely packed pores result from surfactant templating agents, including hydrothermal treatment, salt incorporation, utilizing pore expanding agents like mesitylene, co-surfactants, or tuning the reaction temperature [109].

MSNs possess a solid inorganic oxide framework that is biocompatible and protects the loaded pharmaceuticals inside the pores from exposure to harmful denaturation chemicals and conditions. MSNs have been widely investigated for delivering small drug molecules [110,111], while small proteins with hydrodynamic diameters less than the pore size can also be loaded and released [109]. However, the delivery of large molecular-weight proteins remains a challenge.

SBA-15 and MCM-41 are the most commonly used MSNs for protein delivery. SBA-15 has large pore sizes (5–30 nm), allowing them to accommodate protein and other macromolecules. They also form large particles (800 nm to 2 μm), leading to some limitations, such as large molecular diffusion and adsorption capacity. MCM-41 has tunable pore sizes (2–10 nm), with an average distribution typically centered below 5 nm, which could interfere with optimal loading for large proteins. Unfortunately, only a small number of MSNs have entered clinical trials, and none have received FDA approval [112], due in part to their low colloidal stability, which leads to aggregation, resulting in unpredictable biological interactions.

4.2. Hydrogels

Hydrogels can be used directly as protein delivery systems or as tissue engineering scaffolds supporting cell growth with or without protein release. Protein release from hydrogels can be controlled through a number of mechanisms, including drug diffusion, electrostatic interactions [113], hydrophobic interactions [114], hydrogel degradation, cleavage of degradable protein linkers, guest–host interactions, and dynamic covalent bond formation [115]. Both synthetic and natural materials have been used as hydrogel protein delivery systems. Among the synthetic materials, poly (ethylene glycol) (PEG) is particularly common, but poly(vinyl alcohol) (PVA) and poloxamers are also widely reported [116]. Scaffolds composed of natural polymers, including collagen, silk fibroin, hyaluronic acid (HA), fibrin, self-assembling peptides, starch, fibrin, chitosan, and alginate, have also been well-described. Chemical modification of natural polymers with functional groups or linker molecules has displayed improved binding and release abilities. Collagen is one of the most investigated natural polymers for tissue engineering scaffolds [117]. Using more robust natural materials, improved crosslinking methods [118,119] or natural composites with inorganic or synthetic material such as HAp/chitosan/poly(l-lactic acid) (PLLA) or collagen/PLA can provide greater control over the physical properties [120,121]. Hydrogel preparation methods have already been well-covered in other papers; however, these generally fall into one of two categories, physical cross-linking (e.g., via crystallite formation, polymer chain complexion, hydrophobic interaction, and hydrogen bonding) and chemical cross-linking (e.g., stereo-complexation, inclusion complexation, photopolymerization, Michael addition, and click chemistry) [122,123].

The most common approach to loading hydrogels with drugs is to simply submerge or swell the hydrogel in an aqueous protein-loaded solution, enabling the drug to infiltrate the hydrogel network. Depending on the hydrogel and assembly method, it may also be possible to load the hydrogel at the same time it is produced (for example, via photocrosslinking). In either case, if the protein's hydrodynamic diameter is smaller than the pore size, it will release rather quickly once it is placed in the body or a release solution unless specific elements increase the affinity of the protein for the hydrogel [124]. When the hydrogel pores are smaller than the protein diameter, swelling or degradation is needed to increase the mesh size. Degradation can occur in the polymer backbone or at the crosslinks and is typically mediated by hydrolysis or enzymatic activity. Another strategy to control drug release from hydrogel is to covalently attach the protein to the structural hydrogel component. A variety of covalent linkages have been explored, including esters, amide bonds, and matrix metalloproteinase-degradable peptides, depending on the duration of release that is desired. Electrostatic interaction has also been exploited to form a strong affinity between drugs and the polymer chains. Favorable electrostatic interactions between the hydrogel and protein retards release, while proteins with the same charge as the gel will be accelerated. This strategy is applicable to many drugs and polymers that carry charges; for example, alginate hydrogels with negative charges have been used to deliver cationic, heparin-binding growth factors such as vascular endothelial growth factor (VEGF) [125]. Unfortunately, many of these interactions have the potential to compromise the bioactivity of the encapsulated protein. Hydrophobic and electrostatic interactions can cause proteins to assume non-native conformations while covalent linkages attach extra groups to the protein—often in a permanent way—potentially reducing bioactivity.

In situ-gelling hydrogel is another unique scaffold system that can be injected in liquid form and undergo a sol–gel transition inside the human body. In most cases, the gelation occurs through a physical process, for example, in response to a change in temperature, pH, or salt concentration. Many natural polymers, such as gelatin, form a gel upon lowering of temperature. In contrast, many synthetic polymers such as poly(N-isopropylacrylamide) (PNIPAm) and poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) undergo reverse thermogelation which remain in flowable state at room temperature [126,127]. *In situ* gelation can also be achieved by chemical processes, including charge interaction, stereocomplexation and Michael addition.

4.3. Solid scaffolds

Hydrogels may not be ideal scaffolds for all tissues, especially those that provide some mechanical functions. In those cases, tissue engineers may instead rely on scaffolds composed of solid materials. Since it is often preferable for scaffolds to be fully resorbed by the body after tissue function is restored, porous, biodegradable matrices are commonly used. To support tissue growth and maturation, proteins, such as growth factors (GFs), can be delivered in concert with these scaffolds [128]. Strategies for GFs incorporation into scaffolds include non-covalent or covalent immobilization on or into the delivery system, the selection of which depends on the physicochemical properties and interactions between the GFs, carrier and application.

4.3.1. Solid scaffold composition

Ceramics have been used commonly as biomaterials for orthopedic applications. Calcium phosphates (CaPs), particularly hydroxyapatite (HAp), tricalcium phosphate (TCP), and calcium sodium phosphosilicate (i.e., bioglass), are among the most widely used bone substitute materials due to their compositional similarity to bone mineral and potential to be resorbed by osteoclasts [129]. The porosity of ceramic scaffolds tunes the degradation rate, mechanical strength and degree of GFs entrapment. Due to their

high elastic modulus, robust mechanical properties, and favorable biocompatibility, ceramic scaffolds are popular choices for load-bearing applications. However, because of the extremely high temperatures required to form ceramic biomaterials, these scaffolds are typically only functionalized with proteins on their surface post-fabrication rather than within their bulk during fabrication.

Polymeric scaffolds have emerged as excellent candidates for bone tissue regeneration, primarily due to their versatile and tunable properties. Polymers derived from L- or D,L-lactic and their copolymers with glycolic acid are the most common synthetic polymers used to create solid tissue engineering scaffolds [130]. Nevertheless, numerous other biodegradable synthetic polymers have been employed to achieve the desired scaffold properties [130].

4.3.2. Method of preparing protein-loaded solid scaffolds

Fabrication techniques used to produce solid scaffolds are highly varied and include solvent casting, particulate leaching, freeze drying, thermally induced phase separation, melt molding, phase emulsion, rapid prototyping, *in situ* polymerization and gas foaming [131,132]. Calcium phosphate ceramics are manufactured by a palette of techniques such as polymer foam replication to ceramic foaming, including porogens, 3D printing, and gel casting, and are always followed by a thermal treatment or sintering step. Due to the wide variety of techniques available to produce solid scaffolds, this review will not discuss each one in detail. Instead, the remainder of this section will focus on protein loading techniques appropriate for each scaffold type. Proteins and peptides can be released from solid scaffolds through direct physical entrapment, protein adsorption, or the formation of ionic complexes.

Proteins and peptides can be incorporated directly into scaffolds during fabrication through physical entrapment. This approach is conducive to potentially high levels of protein loading because the protein is interspersed within the volume of the matrix. Unfortunately, the lack of water permeability and potentially harsh fabrication procedures, which sometimes include high heat, organic solvents, or non-specific crosslinking, are typically detrimental to the protein stability [128].

Alternatively, proteins can be added to the surface of solid scaffolds after scaffold fabrication to avoid exposure to harsh environments during fabrication. Adsorption, in which proteins and peptides are added to the surface of a scaffold, often by immersing the preformed scaffold in a protein solution, is one potential approach. This process often leads to rapid protein release but can be extended by varying certain material properties such as surface wettability, roughness, functional groups, and surface charge [133]. In addition to extending release, loading efficiency can also be improved by leveraging electrostatic attraction between proteins (e.g., growth factors) and the scaffold [134]. In any case, protein adsorption to the surface usually results in low loading compared to protein loading during fabrication since the protein is only found on the 2D surface of the scaffold and often leads to an initial burst of protein release, which may or may not be desirable.

When longer durations of protein release are desired, proteins can be attached covalently in the same way that hydrogels accomplish this task. The key difference, however, is that protein loading onto solid scaffolds using these strategies is limited to the 2D surface, whereas these same tools can load protein into the 3D hydrogel since it is water-permeable. Aminosilane chemistry is a common approach for the covalent attachment of growth factors to ceramic surfaces like HAp [135]. Chemical reaction to polymer matrix often requires the modification of proteins to contain reactive functional groups such as thiols, acrylates, azides and Gln tag [134]. This method generally reduces burst release and allows for a prolonged release [136]. However, the covalent binding of proteins is time intensive and costly, and the covalent binding may block active sites on proteins, thereby interfering with protein bioactivity.

Lastly, proteins may be incorporated in nanoparticles and then into the scaffold. For example, the incorporation of PLGA particles that release bone morphogenetic protein 2 (BMP-2) over 30 days has been used to enhance the regenerative potential of bone tissue engineering scaffolds [137]. The encapsulation of proteins within nanoparticles would allow for more precise control of their release and achieve long-term sustained release profiles. Depending on the nature of the nanoparticle, internalized proteins might also be protected from harsh fabrication conditions (e.g., organic solvents). Of course, proteins would instead be subjected to the process of nanoparticle encapsulation, which could cause damage that renders them biologically inactive.

5. Problems of biologics bioactivity/stability in controlled-release systems

5.1. Mechanisms of inactivation

In order to select materials and fabrication methods that promote the stability and bioactivity of peptides and proteins, it is important to understand the nature of these drugs and the mechanisms by which they lose efficacy. Proteins and peptides are composed of some combination of the twenty amino acids native to humans, which (along with environmental conditions) determine their secondary, tertiary, and quaternary structures. Their structures are stabilized by intramolecular and intermolecular hydrophobic interactions, hydrogen bonds between peptide bonds, electrostatic interactions, van der Waals forces, and disulfide bridges. The instability of protein is often a thermodynamic or kinetic event in which an environmental condition (e.g., heat, pH) causes a change in protein structure that can render it unable to interface in the same way with its target proteins, effectively rendering it biologically inactive.

The mechanisms underlying protein instability generally fall into one of two categories: chemical instability and physical instability (Fig. 2). Chemical instability involves processes that form or break covalent bonds, generating new chemical entities. Physical instability does not change the chemical composition, but the higher-order structure of the protein is altered, such as is the case with denaturation, aggregation, precipitation, and adsorption. Both types can suppress protein and peptide activity or render these drugs completely biologically inactive.

5.1.1. Chemical instability

Deamidation is the most common chemical degradation pathway for peptides and proteins and is responsible for much of the heterogeneity observed in mAbs [138]. Deamidation involves the hydrolysis of side-chain amide linkage in asparagine (Asn, especially Asn-Gly and Asn-Ser) and Gln, leading to the formation of free carboxylic acid. This subsequently leads to the conversion of neutral residues to negatively charged residues and potentially a different favored conformation, which may exhibit reduced bioactivity depending on the structural changes resulting from the change. Deamidation is mainly accelerated under neutral/alkaline aqueous conditions but can also occur while the material is in a solid state. As a result, improper storage—even in the dry state—can result in deamidation and potentially ineffective protein drugs. In addition to the effects of pH and protein sequence, other factors such as the ionic strength of the liquid and temperature alter the deamidation rate [139]. From a regulatory perspective, deamidation is also concerning as it generates process-related impurities and degradation products, which may increase immunogenicity

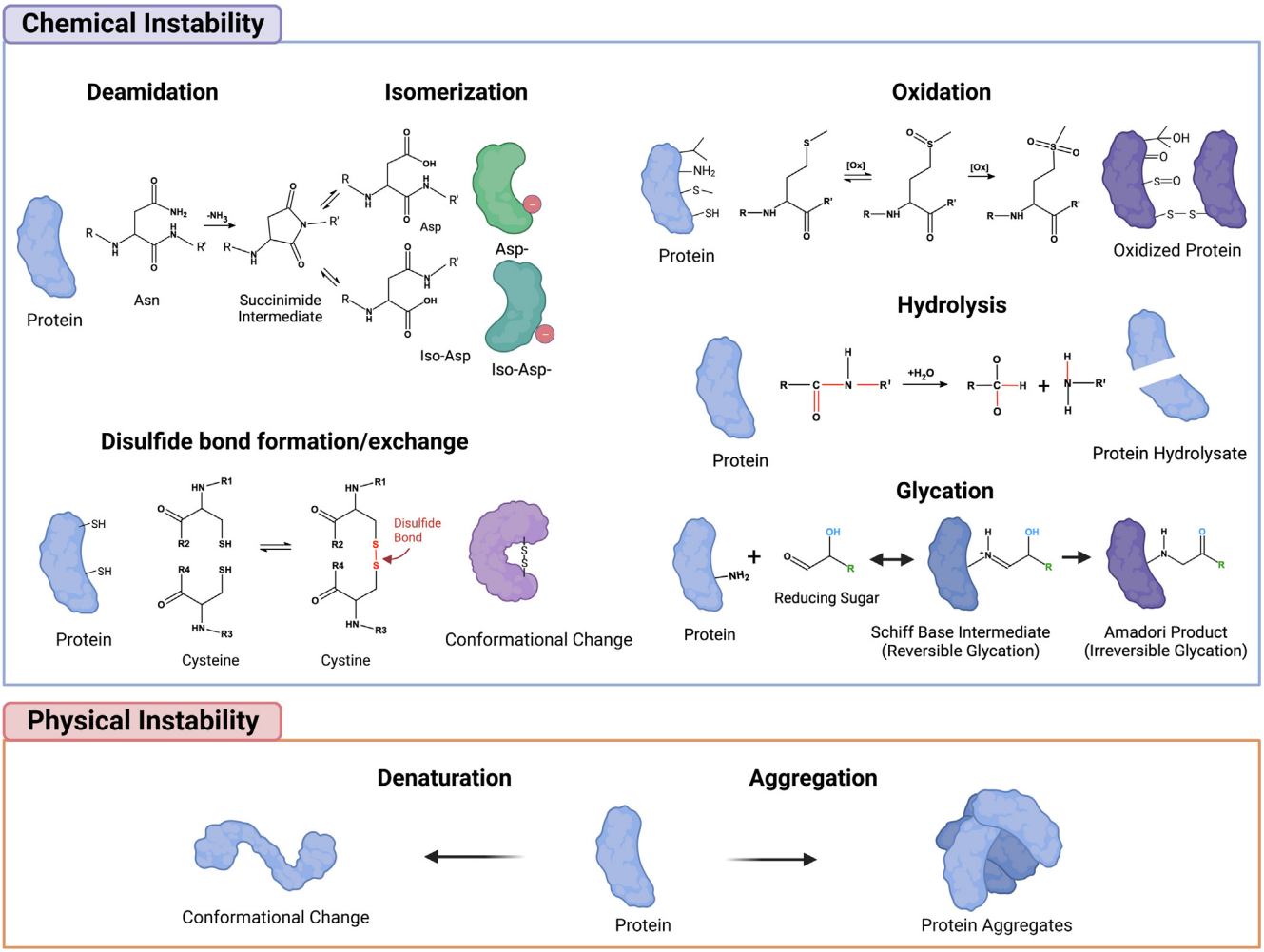

Fig. 2. Inactivation mechanisms of peptides and proteins.

[140]. The most effective approach to slow deamidation is to control the pH; the deamidation rate is the lowest when at pH 3–6 [141]; however, this could deleteriously affect the charge on some amino acids.

Isomerization is also a widely reported mechanism of degradation, especially for mAbs. The most common isomerization in antibodies is *iso*-aspartic acid formation, which results from direct isomerization of Asp and hydrolysis of a succinimide intermediate [141]. Unfortunately, isomerization is prevalent and difficult to control, and, as a result, many purified proteins contain significant amounts of isomerized products, either from deamidation or direct isomerization. The formation of the succinimide intermediate does not require water, which means that it can occur while the protein is stored in a solid state. Asp isomerization is one of the major degradation pathways for a lyophilized mAb, and the degree of isomerization necessarily increases with increasing temperature [142].

Oxidation and reduction are other major protein degradation pathways. Oxidation commonly occurs during protein isolation, synthesis, and storage. Oxidation may occur in Met, histidine (His), lysine (Lys), Trp, and tyrosine (Tyr) residues in proteins. The thioether group of Met is particularly susceptible to oxidation. Under acidic conditions, Met residues can be oxidized by atmospheric oxygen. Typically, the oxidation of amino acid residues is followed by a significant decrease in biological activity, as observed following the oxidation of Met residues in calcitonin, corticotrophin, and gastrin [143]. Glucagon is a notable exception, as it retains biological activity even after oxidation. The rate of oxidation is affected by both intrinsic and extrinsic factors. Intrinsic factors include the flexibility of the peptide backbone and the overall structure of the protein, while extrinsic factors such as pH and buffer type can affect oxidation rates as well [144]. Exposure to light has been recognized as another potential cause of oxidation (photooxidation); however, this can often be prevented by simply using light-attenuating packaging or adding light-absorbing materials to a liquid formulation. Notably, polysorbates (also known as Tweens®) can facilitate photooxidation and therefore may be undesirable for long-term storage purposes [145]. Minimizing oxygen exposure also helps to prevent oxidation, especially for Met. To prevent damage related to oxygen exposure, an inert packaging environment (e.g., nitrogen-filled blister pack or vial) may be appropriate. Limiting the solvent accessibility of oxidation-sensitive side chains is another strategy that has been shown to work for subtilisin and alkaline protease [146].

Acid-catalyzed hydrolysis of amide bonds in the protein backbone is another common source of chemical instability for protein pharmaceuticals, as this cleavage typically destroys or at least reduces protein activity. The vulnerability of peptide bonds to degradation depends on the residues involved. The hydrolysis of peptide bonds such as Asp-Gly and Asp-Pro leads to protein fragmentation [147]. Asn residues are unstable, particularly the Asn-Pro bond, compared to other residues, so some proteins and pep-

tides may be inherently less stable due to their primary structure. Since the reaction also involves intramolecular cyclization, proteolysis shows the same pH-rate profile and sensitivity to buffer catalysis as deamidation. Concerns about the consequences of hydrolysis of protein drugs are not limited to the proteins themselves. Excipients such as polysorbates are susceptible to hydrolysis, which might be due to the heat, acidic or basic conditions, and/or enzymes (e.g., esterase and lipases), thereby promoting hydrolysis of the ester bond [148]. The hydrolysis of sucrose and byproducts has also been shown to negatively affect the mAbs stability [149].

Chemical degradation may also occur due to cross-linking. Disulfide bond formation/exchange is a common crosslinking pathway that leads to chemical aggregation. Disulfide bonds may break and reform with incorrect pairings (e.g., cysteines on different protein chains forming a disulfide bridge instead of forming a bridge within the same protein chain). This typically results in an alteration in the three-dimensional structure followed by a resultant decrease in biological activity. Trimers and dimers can be formed through this mechanism. This reaction is concentration-dependent, particularly for oligomer formation, as higher protein concentrations increase the likelihood of aggregation. This degradation mechanism can occur readily during various controlled-release device processing steps [150]. For example, lyophilization of mAb IgG2a without excipients was shown to reduce the average number of thiol groups from 6.65 to 6.28 per molecule, suggesting a disulfide bridge formation [151]. An increase in environmental pH usually leads to a corresponding increase in disulfide bridge formation. Interestingly, disulfide crosslinking, resulting in the formation of dimers and trimers, has even been observed in a dry state after long-term storage of lyophilized or spray-dried antibodies [152].

Glycation—in which a sugar reacts with a free amino group—occurs when a protein is incubated in the presence of reducing sugars. Glycation has been shown to affect the binding affinity of mAbs, as well as the overall stability of the molecule, potentially due to changes in the charge [153,154]. Thus, formulation scientists tend to avoid using reducing sugars (e.g., glucose, lactose, fructose, maltose) in favor of non-reducing sugars, such as sucrose, trehalose, stachyose, verbascose, and raffinose.

5.1.2. Physical instability

Denaturation refers to protein unfolding and involves loss or disruption of the secondary and/or tertiary structure of the protein. Proteins can denature in response to various environmental insults, including temperature and pH changes, shear stress, and various other conditions, which can occur during the controlled-release device life cycle. Thermal denaturation is the most common stress and causes loss of protein globular structure due to elevated temperatures, typically at exponentially higher rates as the temperature rises. Although not always the case, thermal denaturation is often irreversible as the unfolded proteins rapidly associate to form aggregates. The same phenomenon can affect peptides, though their more limited higher-order structure makes them more likely to be able to return to their native bioactive conformation after heat is removed [155]. Proteins can also denature in the solid state, but their thermostability is often markedly improved when dry, frequently requiring temperatures that are very high, often above 150 °C [156]. Most proteins also display cold denaturation, in which proteins lose their tertiary and quaternary structure at low temperatures. The potential for cold denaturation greatly depends on the glass transition temperature (T_g) of the maximally frozen concentration (usually well below −20 °C), while cold denaturation happens when the environmental temperature reaches the T_g [157].

Protein aggregation is the most common manifestation of physical instability. For example, concentration-dependent antibody aggregation is considered the greatest challenge facing the development of antibody pharmaceutics. Protein aggregation can occur in both liquid and solid states, leading to reduced activity, the potential for enhanced immunogenicity, and the formation of dimers and oligomers. Aggregation mainly occurs as a consequence of protein–protein interactions and advances at a rate that is dependent on the solution's concentration, viscosity, temperature, ionic strength, and pH. It can be further increased through freeze–thaw cycles, shaking, drying, and other processes during protein formulation and storage [158]. Strategies to control protein aggregation include the addition of formulation components, solidification, modification of interaction sites, and the use of filters [159].

If aggregation happens on a macroscopic scale, it is called precipitation and can result in the formation of visible particulates in a solution. Interfacial adsorption may be followed by aggregation and precipitation. This behavior is irreversible, and the protein is partially or completely unfolded, almost certainly rendering it biologically inactive in addition to limiting its mobility after *in vivo* administration [159]. The formation of particulates has now become an important scientific and regulatory focus in the development of protein therapeutics. The extent to which aggregation and precipitation occur is defined by the relative hydrophilicity of the surfaces in contact with the polypeptide/protein solution. The presence of large air–water interfaces generally accelerates this process, so packaging could be designed to limit the impact of this mechanism of protein inactivation [144].

Peptides and proteins are amphiphilic in nature; hence they tend to preferentially distribute at air–water and air–solid interfaces. After adsorbing to one of these surfaces, they form some short-range bonds (van der Waals, hydrophobic, electrostatic, hydrogen, ion-pair bonds) with the surface, potentially resulting in denaturation [142]. The surface-induced instability depends on surface tension, the available surface area relative to the concentration and volume of the solution, surface hydrophobicity, and the intrinsic conformational stability of the protein. To overcome issues of interfacial adsorption, excipients, such as surfactants, can be used to compete with protein for adsorption onto the interfaces and surfaces.

In the pharmaceutical industry, nonionic surfactants, especially polysorbate 20 and 80 (also known as Tween 20® and Tween 80®, respectively), are frequently added to prevent or reduce unwanted adsorption and aggregation. However, it should be noted that nonionic surfactants might be associated with their own set of undesirable consequences. For example, polysorbate 80 can increase both protein oxidation and aggregation during long-term storage, though it has also been shown to prevent shaking-induced aggregation of IL-2 mutein [160].

5.2. Inactivation during controlled-release system preparation

5.2.1. Freezing and thawing protein stocks

Proteins in aqueous solutions are often frozen after they are produced to increase product stability, reduce the possibility of microbial growth, and alleviate foaming issues during transportation (Fig. 3). However, passing proteins through a freeze–thaw cycle is not without its consequences.

Cryoconcentration is one of the most common mechanisms through which protein destabilization can occur during freezing [150]. As the freeze-front advances during cooling, excipients and proteins are excluded from the ice-liquid interface. As a result, the concentration of the liquid bulk close to the ice crystals increases progressively during freezing. Freezing buffered solution can also cause changes in pH due to the selective precipitation of buffer components, which can result in protein destabilization.

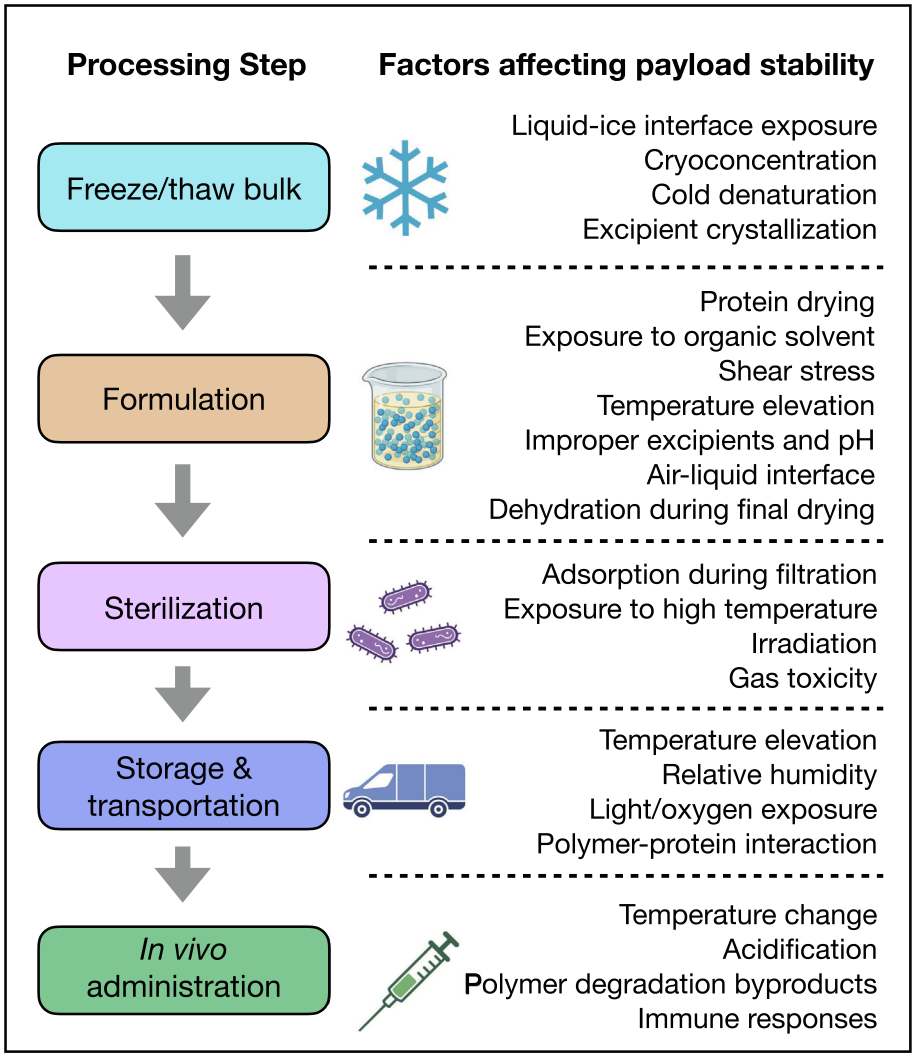

Fig. 3. Stressors during formulation, storage, and *in vivo* administration.

At the same time, increasing protein concentration increases the possibility of molecular collisions and may contribute to protein or adjuvant aggregation or precipitation [161]. The severity of cryocencentration is highest at slow rates of freezing. Therefore, one simple way to minimize freeze concentration effects is to reduce freezing times by increasing heat transfer from the container. Dendritic ice growth is also preferred to minimize consolute exclusion during freezing. This can be achieved by establishing directional heat flow and avoiding mixing during freezing. Mixing can be detrimental as it suppresses dendritic ice growth, making the ice-liquid interface flatter, resulting in an increased cryoconcentration [150].

Unfortunately, very rapid freezing can also be detrimental to proteins. During freezing, protein molecules can concentrate and unfold on the ice-water interface, leading to a loss in protein activity. In contrast, when freezing occurs very quickly—for example, submerging a sealed container in liquid nitrogen—smaller ice crystals are formed, resulting in a large ice-liquid interfacial area. Increased protein aggregation and decreased activity have been reported for liquid-nitrogen-based freezing systems [162]. However, fast freezing can also trap air released during thawing and may cause protein denaturation at air–liquid interfaces.

Inevitably, proteins that are stored in a frozen state must be thawed before they can be formulated into controlled-release systems. Thawing can cause further stress and damage to the protein, so the conditions under which thawing occurs are important for maximizing recovery. Slow thawing rates can result in ice recrystallization with small ice crystals growing into large ones, which can result in protein denaturation at ice-liquid interfaces and a corresponding loss in bioactivity. Like freezing, faster thawing rates are usually preferred for protein stability. While mixing during freezing may be detrimental to proteins, appropriate mixing during thawing is important for minimizing the impact of recrystallization and cryoconcentration.

Most of the stability issues discussed above occur when very slow freeze–thaw rates are applied, which is usually the case for uncontrolled cooling. Polycarbonate carboys are commonly used to freeze and transport bulk drug substances. Freezing is conducted by placing these carboys in walk-in or upright freezers at −30 or −80 °C. As a result, cryoconcentration becomes an important factor governing product quality in these containers. Controlled rate technologies such as Celsius® Paks or Cryovessels, which can accelerate and better control freezing and thawing rates by using a combination of small path lengths and increased flux for heat transfer, represent one promising approach to mitigating the decrease in protein bioactivity [163].

5.2.2. Protein drying

With few exceptions (e.g., non-degradable hydrogels, mechanical devices), controlled-release systems must be dried to avoid pre-

mature degradation for storage. Depending on the fabrication method, this can mean the removal of water and/or organic solvents. The type of drying process and drying parameters, however, can have key implications for protein stability and release system performance.

5.2.2.1. Freeze-drying. The process of freeze drying, or lyophilization, achieves almost complete removal of the water or low-to-mid-boiling point solvents in the drug product, except for the water that is presumably associated with the protein (typically < 1%). Since the presence of water is required for most covalent degradation phenomena, such as residue fragmentation and isomerization events, the process of lyophilization is often an effective approach for mitigating protein instability. However, this process requires the sample first to be frozen and then dehydrated, which are stressful to proteins, potentially resulting in the alteration of protein structure [164,165]. As aforementioned, during the freezing process, the protein solution may become saturated because of ice crystal formation, resulting in changes to solute concentration, pH, and ionic strength. Further, proteins may occasionally denature due to adsorption at ice/water interfaces. Cryoprotectants such as trehalose, sucrose, maltodextrin, and D-sorbitol are normally used to minimize denaturation during freeze-drying. While hydrogen binding partners in the form of water are stripped away from proteins during drying, these carbohydrates can serve as new partners for hydrogen bonding, enabling proteins to retain the same conformation and prevent potentially deleterious protein–protein hydrogen bonding that can cause aggregation. The mass of sugar needed to stabilize a certain mass of a protein can be hundreds or thousands of times greater, depending on the protein and type of sugar used [142]. Even then, the recovery of bioactivity is typically still incomplete. Other excipients such as polyanions, anionic phospholipids, cyclodextrins, amino acids, and salts also help prevent aggregation formation when added to a protein formulation prior to lyophilization, though the mass ratio of excipients to protein required is similarly high. It is important to note that this stabilizing effect would likely be lost if the stabilizing excipients were to crystallize out and not remain in the protein-containing amorphous phase. Non-reducing saccharides, such as sucrose and trehalose, typically remain in the same phase as that of the protein and are thereby able to exert their stabilizing effects [166]. Hydrogen bonding between excipient and protein is also thought to contribute to forming a robust glassy matrix.

5.2.2.2. Spray drying. In addition to being used for biodegradable microparticle preparation, spray-drying has been increasingly used to stabilize proteins by rapid vitrification in the presence of amorphous sugars. It has been used to produce particles for pulmonary and nasal drug delivery and as a potential processing method for vaccines. Spray drying involves several steps, including atomization, drying, and powder separation/collection. Atomization is a process by which the solution, suspension, or colloidal dispersion is sprayed to micron-sized droplets (1–200 μm) at a high velocity [167]. Then the spray comes into thermal contact with a heated, dry gaseous stream (e.g., dry air or nitrogen), causing it to dry before reaching a surface. The resulting powders are separated from a moist gas stream using a cyclone, electrostatic precipitator, or bag filter and collected in a holding chamber.

The atomization step involves not only shear stress but also high surface tension stress due to surface area expansion. Though spray drying can be used to produce stable protein powders, atomization can expose proteins to a larger air–water interface and lead to protein unfolding, resulting in aggregation. However, surface-related stresses can be mitigated with the addition of surface-active excipients or specific ions such as zinc, which have been shown to suppress the aggregation of insulin, hGH, and BSA during spray-drying [168,169].

Protein denaturation has been observed during spray drying due to dehydration. The addition of excipients (e.g., sugars, polyols, amino acids) described above is necessary to replace the hydrogen bonds previously provided by water. Although the drying air temperature may exceed 100 °C in normal conditions, thermal denaturation is most likely not the principal stress because the temperature of the droplet hardly exceeds the wet bulb temperature of water (approx. 40 °C), and temperature elevation is very short-lived [170].

5.2.2.3. Spray freeze-drying. Spray freeze drying (SFD) is a recently developed drying process that involves elements of both spray and freeze-drying. This process has been used to produce free-flowing powder of porous, micron-sized, dense protein particles with a high specific surface area and improved bioavailability. SFD has potential applications ranging from the dermal delivery of vaccines using needle-free ballistic injection devices to pulmonary delivery. The process includes atomization, rapid freezing, and primary and secondary drying. Instead of atomizing into a heated gaseous medium, the feedstock is atomized directly into a cryogenic medium in which droplets rapidly freeze, forming ice particles. The ice is then sublimated to dry out the particles. One key advantage of SFD is that sublimation and secondary drying of the frozen particles occur more rapidly than processes employing conventional freeze-drying due to the expanded surface area-to-volume ratio afforded by frozen particles [171].

5.2.3. Exposure to organic solvents

Organic solvents have been (and are) widely used in the preparation of particulate drug delivery systems and solid scaffolds, including emulsion/solvent evaporation, spray drying, solvent casting, electrospraying/electrospinning, and others. Even in the case of coaxial electrospinning, in which the interaction between the encapsulated protein and organic solvent is minimized, the protein at the interface can exhibit reduced bioactivity [172]. While organic solvents are almost universally problematic for maintaining the bioactivity of proteins due to protein unfolding, choosing the most suitable solvent can help to minimize activity loss when interactions occur. With the exception of nanoprecipitation, the selected organic solvent should be immiscible with water since the protein resides in the aqueous phase. In addition, in comparison to the aqueous phase, using an organic solvent with a higher vapor pressure is important to facilitate organic solvent removal during subsequent processing steps. The most common solvents used to dissolve PLGA are dichloromethane (DCM), acetone, and ethyl acetate, which all have boiling points substantially below the boiling point of water, though acetone is water miscible. Other organic solvents are chloroform and acetonitrile, but they are more toxic and have more limitations [173,174].

The interface between water and the organic solvent is a crucial area where proteins can lose their activity due to protein unfolding that contributes to denaturation and aggregation. The formation of the primary w/o emulsion, and to a lesser extent of the secondary w/o/w emulsion, plays a major role in protein denaturation during particle preparation using that fabrication method. It has been shown that glucose oxidase loses 28% of its activity during the first emulsion and an additional 20% during the second emulsification step, whereas the cumulative activity loss after solidification, centrifugation, and freeze-drying was < 4% [175]. The nature of the organic solvent has an impact on protein stability as well. Ethyl acetate usually induces less denaturation than DCM, depending on protein characteristics [176]. Acetone was proven to have a protective effect to enhance protein C and nerve growth factor stability, and the blending of acetone with DCM also showed improved

stability than DCM alone, owing to the limited contact between DCM and protein C due to the reduction of surface tension between the organic and water phases since acetone is miscible with both DCM and water [177,178].

To reduce solvent-induced protein denaturation, the exposure of protein solutions to organic solvents or hydrophilic/hydrophobic interfaces should be minimized. There are several potential approaches that can be used to achieve this, such as protein stabilization prior to/during microencapsulation involving organic solvents and microencapsulation using water-soluble materials.

Stabilizing protein prior to or during microencapsulation involving organic solvents is one way that formulation scientists have sought to combat the loss of protein bioactivity during solvent interaction. Chemical conjugation or protein fusion designed to increase the *in vivo* half-life of the protein has shown the potential to enhance protein stability due to steric hindrance and/or changes in surface properties. Conjugation moieties include PEG, glycans, and other hydrophilic substances. PEGs are the most widely used conjugation agents and have been found to stabilize proteins against different stresses, such as thermal stress, pH-induced, and protease-induced degradation and oligomerization [179,180]. Similarly, glycosylation of proteins (e.g., maltodextrins, lactose, dextran, and modified trehalose polymer) has been found to inhibit protein aggregation [181,182]. Conjugation of albumin with more hydrophilic substituents alters the intermolecular interactions, which reduces protein aggregation [183]. Of course, it is important that none of these modifications substantially impact bioactivity or deleteriously alter the immune response to these proteins.

Converting proteins to solid particles before encapsulation is an effective way to protect delicate proteins from organic solvent since it limits organic solvent exposure to proteins on the surface of the solid protein, which may be only a very small fraction of the proteins contained in the solid particulate. Protein precipitation using bivalent metal ions is one well-known way to do this. For example, hGH can form complexes with zinc ions in its native form [184]. This effect can be leveraged to introduce a thermodynamically favored state for some proteins but raises structural constraints for others, which has been shown to facilitate the aggregation of other proteins, such as the erythropoietin [184].

The primary encapsulation of proteins into particulates of sugars, polysaccharides, or other water-soluble polymers before microencapsulation has also been widely reported [185–188]. Among these sugar-based protein stabilizers, cyclodextrin and heparin have been suggested as particularly advantageous due to their energetically favorable interaction with proteins [189]. In general, direct lyophilization of proteins with polysaccharides results in larger, irregular, or fibrous particles that must often be broken down into fine particles suitable for secondary encapsulation into a microparticle; however, the process of milling, which can reduce the particulate size, can be hazardous to proteins [190].

To protect proteins from denaturation during emulsification, a variety of excipients can be added into the internal protein solution, such as carrier proteins (e.g., BSA and gelatin) [191,192], sugars (e.g., trehalose, maltose, lactose, and sorbitol) [193,194], and PEG [195]. These excipients either decrease the protein adsorption at the water/organic solvent interface by competitive adsorption from additives (PEG, carrier proteins) or shield the protein from the interface by forming a hydration layer over the surface of the protein of interest. Of course, the addition of carrier proteins may be accompanied by a potential immune response, which may not be desirable.

Various hydrophilic particulate systems have been developed to avoid the protein stability issues raised by the use of organic solvents. Generally, these particles are formed from water-soluble materials and their dissolution is slow downed by the deposition of materials onto the surface, which blocks diffusion or interior crosslinking through covalent, ionic, or hydrophobic interactions. Polyarginine coating with hGH microcrystals helps extend the dissolution time of protein crystals, showing that a once-weekly injection of the crystalline formulation functioned as well as seven daily soluble injections of the traditional formulation in rat and monkey models [196]. Hahn et at. also demonstrated oil-coated hyaluronate MPs for the controlled release of hGH by spray drying an aqueous co-solution containing sodium hyaluronate, hGH, and lecithin [197,198]. These approaches made of water-soluble materials are suitable for protein encapsulation; however, the extension of release has largely been limited to 1 week, which may not be sufficient for many applications. Other approaches are needed for long-term release. ProMaxx® (Epics/Baxter) is another hydrophilic particulate system that has been developed to achieve sustained protein release. These particles are prepared by dissolving proteins in an aqueous PEG solution at an elevated temperature and then cooling the solution [199]. Formation of ProMaxx® particles involves hetastarch (a polyanionic polysaccharide), divalent metal cations, a chemical crosslinking agent, and the addition of heat, each of which could negatively impact protein stability.

Gas antisolvent (GAS) & supercritical antisolvent (SAS) techniques have served as alternative methods of preparing ultrafine protein powders, involving pressurizing a solution into powder using either a dense gas or supercritical fluid. For example, in one study, the GAS CO_2 precipitation method allowed insulin encapsulated in polylactic acid nanospheres to retain greater than 80% of its hypoglycemic function [200]. Another promising approach is to remove the solvent before incorporating the protein into the device. Reinhold and colleagues described a self-healing method of loading microspheres without the use of an organic solvent. In brief, trehalose-doped into PLGA microspheres was leached out to generate pores, which were then back-filled with lysozyme after solvent evaporation. After heating the particles above the T_g (~30 °C) of the polymer, the pores closed, encapsulating the lysozyme [201]. This post-solvent approach has also been used to encapsulate proteins in microfabricated polymeric particles. For example, a method termed the Particles Uniformly Liquified and Sealed to Encapsulate Drugs (PULSED) employs solvent to cast polymer films, but removes the solvent under vacuum and heat during compression into a mold to form a cup-like base, fills a protein solution into the cup under ambient conditions, and uses a non-contact heating method to collapse the top of the cup inward and completely encapsulate protein in a solvent-free manner [202]. This process was used to encapsulate horseradish peroxidase and bevacizumab, a monoclonal antibody, which each demonstrated no loss in activity with or without excipients depending on the polymer-dependent heating temperature required.

5.2.4. Agitation

Insufficient mixing during the addition of excipients could alter product quality due to solution inhomogeneity and result in the final drug product being unable to meet its specifications. Excessive mixing, on the other hand, could create large shear stress that can denature proteins. Shear stress considerably increases the probability that dissolved proteins will encounter air/water and water/organic solvent interfaces, thereby promoting hydrophobic interactions that might lead to further aggregation and/or protein unfolding. Although some fabrication methods avoid substantial agitation of the protein solution, several prominent techniques, like double emulsion/solvent evaporation, add considerable kinetic energy to the system. The sonication of liquids produces acoustic cavitation, in which bubbles rapidly expand and collapse, resulting in a local, extreme increase in temperature and pressure, termed "hot-spots," and the formation of free radicals, which can reduce

protein bioactivity [203,204]. In general, the trend of energy input and therefore, a risk of protein unfolding is that probe sonication induces more damage than homogenization, which induces more damage than vortexing. It has been demonstrated that the energy required to unfold proteins is generally in the range of 5–20 kcal/-mol, which can be easily reached by hydrophobic interactions and water–oil interfacial tension [189]. Relatively low-energy input agitation, such as shaking, has even been shown to induce insulin aggregation [205]. Similarly, IgG1 antibody agitation led to the formation of soluble aggregates and subvisible particles [206], and shaking a solution of recombinant factor XIII or vortexing a solution of hGH both resulted in large populations of non-covalent, insoluble aggregates [207]. However, protein interaction with the air–liquid interface does not always result in protein degradation. The extent of protein aggregation and the species formed as a result of that agitation depend on the individual protein's characteristics, which is ultimately a function of its primary structure as well as the intensity and duration of interface interaction. The surface area available, surface tension, and the surface activity of the protein are all important factors and are affected by various properties, including molecular size, charge, hydrophobicity, stability, and structural features [208].

Mechanical stressors such as stirring and shaking in the presence of various contact surfaces can also induce physical protein destabilization. The very hydrophobic air–liquid interface is ubiquitous during protein encapsulation manufacturing but can be minimized based on the vessel selected. Besides avoiding unnecessary agitation and minimizing the surplus air during fabrication, the addition of excipients can also help reduce agitation-induced aggregation. For example, poloxamer (Pluronic F-68) and polyoxyethyleneglycol dodecyl ether are able to prevent interfacial-induced aggregation of hGH and tetanus toxoid, as summarized in Table 2 [209,210].

5.2.5. Temperature elevation

Generally, most proteins remain fairly stable within a specific temperature range that is dependent on their unfolding temperature (effectively T_m). Thermostability can be assessed by studying the loss of activity over time at a particular temperature of interest. For most proteins, this range is 40–80 °C under aqueous conditions [211], indicating that the proteins denature and form misfolded oligomeric and aggregated species above these temperatures, though the transition is probabilistic rather than instantaneous and uniform. Most fabrication methods used to create biodegradable polymeric systems that do not use organic solvents use heat and/or pressure instead. The T_m and T_g of PLGA generally range from 80 to 160 °C and 33 to 60 °C, respectively [212,213], depending on the stereochemistry of the lactic acid units and the percentage of glycolic acid in the copolymer. PLGA with a 50:50 ratio of lactic acid-to-glycolic acid exhibits the lowest T_m and T_g and is most widely used. Molecular weight also affects the thermal properties of a material; higher molecular weights typically require higher temperatures to mobilize. One approach used to encapsulate drugs within a polymer matrix is melt extrusion, which usually requires a high temperature to melt the polymer and potentially high levels of shear imparted by the conveying screw. These temperatures are typically in the range of 80–105 °C, which would denature most proteins when in solution [179,214]. Viscosity modifiers such as PEG or sugars can be used as plasticizing agents to lower the temperature required for thermal processing [215,216], but may also affect protein release kinetics. Additionally, it is generally desirable to have biodegradable materials remain solid *in vivo*, effectively setting a lower bound on thermal transition temperatures at 37 °C. Processing proteins in a solid state rather than in solution improves thermal stability and allows the proteins to be processed at the elevated temperatures necessary for the melt encapsulation [217]. In the absence of the plasticizing water molecules, solid protein exhibits T_g values of 130–185 °C, which is above the T_m and T_g of most synthetic biodegradable polymers. However, proteins can still exhibit denaturation and aggregation depending on the properties of the proteins and additives used.

The aggregation of proteins during melt processing can also lead to uneven distribution within the polymer matrix, potentially resulting in increased burst release and decreased protein stability [218]. Modifying the protein surface properties with amphiphilic or hydrophobic moieties has increased the release rate relative to the unmodified proteins [219]. The aggregation of proteins within the PLGA matrix can be mitigated by controlling the initial particle size of the solid proteins before melting extrusion with PLGA. A smaller protein particle size generally leads to less aggregation and a more even distribution of proteins within the matrix. The protein particulate size can be controlled by breaking up particles after drying [213]. Spray drying with stabilizing additives during protein preparation enhanced thermal stability and sustained release after melt extrusion with PLGA. Ball milling has also been used to mechanically break protein particles and reduce the aggregation of BSA [220], lysozyme, and glucose oxidase within polymer matrix [217,221]. Appropriately milling proteins can enhance the retention of secondary structure and enzymatic activity after melt extrusion, while aggressive milling generates excess physical stress and thermal energy, resulting in protein denaturation and a potential reduction in protein bioactivity.

Table 2
Common excipients used to stabilize protein and peptide drug formulations.

Purpose	Type	Common Excipients
Cryoprotection/ lyoprotection	Sugars and polyols	Trehalose, sucrose, lactose, mannitol, sorbitol
	Polymers	Dextran, PVP, starch derivatives
	Surfactants	Polysorbates 20 and 80
	Proteins	Albumin
	Amino acids	Arginine, valine, threonine
Anti-adsorption/ aggregation	Surfactants	Poloxamer, polysorbate 20 and 80, brij 35
	Polymers	Dextran, Poly(ethyleneglycol)-b-poly(L-hisditine)
	Polyalcohols	Glycerol
	Proteins	Albumin, lactalbumin
Oxidation protection	Antioxidants	Ascorbic acid, ectoine, glutathione, monothiogycerol, morin, poly (ethylenimine), propyl gallate, vitamin E
	Chelating agents	Citric acid, EDTA, hexaphosphate, thioglycolic acid
Controlling pH	Buffer agents	Phosphate, bicarbonate, sulphate, nitrate, acetate, chloride, pyruvate, Tris, glutamate, glycine
	Antiacids	$Mg(OH)_2$, $MgCO_3$, $ZnCO_3$
Stabilizer	Amino acids	Alanine, arginine, aspartic acid, glycine, histidine, lysine, proline
	Sugars	Glucose, sucrose, trehalose
	Polyols	Glycerol, mannitol, sorbitol
	Salts	Potassium phosphate, sodium sulphate
	Chelating agents	EDTA, hexaphosphate
	Ligands	Phenol, zinc
	Polymers	Cyclodextrin, dextran, PEG, PVP

5.2.6. Drying in controlled release systems

The last stage in microparticle manufacturing is typically drying, during which residual water and/or organic solvents are removed. This step is especially essential when using hydrolytically degradable materials in order to prevent on-the-shelf degradation and premature release due to high residual water content. Depending on the previous processing steps, drying can be achieved by air-drying, vacuum-drying, or lyophilization. Lyophilization is most commonly used when water is used in

one or more previous stages of the preparation process. The effects of lyophilization on protein integrity and the excipients used for preventing protein denaturation and aggregation have been discussed in Section 5.2.2. Another way to remove water/solvent from products containing components sensitive to heat or oxidation is vacuum drying, which avoids freezing and excessive thermal stress by lower boiling point temperature. However, if a protein payload is already dried before formulation, thermal denaturation is not usually a problem. Dehydration is the ultimate result of any drying method; therefore, sugars are also often added as a "water substitute" to preserve the protein's native structure upon water extraction [222].

Besides the direct effects on protein stability, drying processes also have an influence on the physical properties of microspheres, especially surface morphology and internal structure [223,224]. One study showed that freezing the final microspheres either during lyophilization or vacuum drying resulted in cracking of the microspheres, while air drying at 2–8 °C minimized structural defects as well as reduced the initial burst release of the subunit vaccine gp120 [225].

5.2.7. Sterilization

Proper sterilization is critical to the safe *in vivo* use of drug delivery systems. When sterilizing a solution, sterile filtration can be performed with a 0.22 μm filter to ensure that the bulk is free from micro-organisms. However, this approach is impossible for most delivery systems, which are larger than 220 nm in diameter. Instead, devices must be either prepared in a sterile manner, which is very expensive, or sterilized after the device has been fabricated, which has the potential to damage encapsulated proteins.

The FDA acknowledged four main categories of sterilization methods applicable to polymers: Class A, class B, novel, and aseptic approaches. Classes A and B and novel methods utilize terminal sterilization applied to the completed product, which includes gamma-irradiation, electron-beam irradiation, heat and steam sterilization, hydrogen peroxide gas plasma, and ozone treatment. In addition to causing encapsulating proteins and peptides to denature, high sterilization temperatures like autoclaving and dry heat sterilization could result in melting, plastic deformation, and degradation of polymeric delivery systems. Similarly, gas sterilization is typically not appropriate for protein-containing delivery systems due to the high toxicity of residual elements in porous materials and protein inactivation since this is the mechanism by which this process imparts sterility [226]. Physical sterilization, like gamma or beta irradiation, is more commonly used for proteins and peptides since these impart sterility primarily by damaging nucleic acids. This approach can partially degrade encapsulating polymers, such as PLGA, which affect the drug release profile; however, if this degradation is reproducible, an appropriate version of the polymer (i.e., higher molecular weight) could be selected in anticipation of partial degradation during sterilization [227–229]. Other research has shown that gamma irradiation could form hydroxyl radicals in the presence of water and oxygen, potentially leading to changes in protein function [230].

Supercritical CO_2 ($scCO_2$) is an emerging method of sterilization that has been explored for scaffold and implant sterilization [231]. CO_2 has been used as a green industrial extraction solvent due to its chemical stability, relatively low toxicity, and low environmental impact. Some studies have shown that the $scCO_2$ can penetrate microbes' cellular compartments, thus perturbating the intracellular pH and enzyme structure [232]. However, the exact mechanism of action for microbial destruction has not been fully elucidated. To achieve better bactericidal effects, $scCO_2$ is often combined with one or several additives, such as peracetic acid (PAA), H_2O_2, or acetic anhydride, to reduce the heat and pressure required [233]. However, these additives are likely to negatively affect protein bioactivity. Water can also be added to enhance $scCO_2$ diffusion if compatible with the target polymers [232].

Another potential approach is using materials that exhibit antimicrobial properties, e.g., chitosan and its derivatives, as well as quaternary ammonium functionalized polymers [234,235], though the ability to fully eradicate the presence of microbes through simple material selection is unclear. For many parenteral drug delivery systems, the upstream sterilization of individual formulation components via sterile filtration or gamma sterilization prior to assembly and subsequent aseptic preparation may present the only viable path to ensuring sterility without damaging encapsulated biologics.

5.3. Inactivation during sample storage

5.3.1. Temperature

Low storage temperatures reduce the likelihood of protein refolding and slow the rate of most reactions, which may be relevant for both protein stability and premature encapsulant degradation, making them desirable for delivery systems storage. However, repeated freeze–thaw cycles should be avoided since they induce protein adsorption to container surfaces, interact with air–water and ice-water interfaces, and can cause buffers to crystallize, leading to subsequent pH alterations. Additionally, it may be the case that some delivery systems cannot go through a freeze–thaw cycle without compromising their function. The rate of freeze and thaw should also be considered, as described in the discussion of the bulk protein preparation. Lyophilization offers an alternative long-term storage strategy for protein delivery systems but is often associated with an immediate decrease in protein bioactivity during the transition. Once lyophilized, however, there should be little damage to the protein or encapsulating material during storage, assuming it is maintained in a dry environment free of reactive gasses and shielded from light. Aside from the compatibility of the protein and delivery system with freezing, the duration of storage should also be factored in since short-term storage at 4 °C might be favored to avoid freeze–thaw damage whereas it is "worth" subjecting the encapsulated protein to a freeze–thaw cycle in exchange for a lower rate of bioactivity loss while sitting on the shelf.

Most vaccines are temperature sensitive and require cold-chain maintenance, which remains a major challenge facing distribution in remote regions and low- and middle-income countries. Developing technologies that could notably minimize resources needed for the distribution and administration of vaccines would likely reduce rampant undervaccination and the 1.5 million deaths that occur from vaccine-preventable infectious diseases each year [236]. Like other proteins, protein vaccines show significantly increased thermal stability in the dry state. For example, a lyophilized vaccine for anthrax and ricin retained its full immunogenicity after 15–16 weeks of storage at 40 °C [237,238]. Spray-dried and foam-dried vaccine formulations were also stable for more than 1 year at 37 °C [239]. Another common approach to thermally stabilize vaccines in solution is through the addition of stabilizing adjuvants. For instance, the immunogenicity of liquid adenoviral vaccine formulations could be maintained up to 10 days at 37 °C by adding PEG, gold nanoparticles, and sucrose [240]. The combination of pullulan and trehalose also provided long-term stabilization for dried viruses, which can be preserved for up to 3 months at 40 °C [241]. Irnela et al. evaluated 30 combinations of buffers and excipients on the recovery of adenovirus-based Ebola vaccine upon reconstitution of a peelable film matrix for sublingual and buccal delivery [242]. They found that preparations made with tris buffer with low and medium base concentrations showed the highest recoveries—greater than 90%—while the formulation using sorbitol as the binder increased that to 97%. Rehydrated films

containing surfactant retained 100% of the original titer after 84 days of storage at 4 °C and 56 days at 20 °C.

In addition to potentially compromising the activity of the protein payload, the storage temperature may also affect the stability of the materials controlling release, which is potentially problematic for drug delivery systems to retain their expected performance. The T_g and the polymer's enthalpy of relaxation are affected by PLGA MPs storage at high storage temperature, which changes the polymer's glassy state from amorphous to crystalline, resulting in shape deformation that would likely alter the drug release profile [243]. Physical aggregation and shape deformation were observed following storage at 40 °C/75% RH for 3 months. Surface scaling and melting were also observed in PLGA MPs at high temperatures (37 °C and 45 °C), whereas this was not observed when they were stored at 25 °C [244].

5.3.2. Humidity and moisture level

In addition to temperature, relative humidity (RH) is an important parameter for storage conditions. Water molecules absorbed into an amorphous polymeric matrix plasticize the system, leading to an increase in polymer chain mobility and a decrease in the effective T_g of the polymer [245]. A significant decrease in the T_g of PLGA/PVA hydrogel composites was observed upon incubation at 40 °C/75% RH for one week but not observed in the solid PLGA [246]. This difference was attributed to the relative hydrophobicity of PLGA, which absorbs water much slower than a hydrogel. However, at the same moisture level (75% RH), the composite showed a lower T_g when stored at 40 °C than at 25 °C, revealing that the water absorption strongly depends on storage temperature.

In addition to the plasticization effect, the presence of moisture also induces the hydrolytic degradation of PLGA and other hydrolytically debatable polymers. After storage at 40 °C/75% RH for 1 month, the T_g of PLGA/PVA composite quickly decreased to a value below 40 °C, and the microspheres were rubbery. Protein and particle stability are worst when stored in a humid environment and at high temperatures. Hydrolytic degradation progresses slowly at low temperatures, and dry formulations offer favorable stability even at high temperatures, but in combination, heat and humidity can both rapidly degrade the particle and cause the protein to lose its activity.

5.3.3. Concentration

During long-term storage of dispersions, aggregation can occur [247]. It has been shown that particle dispersions at high concentrations resist aggregation during storage, whereas low-concentrated ones aggregate more readily. One explanation that has been proposed for this counterintuitive finding is that in low-concentrated particle dispersions, the particles are freely diffusible, enabling them to collide and aggregate. In the highly concentrated dispersions, the particles are fixed in the pearl-like network diffusion, and subsequently, aggregation is reduced [248].

5.4. Inactivation after administration

Even after administration, there are many potential insults that can compromise the clinical value of controlled-release protein delivery systems, including elevated temperature (i.e., body temperature) and pH due to polymer degradation products. After administration, excipients may lose their stabilizing effect due to dilution or separation from the therapeutic protein. The latter may occur due to rapid tissue distribution and elimination at the injection site or result from differences in the duration of release between excipient and payload release owing to unequal molecular weights, water solubility, or charge. This can leave the protein, which is typically larger than excipients, unprotected in the delivery system at 37 °C.

5.4.1. Polymer degradation

Proteins microencapsulated in biodegradable polymers must survive potentially hazardous conditions associated with the degradation of the encapsulating material. For example, inside sustained-release PLGA and other polyester microspheres, the hydrophobic environment of the polymer matrix and acidic polymer degradation products can alter the environment and cause proteins to favor non-native conformations. Stabilizers, such as sugars or salts, are generally soluble and rapidly diffuse out of the MPs, leaving those proteins unprotected in a hydrophobic polymer matrix. PLGA degrades into oligomers and monomers of lactic and glycolic acid, which depending on particle size, can be entrapped within the matrix or slow to diffuse out, resulting in a substantial local drop in pH. This acidic environment has been reported to be as low as pH 1.5 for larger particles where degradation products must travel a longer distance to escape, further adding to the potential for protein inactivation. The addition of magnesium hydroxide and other antacids (e.g., $MgCO_3$, $ZnCO_3$) within the microsphere has been shown to negate the adverse effects of low pH [249]. Magnesium hydroxide is particularly interesting because it is relatively insoluble in water at neutral or basic pH, but is soluble at acidic pH and effectively acts as a buffer against polymer degradation product-mediated acidification. This is potentially important because it prevents solid magnesium from escaping the particle due to diffusion prior to protein release—as many readily water-soluble salts would. Aside from salt buffer solutions, other protein stabilizers have also shown abilities to inhibit pH changes, for example, dermatan sulfate (DS), a glycosaminoglycan, has been shown to shield proteins at a pH of 3 due to the polysaccharide's favorable interaction with positively charged proteins below their isoelectric point [250].

Another approach to avoiding degradation-associated pH changes is to blend hydrophilic polymers into PLGA matrix. This increases the permeability of the PLGA matrix during protein release and helps release or alleviate the acids generated by degradation. It has also been reported that blending PEG into PLGA MPs helped reduce entrapped acidity [251]. The use of cationic polymer excipients Eudragit E, poly(l-lysine) (PLL), and branched polyethylenimine (bPEI) has also been reported to help stabilize IPV antigens in PLGA microspheres [252]. Moreover, since hydrophilic polymers are not released from PLGA MPs as rapidly as small molecular sugars, they are retained in the matrix during the prolonged release period and protect proteins from adsorption onto PLGA. Dispersing solvent-insoluble polysaccharide particles into PLGA microspheres is another method to blend hydrophilic polymers into a hydrophobic matrix [253]. Of course, the effect of these methods on protein release kinetics must also be considered.

Although the most well-studied degradable polymeric remains PLGA, there are other biodegradable and biocompatible materials that can be used to avoid or mitigate pH issues that undermine protein stability. For example, poly(lactide-*co*-hydroxymethyl glycolide) (PLHMGA) is more hydrophilic than PLGA, enhancing the diffusion of water into the polymer matrix [254]; however, it will also release the protein cargo more quickly. Another choice is to replace PLGA with highly degradable but comparatively water-impermeable polymers, such as polycaprolactone or polyanhydrides. Exceptionally hydrophobic materials will degrade exclusively at their surface through a process known as surface erosion rather than uptaking water throughout their volume and undergoing bulk degradation. The benefit to these surface-eroding particles is that, even if degradation products are not neutral, they will be produced only at the interface with the well-buffered body, thereby avoiding any potential for the internal accumulation of degradation products that change local pH.

5.4.2. Temperature

As with delivery system preparation and storage, post-administration damage due to temperature is also an issue. Unlike these previous stages, however, there is, at present, no viable strategy to prevent the temperature of an internal device from approaching body temperature over a period of time relevant for controlled-release systems. Because long-term *in vivo* residence prior to release at elevated but not extreme temperatures is inevitable, it is important to assess strategies and formulations that promote protein stability. Over time, proteins stored at 37 °C will degrade, though the rate at which this occurs is highly variable [255]. Some studies have shown that a significant amount of degradation occurs *in vivo* prior to the release of the protein from microspheres [256]. In general, the degradation half-life of a protein decreases with increasing temperature. Unfortunately, as mentioned in the previous section, excipients added to stabilize the protein against *in vivo* degradation may diffuse out the microspheres faster than the protein. PEG or polysialic acid can be modified to improve the circulation time of fast-degrading proteins; however, there would need to be confirmation that modified proteins retain their bioactivity [257]. Additional methods for improving *in vivo* thermostability include hyperglycosylation or introducing mutations within the protein to alter its structure.

5.4.3. Immunogenicity and injection-site

As with the long-term delivery of therapeutic agents, which might last from days to months or even years, controlled-release systems are engineered to reside in the body for an extended duration, which increases the risk of adverse reactions, while the generation of antibodies is essential for a subunit vaccine, antibodies to a therapeutic protein may result in a loss of efficacy, autoimmune responses, and other adverse side effects. If a heterologous protein is administered to humans, which has different chemical composition from the human form, it will invoke an antibody response. A microsphere formulation containing the heterologous protein may further enhance this response by providing a constant stimulus. Naturally occurring antibodies to phospholipids and cholesterol are widely spread, leading to antibody reactions to lipid-based carriers [258].

Polymers contain repeat units, which may trigger B cell activation and hence antibody production. Previous studies also have indicated that PLGA or PLA microspheres may act as an adjuvant [259]. Anti-PEG antibodies have been observed in patients treated with PEGylated proteins and found to be correlated with accelerated clearance and reduced activity of the drugs [260].

However, there are limited data on the immunogenicity of controlled-release systems, largely because a relatively small number of products have reached the clinical stage, and most of the ones that have are anticancer drugs, which are unlikely to yield strong antibody responses. It's hard to predict the immunogenicity of the drug delivery system. Nevertheless, scientists should consider the risk of antibody formation in the design of drug delivery systems, especially for immunosuppressive drugs. Multiple epitope arrays clustered at the surface of the formulation is a particular risk factor. Ligands such as mannose that are recognized by APCs should especially be avoided, except for vaccination purposes [261].

The route of administration is also a major influence on the risk of immune responses. Subcutaneous administration represents the easiest way to generate antibodies, complement activation and platelet activation are also clinically relevant to intravenous administration. The dose administrated also influences the local events at the site of injection. In particular, the mass of administration is often limited for subcutaneous injection. Dilatancy effects might occur during the microspheres' flow through the needle, causing the microspheres to clog the needle with incomplete injection. Excipients in the suspending vehicle, such as CMC, dextran, or sorbitol, are often useful in preventing the agglomeration of microspheres [262].

6. Perspectives and conclusions

6.1. Protein and peptide stabilization overview

At present, there is no one-size-fits-all strategy to maintain protein and peptide bioactivity through delivery system preparation, storage, and *in vivo* release. Instead, the selection of processes, materials, and storage conditions are dependent on the properties of the specific protein or peptide as well as the release kinetics that are desired. Nevertheless, there are some core principles that can be gleaned from previous studies that can increase the likelihood of maintaining protein stability throughout a controlled-release system's product life cycle.

6.2. Inherent protein and peptide stability

The selection of what protein to deliver is critical due to inherent differences in protein stability. Due to their short length and reduced higher-order organization, peptides are typically more robust than proteins. Unlike protein conformations, which can readily become locked into a new conformation that is not readily reverted back into its normal structure when returned to a favorable aqueous environment, peptides may more readily revert to their bioactive conformation. Nevertheless, maintaining the primary structure can still be a concern. Peptides (and proteins) containing high degrees of Asn, Gln, Asp, Met, His, Lys, Trp, and Thr are more prone to deamidation, oxidation, isomerization, and peptide bond hydrolysis, which could compromise primary structure and function, especially if they are in the region known to interact with another biological molecule (e.g., a binding pocket).

Proteins and peptides whose native conformation is highly energetically favorable relative to the alternative, biologically inactive conformations will require more significant changes in the environment to cause the protein to change its folding pattern. Proteins with alternative conformations that are readily favored will be more difficult to stabilize. Additionally, those that rely on disulfide bonds will be difficult to stabilize in reducing environments. Although proteins are less stable than peptides, multiprotein assemblies are even more unstable as they typically require the retention of multiple proteins in their native conformation to retain their activity. The poliovirus vaccine is a good example of this since there are sixty copies of each of the four structural proteins that must remain in their native conformation to confer protection against the wild-type pathogen. Environmental insults that cause the structure to change hide epitopes that are necessary for neutralizing antibody formation and render the vaccine effectively useless. However, if there is one favorable aspect of the inactivated poliovirus vaccine, it is that the structure is crosslinked, which can provide some additional resistance to conformational changes.

Of course, the choice of peptide or protein will largely depend on the target application. In some cases, multiple proteins can serve similar purposes—perhaps by acting at different points along a pathway—in which case the more stable protein might be preferred. However, this is likely not true in all instances, in which case protein engineering could be employed to make a protein inherently more stable may be possible in these cases. However, even if there is only one protein or peptide appropriate for a target application, there are other processing and material modifications that can be made to improve protein stability through *in vivo* release.

Finally, in some instances, the conformation may not be very important at all. For example, the antigen processing pathway in the adaptive immune system readily digests proteins and peptides into shorter peptide fragments, which are then presented to T cells. Therefore, for applications that primarily require a cellular immune response (e.g., vaccines for cancer and intracellular pathogens), excessive measures to maintain native peptide or protein primary structure and conformation may not be necessary.

6.3. Processing stability

Each controlled-release system preparation method presents a unique combination of stressors that can compromise protein and peptide function. Exposure to organic solvents, heat, and physical agitation are among the most common of these stressors. The only device preparation method that fully avoids these stressors are aqueous systems, such as hydrogels. However, hydrogels are limited in other ways downstream of preparation. Specifically, because of their hydrated nature, hydrogels also provide paths for proteases to infiltrate and degrade proteins prior to release while simultaneously posing challenges for the retention of excipients since these can often readily diffuse out of these same paths. Additionally, it can be difficult to extend protein release over long periods of time when using diffusion alone, though favorable electrostatic interactions, hydrophobic interactions, hydrogel degradation-mediated release, and cleavable covalent linkages can be used to extend release. The PRINT technique also avoids heat and classical organic solvents, though the protein is exposed to a prepolymer solution that may function similarly to organic solvents from the perspective of protein damage, albeit for a potentially short period of time until crosslinking.

Organic solvents, used in double-emulsion, spray drying nanoprecipitation, phase separation, solvent-casting of scaffolds, and other methods, create an atypical environment for proteins, which can cause them to assume different conformations. When one of these preparation methods is preferred, several strategies can be employed to mitigate the loss of protein activity. The duration of exposure can be minimized, solvents that are more compatible with proteins (e.g., ethyl acetate) can be employed, excipients can be added to protect the protein, and the surface area of interaction can be minimized (e.g., through lower-energy sonication or homogenization to keep emulsion droplets large and with a lower surface area-to-volume ratio) to limit damage to the protein. Alternatively, the self-healing technique in which the protein is only present after the initial MPs have been fabricated can also be used to avoid organic solvent exposure, though slightly elevated temperatures are required to seal pores on the MPs to fully encapsulate the protein payload. The interface of proteins and their encapsulating materials also presents a non-native interaction in much the same way a solvent does, albeit only in two dimensions, potentially causing proteins to assume undesirable conformations.

Thermal stressors will be present at different temperatures, for different durations, and in different contexts, for many fabrication techniques. For techniques that require an emulsion step, both agitation and cavitation are potential sources of bioactivity loss. As with minimizing solvent exposure, lower-energy agitation would be preferred; however, this will make the inner aqueous emulsion droplets larger and the particles larger as well, which can have downstream consequences for release and stability (see the following section). Other techniques, such as melt extrusion and PULSED, introduce heat to encapsulate and prepare controlled-release devices, but in both cases, the encapsulated protein is in a dry state, which is known to greatly improve its thermostability. Non-reducing sugars and other excipients can also be used to further enhance this effect.

6.4. Stability from preparation to use

Except for non-degradable release systems, which are generally not preferred due to their *in vivo* permanence, delivery systems will need to be stored in a dry state or frozen to be useful over any clinically relevant time period since the encapsulating polymer will otherwise begin to degrade or change. Because proteins are already incorporated into the device at the time of drying or freezing, it is important that relevant excipients, such as cryoprotectants, be added during formulation. Beyond that, drying and freezing conditions, such as the rate of temperature change, storage vessel, and the gaseous environment above the formulation, can be studied to optimize recovery.

6.5. Post-administration stability

Elevated temperature in the body after administration is currently unavoidable, and therefore thermostability should be addressed regardless of the device preparation method and materials used. The desired duration of release will also determine how necessary thermostabilization will be, with longer durations necessitating greater stabilization. However, there are ways in which release systems can be tuned to minimize the effects of elevated temperature on encapsulated protein activity. One simple approach is, of course, to encapsulate excipients like non-reducing sugars and other carbohydrates. Another is to keep the protein dry. Although this is not possible when the polymer is encapsulated in a bulk-degradation material, like PLGA, which allows water to readily infiltrate the device and interact with the protein, surface-eroding polymers, such as polyanhydrides and polycaprolactone, can exclude water due to their hydrophobicity. As a result, the protein encapsulated in these materials remains dry and dry proteins are far more thermostable than the same proteins in an aqueous solution. These materials are also promising because, unlike PLGA and other bulk-degrading materials, their potentially acidic degradation products are confined to the surface of the particle, where they can be readily buffered by the body, eliminating the potential for potentially damaging pH inside the particle. In contrast, hydrogels provide a similar ability to prevent local pH changes within the device using the opposite mechanism. Instead of preventing water from entering, they freely allow water to enter, enabling the rapid diffusion of degradation products outside of the hydrogel.

When PLGA or another bulk-degrading polymer is desired, pH changes should be mitigated to enhance the recovery of bioactive protein. Because larger particles provide longer concentration gradients down which acidic degradation products must be transported, creating smaller particles is one way to minimize particle acidification that might otherwise compromise protein activity. Of course, this typically comes at the cost of faster sonication and homogenization speeds, which can lead to more damage during fabrication. Another strategy is to incorporate basic excipients. The effectiveness of this strategy largely depends on the nature of those excipients and the release system. For example, water-soluble buffering agents will be rapidly released from hydrogels, while they might be retained for considerably longer when encapsulated in a solid material. However, there is a good likelihood that hydrophilic excipients will be released from solid materials prior to proteins due to their difference in size between small molecule excipients and proteins, which would leave the protein unprotected. In this case, buffers that are insoluble at neutral pH, such as $Mg(OH)_2$ can be employed. Additionally, trehalose polymers have been shown to stabilize proteins better than their trehalose counterparts while potentially remaining in the device longer due to their macromolecular size [263].

6.6. Concluding remarks

The use of proteins and peptides for pharmaceutical applications has opened up new possibilities for highly specific and potent drugs. The potential benefits of controlled-release biologics are numerous, including enhanced efficacy, reduced side effects, improved convenience, accessibility, and patient adherence, and dose sparing, among others, which provides ample motivation to pursue the development of these formulations. Unfortunately, the development of protein delivery systems is made more challenging by the challenge of preserving drug stability through device preparation, storage, and release, especially when using a manufacturing process that involves heat, pressure, or organic solvents. As a result, employing a system that avoids these conditions, such as hydrogels prepared under aqueous conditions, can minimize losses in bioactivity. Another promising approach for maximizing the bioactivity of the protein or peptide through release is to incorporate thermostabilizing and buffering excipients into the formulation. Alternatively, or in addition to using excipients, keeping protein in a dry state protected from water will also serve to reduce thermal stressors and prevent pH alterations. However, although formulations should strive to maintain protein bioactivity through encapsulation and release, incomplete recovery may still be acceptable in many cases owing to the benefits conferred by controlled release. Nevertheless, a growing understanding of environmental stressors that undermine the biological activity of proteins and the development of new techniques to eliminate or mitigate exposure to those stressors suggests that it is only a matter of time before controlled-release systems for proteins and peptides become a clinical reality.

Data availability

No data was used for the research described in the article.

Declaration of Competing Interest

K.J.M. holds several current and pending patents related to controlled releases that are potentially suitable for biologic delivery.

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