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Issues in long-term protein delivery using biodegradable microparticles

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ABSTRACT

Recently, a variety of bioactive protein drugs have been available in large quantities as a result of advances in biotechnology. Such availability has prompted development of long-term protein delivery systems. Biodegradable microparticulate systems have been used widely for controlled release of protein drugs for days and months. The most widely used biodegradable polymer has been poly(D,L-lactic-co-glycolic acid) (PLGA). Protein-containing microparticles are usually prepared by the water/oil/water (W/O/W) double emulsion method, and variations of this method, such as solid/oil/water (S/O/W) and water/oil/oil (W/O/O), have also been used. Other methods of preparation include spray drying, ultrasonic atomization, and electrospray methods.

The important factors in developing biodegradable microparticles for protein drug delivery are protein release profile (including burst release, duration of release, and extent of release), microparticle size, protein loading, encapsulation efficiency, and bioactivity of the released protein. Many studies used albumin as a model protein, and thus, the bioactivity of the release protein has not been examined. Other studies which utilized enzymes, insulin, erythropoietin, and growth factors have suggested that the right formulation to preserve bioactivity of the loaded protein drug during the processing and storage steps is important. The protein release profiles from various microparticle formulations can be classified into four distinct categories (Types A, B, C, and D). The categories are based on the magnitude of burst release, the extent of protein release, and the protein release kinetics followed by the burst release. The protein loading (i.e., the total amount of protein loaded divided by the total weight of microparticles) in various microparticles is 6.7 ± 4.6%, and it ranges from 0.5% to 20.0%. Development of clinically successful long-term protein delivery systems based on biodegradable microparticles requires improvement in the drug loading efficiency, control of the initial burst release, and the ability to control the protein release kinetics.

Contents

Abbreviations: AFTV, autologous fixed tumor vaccine; BDNA, brain-derived neurotropic factor; BSA, bovine serum albumin; bSOD, bovine superoxide dismutase; CD, cyclodextrin; DCM, dichloromethane, methylene chloride; dex-HEMA, hydroxyethyl methacrylated dextran; DGR, staphylokinase variant K35R; DMSO, dimethyl sulfoxide; DOPC, dioleoyl-sn-glycero-3-phosphocholine; DW, distilled water; EE, encapsulation efficiency; EO, ethylene oxide; EtAc, ethyl acetate; FITC, fluorescein isothiocyanate; GDNF, glial cell-line derived neurotropic factor; GFP, green fluorescent protein; GM-CSF, granulocyte macrophage colony-stimulating factor; HAp, hydroxyapatite; hCG, human chorionic gonadotropin; HGF, hepatocyte growth factor; HPβCD, hydroxypropyl-β-cyclodextrin; HSA, human serum albumin; IgG, human immunoglobulin G; IL, interleukin; INF, interferon; LC, loading capacity; LHRH, luteinizing hormone-releasing hormone; MβCD, methyl-β-cyclodextrin; MW, molecular weight; OMCTS, octamethylcyclotetrasiloxane; PC, posphatidylcholine; PDMS, poly(dimethyl siloxane); PE, petroleum ether; PEG, poly(ethylene glycol); PEG-PHis, poly(ethylene glycol)-b-poly(L-histidine); PGA, poly(glycolic acid); PHBV, poly(3-hydroxybutyrate-co-3-hydroxyvalerate); PHis, poly(L-histidine); PLA, poly(D,L-lactic acid); PLGA, poly(D,L-lactic-co-glycolic acid); PLHMGA, poly(lactic-co-hydroxymethyl glycolic acid); PLL, poly(L-lysine); PMMA, poly(methacrylic acid); PPF, Precision Particle Fabrication; PRINT, particle replication in non-wetting template; PVA, poly (vinyl alcohol); PVP, poly(vinylpyrrolidone); rhEGF, recombinant human epidermal growth factor; rhEPO, recombinant human erythropoietin; rhGH, recombinant human growth hormone; rhIGF-1, recombinant human insulin-like growth factor-1; rhNGF, recombinant human nerve growth factor; rhVEGF, recombinant human vascular endothelial growth factor; RSA, rat serum albumin; SAIB, sucrose acetate isobutyrate; SBE-CD, sulfobutyl ether β-cyclodextrin sodium salt; SOF, spinning oil film; S/O/O, solid-in-oil-in-oil; S/O/W, solid-in-oil-in-water; TPGS, α-tocopheryl polyethylene glycol 100 succinate; TT, tetanus toxoid; W/O/O, water-in-oil-in-oil; W/O/W, water-in-oil-in-water.

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1. Introduction

The traditional way of delivering a protein drug requires daily, sometimes multiple, injections to achieve its therapeutic effectiveness. To improve patient compliance and convenience, sustained release dosage forms have been developed [1–3]. In the last three decades, many therapeutic proteins and peptides have been microencapsulated in biodegradable polymers, mainly poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(lactic-co-glycolic acid) (PLGA) [4–7]. The principle behind using biodegradable polymer is that the release of a loaded protein drug depends mainly on the degradation kinetics of the polymer. Thus, it has been assumed that a loaded protein drug is released gradually following the PLGA degradation kinetics which can be adjusted by changing the lactide/glycolide ratio and molecular weight (MW) [2,8]. This, however, may not be always true, because other factors of the formulation can also affect the drug release kinetics, and sometimes they are more dominant than the degradation kinetics of a polymer.

An ideal microparticle formulation should have reasonably high protein encapsulation efficiency, loading capacity, and sustained release of the loaded protein with retained bioactivity [2,9]. The high protein loading and high encapsulation efficiencies are most critical simply due to the extremely high price of therapeutic proteins [9]. For an injectable formulation, the size of microparticles should be small enough for going through a fine needle. Usually, needles of 22–25 gauge (inner diameters of 394–241 μm) are used for quick intravenous infusion as well as intramuscular and subcutaneous injections. Microparticles with the diameter much smaller than that of a needle are preferred, in order to minimize potential blockage of the needle by them. The particle size and size distribution are also important for protein release rate as the total surface area for protein delivery depends on the particle size [10]. Preparing microspheres with all desirable properties has met with only limited success. This article examines the properties of protein-loaded microparticles, in particular, protein loading and release properties from PLGA microparticles.

Table 1
Methods for making protein containing microparticles.

Double emulsion methods
1. Water/oil/water (W/O/W) method
2. Solid/oil/water (S/O/W) method
3. Water/oil/oil (W/O/O) method (Coacervation method)
4. Solid/oil/oil (S/O/O) method
Other methods
5. Spray drying and spray freeze-drying method
6. Ultrasonic atomization method
7. Electrospray method
8. Microfluidic method
9. Pore-closing method and thermoreversible-gel method
10. Microfabrication method

2. Microencapsulation methods

Understanding the protein loading and release properties requires understanding the microencapsulation methods used for protein drugs. The preparation methods commonly used for making protein-loaded microparticles are listed in Table 1. Compared to double emulsion methods, ultrasonic atomization method, electrospray method, microfluidic method, pore-closing method, thermoreversible-gel method, and microfabrication are relatively new and still under investigation. All methods, except microfabrication technique, listed in Table 1 produce microparticles in the spherical form, and so the terms “microparticle” and “microsphere” have been used interchangeably. Easy comparison of properties of microparticles prepared by different methods requires several parameters related to microencapsulation and microparticle. Of those, the two parameters, the protein loading capacity and protein encapsulation efficiency, requires definitions as they are not intuitive in their meanings. In this review, the protein loading capacity (LC) and the protein encapsulation efficiency (EE) are defined as follows [11]:

$$\textit{Loading capacity} = \frac{\textit{Weight of encapsulated protein}}{\textit{Weight of microparticles}} \times 100\%$$

$$\textit{Encapsulation efficiency} = \frac{\textit{Weight of encapsulated protein}}{\textit{Weight of the total protein used for encapsulation}} \times 100\%$$

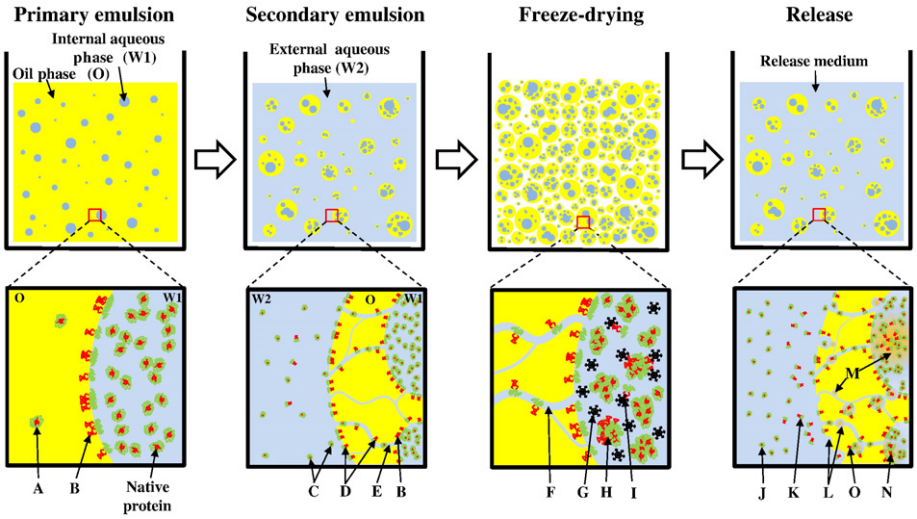

Fig. 1. Typical W/O/W double emulsion method to prepare microspheres containing protein drug (upper panel) and microscopic events during fabrication process (lower panel). The sequence of fabrication is primary emulsion, secondary emulsion, solvent extraction/evaporation (not shown), (freeze-)drying, and drug release test. With negligible partition of protein into oil phase (A), the organic solvent–water interface during W1/O emulsion results in protein denaturation (B). During generation of secondary emulsion, water channels connecting internal (W1) and external (W2) aqueous phases (E) allow proteins to escape from droplets (C), and provide more chances of protein denaturation by increased surface area of the oil–water interface (D). The water channels become pores (F) of microspheres hardened by freeze-drying. Ice crystal (G) is known to provide a hazardous condition inducing protein denaturation (I). Irreversible aggregation (H) between protein molecules can be formed if stabilizer or cryoprotectant is not added. Normally, microspheres made by double emulsion have a broad range of particle size distribution as well as different protein amount in each microparticle. In a release test, a burst release of protein at the initial period (<24 h) is mostly due to the protein release (K) from the proteinaceous film on the particle surface (D). With time, proteins are release from particles (J) by diffusion and degradation (L) of polymer (e.g., PLGA). Microparticle degradation cumulates acidic products inside particles (M), which further facilitates protein denaturation (N). Protein adsorption on hydrophobic polymer surface (O) often leads to incomplete release of protein drugs.

The loading capacity indicates the percentage of the microparticles occupies by the loaded protein drug, and the encapsulation efficiency describes how much portion of the initial protein drug is present inside the microparticles. Obviously, the higher the values, the better, as long as the protein release profiles meet the intended goals.

2.1. Water/oil/water (W/O/W) double emulsion method

The W/O/W double emulsion methods have been most widely used because of their relatively simple process, convenience in controlling process parameters, and ability to produce with inexpensive instrument [12,13]. In W/O/W double emulsion methods, aqueous protein solution is dispersed in a polymer-dissolved organic solution, e.g., PLGA in dichloromethane (DCM) or ethyl acetate (EtAc), to form a primary W/O emulsion. Then, the primary emulsion is further dispersed into a large volume of water containing an emulsifier, such as poly(vinyl alcohol) (PVA), to form a W/O/W double emulsion. Hardened microparticles are formed by removing organic solvent from the polymer phase [14–16]. The organic solvent is removed by either solvent extraction or solvent evaporation. In solvent extraction, W/O/W double emulsion is exposed to a large amount of water or a cosolvent, such as acetone or alcohol, added into the aqueous bath. For solvent evaporation the temperature is increased, often under reduced pressure.

As a modification of traditional W/O/W method, membrane emulsification makes relatively uniform microspheres (less than 14% coefficient of variation) by forcing the primary emulsion through the uniform pores of a glass membrane into the external water phase under the pressure applied by nitrogen gas [17,18]. The particle size and distribution can be controlled by the pore size of the glass membrane and the emulsifier concentration in the external water phase.

The properties of microparticles (such as LC, EE, release kinetics, and particle size) depend on the parameters of protein (type and concentration), polymer (composition, MW, and concentration), volume ratio between protein and polymer solutions, emulsification method (time and intensity), and surfactant (type and concentration) [19]. Analysis of the available information in the literature, however, suggests that the properties of the microparticles are not easy to control. One of the most critical factors to keep in mind in the W/O/W double emulsion methods is the step for removal of solvent from emulsion particles. W/O emulsion droplets are exposed to a huge amount of water to remove solvent and make the solid microparticles. This process takes time, and it is this step that may cause lower loading capacity and encapsulation efficiency, as well as the large initial burst release properties. While solvent is removed from the emulsion into aqueous bath, protein molecules can diffuse out from the emulsion into the aqueous bath and also can cumulate on the surface of microparticles, as they become hardened, resulting in the high initial burst release. Fig. 1 shows the major problems that have limited the success of W/O/W method for protein drug delivery. Each issue will be discussed in Section 3 in detail. Recent methods to prepare microparticles containing proteins drugs have been developed to figure out those problems, and are briefly described below.

2.2. S/O/W method

Protein adsorption and denaturation at the water/solvent interface is one of the major factors for decreased protein bioactivity occurring during the microencapsulation process [20–22]. To avoid the protein denaturation during formation of W/O emulsion, S/O/W method has been developed, because proteins in the solid state are believed to maintain their bioactivity by drastically reducing conformational mobility in comparison to the large structural change found in the dissolved state [23]. In the S/O/W method, solid protein particles are dispersed in the polymer solution to form the primary emulsion. Then the solid dispersion is introduced into a large volume of aqueous solution containing emulsifying agent, such as PVA or poly(ethylene glycol) (PEG) [23,24]. It is, however, noted that making dispersion of protein particles in organic solvent is not easy. Protein particle micronization is one of the major issues in the S/O/W method. Micronization methods include lyophilization, spray drying, and spray freeze-drying [22,25]. Spray freeze-drying collects atomized protein microdroplets in a frozen form and followed with the ice sublimation

under reduced pressure. Thus, the temperature-related protein denaturation and deactivation experienced in spray drying is circumvented [22]. The low operational temperature processes, such as spray freeze-drying and lyophilization, are widely used to micronize protein particles [26]. It is also significant to control the size of micronized protein. Research of recombinant human growth hormone (rhGH) encapsulation indicated that the protein particle size significantly affected the protein encapsulation efficiency and *in vivo* release profile [5].

The S/O/W method can be modified for peptides and some low-molecular-weight proteins. It is expected that peptides and low-molecular-weight proteins can be dissolved in dimethyl sulfoxide (DMSO) or methanol without inducing the loss of activity because they do not have advanced tertiary or quaternary structures. Based on this assumption, a method named "*in-situ* S/O/W method" was applied to insulin encapsulation. In this method, insulin is dissolved in DMSO first. The S/O emulsion is then dispersed into the PLGA-dissolved DCM solution to generate microspheres [27]. Similarly, orntide acetate and leuprolide (peptides, luteinizing hormone-releasing hormone antagonist) is dissolved in methanol and dispersed in PLGA solution to obtained microspheres [28,29]. This modified method usually generates microspheres with a high EE.

2.3. W/O/O method (coacervation method)

In the W/O/O (coacervation or phase separation) method, two steps replace the secondary emulsion formation step in the W/O/W method. The first one is to add the primary emulsion into a nonsolvent in which polymer (e.g., PLGA) has no or negligible solubility. Commonly used nonsolvents for PLGA are poly(dimethyl siloxane) (PDMS), known as silicon oil, and petroleum ether [30]. Under stirring, the polymer undergoes phase separation to form the coacervate phase. With the solvent gradually extracted from the coacervate phase, the polymer enriched, physically stabled coacervate droplets are formed. The second step is to add a large volume of hardening agent, which is miscible only with the oil phase, i.e., solvent for the polymer and nonsolvent, into this two-phase system. The commonly used hardening agents are hexane and octamethylcyclotetrasiloxane. Extraction of solvent and nonsolvent results in formation of hardened microspheres [31,32]. The W/O/O method has been used to make many PLGA/PLA microspheres containing different proteins and peptides, including bovine serum albumin (BSA) [33,34], vapreotide acetate (somatostatin analogue) [35], and MVFMF2 (peptide epitope for HTLV-1) [8].

2.4. S/O/O method

The non-aqueous approach to make microspheres is thought to have advantages in protein stability. It is assumed that the procedure-induced protein structural change can be minimized and, thus, the protein bioactivity can be retained by dispersing protein powders into organic solvent to restrict the conformational rigidity of proteins and by excluding water in the whole manufacturing process [36]. In the S/O/O method, solid protein is directly dispersed either in an organic solvent and then mixed with polymer solution, or in the polymer solution to form the primary emulsion followed by the standard coacervation procedure [36,37].

In a modified method known as the spinning oil film (SOF) method, the coacervate droplets are introduced into a spinning cottonseed oil film at a controllable flow rate so that the shear generated by the spinning film separates the droplets to produce uniform microspheres [38]. This method resulted in more uniform microspheres, higher encapsulation efficiency, and lower initial burst release of protein as compared with microspheres produced by a conventional emulsification technique.

2.5. Spray drying and spray freeze-drying method

In spray drying method, protein solution or emulsion (W/O or S/O) is sprayed into the air for atomization, usually at an elevated temperature to evaporate the organic solvent [31]. The properties of final microspheres depend on the nature of the feeding flow (solution or W/O emulsion) as well as the operational parameters such as flow rate and inlet temperature [2]. As a one-step method, the main advantages of spray drying include the easy control of microsphere properties by changing the operational parameters, and the convenience in scale-up [39]. However, high operational temperature, separation of final particles, and product loss in commercial lab-scale spray-dryer are the problems [2,40]. The spray drying method was used to make microparticles containing insulin [41,42], tetanus toxoid (TT) [43,44], vapreotide [45], recombinant human erythropoietin (rhEPO) [39,46], and BSA [39,40].

As a way to avoid high temperature associated with the spray drying process, spray freeze-drying method have been studied to make PLGA microparticles of human growth hormone [47–49], nerve growth factor (rhNGF) [50], human vascular endothelial growth factor (rhVEGF) [51], and insulin-like growth factor-1 (rhIGF-1) [7]. The first step in the freeze-drying method is to spray a protein solution into liquid nitrogen, followed by lyophilization. Then, the protein particles are suspended in the polymer solution to form S/O emulsion. An ultrasonic nozzle is used to spray the S/O emulsion into a vessel containing frozen ethanol overlaid with liquid nitrogen. After submerged in liquid nitrogen bath to keep ethanol frozen, the vessel is transferred to a −80 °C freeze where the ethanol melts and organic solvent extraction occurs. The microspheres are solidified for three days and ready to be dried by passing through nitrogen gas at 2–8 °C [52].

2.6. Ultrasonic atomization method

Preparation of microparticles by the ultrasonic atomization method is relative new. However, it has shown some advantages in generating microparticles using ultrasonic transducers, which include simplicity in operation ("one-step" operation), aseptic processability, and continuous production. The Precision Particle Fabrication (PPF) was developed by combining ultrasonic nozzle (single or coaxial needle) and a carrier stream (nonsolvent of the polymer) flowing around the nascent microdroplets. As the polymer solution passes through the vibrating nozzle, the mechanic excitation launches a wave of acoustic energy along the liquid flow, generates the periodic instabilities, and consequently the stream is broken to form uniform microdroplets. These microdroplets are surrounded by the carrier stream at the nozzle and travel with it toward the aqueous bath. With the solvent evaporation, the microdroplets are solidified to form microspheres [10,53]. The major advantage of this method is that the different sizes of uniformed microspheres can be generated by changing the nozzle diameter, vibration frequency, the flow rate of the polymer and the carrier stream. It should be noted that by the mixing the microspheres of different sizes, the protein release pattern can be finely tuned. With the correct combination, the liner release kinetic can be achieved [54]. By pumping two different polymer solutions through the coaxial nozzle, furthermore, uniformed double-walled polymer microcapsules with controlled shell thickness can be fabricated [55].

The ultrasonic system developed by Freitas et al. consists of three major units [56]. The first unit is a simple mixer that generate W/O coarse emulsion. The second unit is ultrasonic flow-through cell which has a high pressurized water jacket so that the high-intensity ultrasound can be transmitted to the flow-through coarse emulsion. The pressure in the transmitting water is maintained above the threshold to guarantee the breakage of flow-through coarse emulsion into fine emulsion under the acoustic energy [57]. Then the fine

emulsion is transferred to the third unit, a static micromixer, to form the W/O/W double emulsion by introducing the extraction fluid though the microchannels in the micromixer. Two flows, emulsion and extraction fluids, alternatively pass through the microchannels and become mixed at the outlet. Owing to the much faster flow rate of extraction fluid, the emulsion flow disintegrated into droplets. Finally, the double emulsion is collected and further solidified to form microspheres. The mean diameter of the microspheres can be tuned by changing the flow rate of two liquids, the sonication power and time. However, the particles size distribution is relatively broad [56,58,59].

The microdispenser system, developed by Yoon et al., contains two inkjet nozzles [60,61]. After individually pumping the protein and the polymer solutions into separate nozzles, uniformed droplets are generated at the tip of each nozzle by the high frequency vibration induced by a frequency generator. The setup of the two nozzles is finely tuned to make these two kinds of droplets collide in the air, so that organic solution spreads on the surface of the aqueous droplets owing to their differences in surface tension. The incipient microcapsules are formed by solvent exchange and collected in the aqueous bath containing emulsifier. The dual microdispenser system, however, has an inherent limitation in generating large quantity of droplets. The setup of this system requires high precision alignment, which is complicated for scale-up production. This problem can be overcome by using an ultrasonic atomizer to generate microdroplets in large quantities. When the ultrasonic atomizer vibrates in a direction parallel to the central axis of the transducers, the liquid spreads on the tip of the front horn and absorb energy to break into a large amount of microdroplets [62]. Obviously, the ultrasonic atomizer system is easy to setup and to scale-up for mass production. Felder et al. used ultrasonic atomizer to generate microspheres by introducing protein containing W/O emulsion into the atomizer [63]. The atomized droplets are collected in a defined volume of hardening agent. The encapsulation study of BSA, thymocartin, and vapreotide pamoate (somatostatin analogue) showed low encapsulation efficiency of water-soluble proteins. The so-called "coaxial nozzle" design, in which two concentric nozzles with the different inner diameters share the same axis, can atomize protein and polymer flow at the tip without any premixing. Upon atomizing these two solutions by vibrating at a certain frequency, organic polymer and aqueous droplets are formed and collide. Due to their differences in surface tension, organic solution spreads on the surface of the aqueous droplets and the incipient microcapsules are formed, just like the way that the microcapsules are formed by the microdispenser method. These microcapsules are collected in aqueous bath containing an emulsifier [64,65].

2.7. Electrospray method

A well-known application of the electrospray is mass spectrometry. It can successfully produce a gas phase consisting of multiply charged ions from biological macromolecules (DNA, protein) existing in an aqueous phase [66]. One of the attractive features of this method is the ability to easily produce monodisperse droplets in different sizes [67]. Basically, the electrospray system includes a liquid delivery system (pump), a needle with high electric potential, and a grounded electrode which is in a short distance away from the needle. In the absence of electric field, a drop at the tip of needle grows until its mass exceeds the surface tension of the liquid at the needle-drop interface. When a high electric field is applied, the solution at the tip of the needle forms a conical meniscus (Taylor cone). Then, the jet emerging from the apex breaks into monodisperse droplets. There is no coalescence between these highly charged droplets because of electrostatic repulsion [68,69]. The advance in application of electrospray for microparticle generation has made the protein encapsulation possible, and the examples are insulin nanoparticles [70], β-tricalcium phosphate (β-TCP) in chitosan microspheres [71], and PLGA microparticles [55]. Currently, only BSA has been successfully encapsulated into PLA and PLGA polymers by passing primary W/O emulsion through the charged nozzle. Applied voltage, emulsion flow rate, polymer concentration, and type of solvent affect the property of microspheres [68,69,72]. Micro- or nanoparticles can be produced by applying several kilovolts electrical potential to charge the fluid filled in the outer nozzle of the two concentrically located nozzles. The size of microcapsules is determined by the potential difference of inner and outer needle and the flow rate of two flows. These results indicate the possibility to generate protein-encapsulated microcapsules by pumping through protein and polymer solution simultaneously through the charged concentric nozzles [67].

2.8. Microfluidic methods

Recently, microfluidic devices have gained interests owing to the flexibility to generate microdroplets with controlled internal morphology. Nowadays, most studies focus on the possibility to form oil microdroplets which contain different numbers of aqueous cores. Tan et al. showed the encapsulation of green fluorescent protein (GFP) in dioleoyl-sn-glycero-3-phosphocholine (DOPC) vesicle. However, there is no further information regarding to particle size and EE [73]. There are two strategies prevailing in designing microfluidic systems. One maintains the traditional simple channel design but with different hydrophobic or hydrophilic coating on the inner channel wall. By controlling diameter, hydrophobicity of the channel, sequence of aqueous or organic flow going through the channels, and flow rate, the polymer droplets with controlled size and aqueous core can be obtained [74–76]. The other strategy is to force three flows (aqueous, organic, and bath) to enter a well-designed device at the same time. By controlling the flow rate of each stream, desired size of the microparticles can be obtained [77–81]. The setup of this kind of device is much complicated compared with the previous methods, but the control of the system seems easier to achieve, and microdroplets with one or more aqueous cores can be easily obtained.

2.9. Pore-closing method and thermoreversible-gel method

Kim et al. created a novel technique to close microsphere pores for rhGH encapsulation. The first step is to make PLGA porous microspheres. The blend of Pluronic® F127 and PLGA is dissolved in DCM and form O/W emulsion in PVA solution. After microspheres are hardened and freeze-dried, the rhGH is loaded by dipping these microspheres in rhGH solution. The following pore-closing procedure can be accomplished in an aqueous environment or in ethanol vapor condition [82].

Thermoreversible-gel method employs the sol–gel transition of Pluronic F127 solution to encapsulate protein into microspheres. For example, when F127 solution of more than 20% concentration is warmed up to 10 °C, the liquid changes to gel. To make microspheres, first step is to dissolve BSA in 25% F127 solution at 4 °C, then the solution is heated to 37 °C for gelation. PLA solution in acetone is dispersed in gel at 37 °C using homogenizer and then is cooled down to 4 °C to have the gel to sol transition. Acetone diffuses to the aqueous solution, and a large volume of water is added into the system to completely extract acetone. Finally, solidified microspheres (TG-MS) are collected and washed. The confocal microscopy images indicated that the protein was distributed in the core of the polymer matrix. Due to reduced protein association on particle surface, the TG-MS showed a higher EE (93% vs. 72%) and lower initial burst release (about 30% vs. 50%) as compared with microspheres prepared by W/O/W methods. The protein in the microspheres was sustained its release for 70 days [11].

Table 2
Characteristics of protein-encapsulated microspheres prepared by the W/O/W method.

Release profile type and characteristics		Burst release (%)	Particle size (μm)	Protein loading (%)	Protein encapsulation efficiency (%)	Polymer and concentration (mg/ml)	Protein-encapsulated	Activity of released protein	Fabrication condition or formulation highlights	References
A	No release after the burst release in first 48 hours	20~60	107	1.2	72	PLGA, 129	Lysozyme	Retain 42% of activity after 24 hours	Sucrose coencapsulated with lysozyme at molar ratio of 37:1 (sucrose to lysozyme)	[111]
A	No further release up to 60 days	40	35	0.5	90	PLGA, 304	rhEPO		10% CD coencapsulated with rhEPO.	[4]
A	Slow release to 55% in 15 days	38	75	0.7	78	PLGA, 80	DGR	Retain ~85% activity on day 15	2% PVA coencapsulated with DGR, and 2.5% NaCl in the external aqueous phase.	[105]
A	Slow release to 65–70% in 14 days	20–55	0.2–167		82–87	PLGA	BSA, VEGF		Alginate hydrogel as a matrix for PLGA microspheres suppressed the initial burst and improved the release kinetics.	[112,113]
B	No release after the burst release in first 48 hours	7	15~30	6.9	76	PLGA/PLA	Lysozyme	Retain 80% of activity on day 60	2.5% RSA, 2% $NaHCO_3$ and 10% sucrose in internal aqueous phase, 10% sucrose and 1% PVA in external aqueous phase.	[114]
B	Extremely slow release (linearly) to 12% in 30 days	3	46	0.9	91	PLGA, 200	BSA		Solvent was rapidly removed by extraction with 100 ml of 2% aqueous isopropyl alcohol solution.	[106]
B	Extremely slow release (linearly) to 16% in 30 days	9	16	0.9	93	PLGA, 200	Lysozyme		Solvent was rapidly removed by extraction with 100 ml of 2% aqueous isopropyl alcohol solution.	[106]
B	Extremely slow release to 7.5% in 25 days	5	15	6.6	72	PLA, 60	Ovalbumin		5% NaCl in 5% PVP external aqueous phase.	[96]
B	Slow release to 29% in 120 days	10	2~8	3.9	69	PLA, 50	TT	*In vivo* serum highest anti-TT antibody tires 164 μg/ml	2% $NaHCO_3$ and 10% sucrose in internal aqueous phase, 1% PVA and 10% sucrose in external aqueous phase.	[12]
B	Fast release to 60% in 5 days, slow release to 80% in next 20 days	23		9.1	81	PLGA, 50	Swine insulin		200 μl 50 mg/ml swine insulin as internal aqueous phase. The primary emulsion was added into 200 ml 1% PVA solution.	[27]
B	Slow release to 12–50% after the burst	6–45	8–29	3–40	50–100	PLGA/PEG	GDNF	Active at least for 7 days	Internal aqueous phase was PBS (pH 7.9) containing 5% HSA and different concentrations of PEG	[115]
C	Slow release to 35% in 40 days	7		1.4	72	PLGA/PLA, 50/50	Insulin		Insulin 1% acetate acid solution (pH 2.8) was internal aqueous phase, and the primary emulsion was filtered through a porous glass membrane (pore size 2.8 μm) to 1% PVA.	[18]
C	Gradual release to 40% in 22 days	2		3.5	73	PLA, 265	BSA		10% NaCl in inner and external aqueous phase.	[116]
C	Gradual release to ~55% in 60 days	20	2	4.9	97	PLGA, 12	hCG	Serum IgG antibody responses up to 12 weeks	20% (v/v) glycerol coencapsulated with hCG and DCM:acetone (6:4) as PLGA organic solvent.	[104]
C	Gradual release to ~85% in 35 days	33	28	9.3	93	PLGA, 200	BSA		Second emulsion was formed in DCM-saturated 6% PVA solution at 4 °C. The solvent extraction phase contains 3.6% NaCl.	[14]
C	Slow release for 15 days after burst and gradual release to 95% for next 30 days	5	15			PLGA/PLGA-PLL-PEG, 28.5	BDNF	Active up to 65 days based on PC12 cell morphology	Internal aqueous phase was 0.1% BSA aqueous solution	[117]

C	Gradual release to 65% in 18 days	10–28	32–72	25–82	90–95	PLGA/HAp, 100	BSA		Internal aqueous phase contained 8 or 16 mg HAp and 1 mg BSA in 250 μl BSA. MW of PLGA was varied (10, 25, and 90 KDa)	[118]
C	Gradual release to 90–100% in 24 days	25–55	17–20	0.7–9.7	53–76	PLGA, 200	BSA	Dematan sulfate reduced insoluble BSA formation	External aqueous phase was 0.5% PVA containing 0.9% NaCl (pH 7.4). Dermatan sulfate enhanced drug loading amount and efficiency.	[119]
C	Gradual release to 45–70% in 34 days	20–40	17–24	2.2–2.6	72–88	PLGA, 200	Insulin	Condroitin sulfate A preserved secondary structure of insulin up to 20 days	Condroitin sulfate A enhanced drug loading amount and efficiency.	[120]
C	Gradual release to 95% in 24 days	55	194		86	PLGA, 50	rhEGF	*In vivo* activity was maintained up to day 21	Internal aqueous phase was PBS (pH 7.4) containing 2% protein. Oil phase was PLGA solution in acetonitrile/DCM (1:1) and external aqueous phase was 0.3% F68 in water.	[121]
C	Gradual release to 63–84% in 30 days	25–43	46–110			PLGA, 150	INF-α2b		Internal aqueous phase was PBS (pH 7.0) containing poloxamer 118. PLGA with higher MW lowered initial burst and endpoint of drug release.	[122]
D	Gradual release to 60% in 11 days	22	75	1.8	93	PLA, 50	rhEGF	Gastric ulcer was cured 82% at day 11 after administration with dose of 220 μg/kg	Emulsified under ice cooling. 0.3 M phosphate buffer in exteranal aqueous phase.	[123]
D	Continuous release to 78% in 35 days	10	27	15	90	PLGA, 100	Lysozyme	Retain 95% of bioactivity on day 28	40% glycol chitosan was coencapsulated with lysozyme, 1% PVA bath with 0.9% NaCl.	[110]
D	Continuous release to 52–73% in 60 days	<7	6.6–13.0	3.7–7.5	66–85	PLHMGA, 100–200	Lysozyme	Retain 100% bioactivity of released lysozyme after 60 days	Release profile of lysozyme was essentially controlled by polymer degradation, initial polymer concentration, and volume ratio between disperse and continuous phases.	[124]
D	Continuous release to 90% in 60 days	20	34	1.7	88	PLGA, 50	BSA		1 mg/ml PEG coencapsulated with BSA in PH 5.5 buffer with ionic strength 0.15 M. The w/o/w emulsion was formed in 0.5% PVA containing 0.5% NaCl.	[125]
D	Nearly zero order release to 95% in 65 days	<5	24	16.4	97	PLGA, 100	Lysozyme	Retain 90% of bioactivity on day 60	2.5% soluble starch, 5% PEG2000, and 10% SBE-CD coencapsulated with lysozyme, 3.3% SAIB and 83.3 ppm sorbitan monooleate 80 in polymer solution and 0.9% NaCl in external aqueous phase.	[109]
D	Continuous release to 70–80% in 25–75 days	<5	1.5–2.0		55–95	PLGA/DDA and/or TDB, 30	Ag85B-ESAT-6, a fusion protein of antigen subunit for tuberculosis		Concentration of the adjuvants, dimethyldocadecylammonium bromide (DDA) and trehalose 6,6′-dibehenate (TDB) was 20 and 2%, respectively	[126]
D	Continuous release to 45% in 45 days	<1	78	2.6		PLGA, 100+ chitosan, 30	BSA		On PLGA microspheres, shell structure was formed by dropping chitosan solution in acetic acid into liquid paraffin/Span 80 and curing by 5% TPP aqueous solution.	[127]
D	Continuous release to 87–95% in 20 days	4–26	35–105		68–90	Acetylated pullulan, 7.7	Exenatide	No peptide degradation up to day 16	Internal and external aqueous phases were 2 mg peptide in 0.1 ml water and 30 ml 0.5% PVA solution, respectively. Oil phase was acetylated pullulan solution in DCM.	[128]
D	Continuous release i) to 90% in 95 days, ii) to 50% in 110 days	i) <1 ii) 18	181	i) 5 ii) 0.002	i) 91 ii) 89	PLGA/PHBV, 80	i) BSA ii) HGF	Active up to day 40 based on cell proliferation assay	Internal aqueous phase was 10 mM PBS (pH 7.4)	[129]
C, D	i) Gradual release to 80% in 190 days, ii) No release for 20 days after burst followed by fast release to 95% for next 70 days	i) 41 ii) 23	1.6–2	7–8		PLGA i) 75:25 and ii) 50:50, 50	SPf66 malarial antigen	Retain *in vivo* activity up to week 27	First emulsion was formed by sonication (30 sec, 50 W) and second emulsion was prepared by homogenation in 8% PVA aqueous solution. 2% isopropanol in water was used to extract solvent.	[130]
C, D	Gradual release i) to 75% in 150 days and ii) to 85% in 150 days	i) 30 ii) <1	50–70	0.005	12	PLGA/PLLA (50:50), 200	Lysozyme	Retain 100% activity up to day 53	Lysozyme was lyophilized i) with or ii) without docusate sodium salt (AOT) before emulsion	[131]

Table 3
Characteristics of protein-encapsulated microspheres prepared by the S/O/W method.

Release profile type and characteristics	Burst release (%)	Particle size (μm)	Protein loading (%)	Protein encapsulation efficiency (%)	Polymer and concentration (mg/ml)	Protein-encapsulated	Activity of released protein	Fabrication condition or formulation highlights	References
A Fast release to 90% in 6 days, no further release till day 24	40		9.3	91	PLGA 50	Insulin		Insulin was dissolved in DMSO and dispersed into DCM to form fine particles	[27]
B No further release after burst release	~15	111	4.3	85	PLGA 46	IgG		Zn:IgG ratio was 1:1	[22]
C Gradual release to 50% in 30 days	~26	89	3.4	68	PLGA 80	Insulin	*In vivo* glucose concentration was below 20 mmol/l for 48 hours	Inner aqueous phase contained PMAA/insulin complex suspension with ratio of 68:32 at pH 3.0	[134]
C Gradual release to 90% in 35 days, no further release in next 25 days	~38	5	9.1	91	PLGA 180	α-Chymotrypsin		α-chymotrypsin co-lyophilized with MβCD at 1:4 mass ratio, s/o emulsion was homogenized in 50 ml 10% PVA	[135]
C Gradual release to ~95% in 16 days	~35	31	6.5	93	PLGA 685	rhGH		Co-lyophilized with ammonium acetate at molar ratios of 20 times of rhGH, coencapsulated with 0.5% ZnO in PLGA.	[5]
C Gradual release to 100% in 62 days	30–35	40–100	1.75–2.5	39–55	PLGA, 100	BSA, horse myoglobin		Protein particles were prepared by lyophilization with 1% dextran and 8% PEG, followed by vacuum drying after removing PEG with DCM. Oil phase 1 and 2 were PLGA solution and hydrophilic oil (72.7% glycerol, 18.2% 2-isopropanol, 9.1% water, 1% PVA, 5% NaCl), respectively (S/O/O/W method).	[136]
D Gradual release to 82% in 30 days, slow release to 100% in next 50 days	~22		7.6	76	PLGA 360	γ-Chymotrypsin	Retain 40% activity on day 7	γ-chymotrypsin co-lyophilized with PEG at 1:4 mass ratio, PEG co-dissolved with PLGA at 1:1 mass ratio, s/o emulsion is homogenized in 50 ml 10% PEG	[23]
D Gradual release to 60% in 15 days	~10		3.0	100	PLGA784	Insulin	*In vivo* blood glucose level drop to normal between days 8 and 10	Glycerol was added to PLGA-insulin-ZnO DCM suspension until high transparent and viscous solution was obtained. Then, it was poured to 1% PVA and 7% zinc acetate dehydrate solution to form secondary emulsion	[137]
D Gradual release to 96% in 25 days	~18	7	7.7	85	PLGA 80	BSA		BSA co-lyophilized with trehalose at ratio of 1:4, s/o emulsion was homogenized in 50 ml 10% PVA	[133]
D Gradual release to 97% in 57 days (Measured at pH 4.0 with dialysis method)	7	~11	10.8	83	PLGA	Ornitide acetate		Ornitide acetate methanol solution was added to PLGA DCM solution to form clear solution. Then the solution was injected into1 L 0.35% PVA phosphate buffer (pH 7.2) solution and homogenized at 5500 rpm. Vacuum dried.	[28]
D Nearly zero order release to ~75% in 28 days	1	<10	2.2	88	PLGA/PLA 7/28[a]	bSOD	Retain 100% activity in dried microspheres	Co-lyophilized with PEG 70 K at ratio 1:1, then add PLGA/PLA with a final ratio of bSOD:PEG70K:PLGA:PLA (2.5:2.5:19:76) with the total weight of 500 mg. Then 1350 mg DCM was added to dissolve the polymers. The primary emulsion was added in 0.25% methylcellulose solution at 15 °C to form secondary emulsion. Finally 400 ml DW was added and stepwise increased to 30 °C for 3 h	[132]
D Nearly zero order release to ~90% in 150 days (Measured with dialysis method)	~10	52	13.4	81	PLA 340	Leuprolide	*In vivo* testosterone levels were suppressed to 0.5 ng/ml from day 4 to day 50	Leuprolide methanol solution was added into a 34% (w/w) PLA solution in DCM to form the clear solution. The resulting solution was then slowly injected into 0.35% PVA solution homogenized at 7000 rpm. Vacuum dried.	[29]
D Gradual release to 100% in 130 days	20	21–24	7.2–7.8	37–40	PLGA	Bovine insulin		Alginate particles containing protein were suspended in PLGA solution (DCM). After sonication, it was emulsified in 6% PLA aqueous solution.	[138]

[a] PLGA/PLA concentration is 7/28 mg/mg DCM.

Compared with traditional microencapsulation methods, these novel methods can generate microspheres with high EE with improved *in vitro* release kinetics. However, there are limited studies on bioactivity of released protein. Following these initiative studies, the studies of process parameters optimization, formulation, variety of proteins encapsulation and the following release and activity study are demanded.

Table 4
Characteristics of protein-encapsulated microspheres prepared by the W/O/O (coacervation) and S/O/O method.

Release profile type	and characteristics	Burst release (%)	Particle size (μm)	Protein loading (%)	Protein encapsulation efficiency (%)	Polymer and concentration (mg/ml)	Protein-encapsulated	Activity of released protein	Fabrication condition or formulation highlights	References
A	No further release after initial burst release	~90	1~3	6.2	83	PLGA, 38.9	Carbonic anhydrase	Retained 17% activity at 30 min	6% lecithin was added in protein phase. The primary emulsion was lyophilized and then dissolved in DCM. The PE nonsolvent volume to DCM was 50:1.	[30]
B	No further release after initial release in first five days (to 40%) during a month long release study	~16	6	6.8	75	PLGA, 40	BSA		10 ml petroleum ether (PE69) containing 10% Span 80 as coacervating agent with a addition rate of 5.0 ml/min.	[108]
B	Very slow release to 17% in 50 days	12	44	1.1	35	PLGA, 100	TT	*In vivo* tetanus toxin IgG antibody remained ~2 AU/ml for 4 weeks	50 mg poloxamer 188 in TT solution was lyophilized to made TT particles. Then TT particle was suspended into PLGA acetonitrile solution. Cottonseed oil containing 0.05% soybean lecithin was used as nonsolvent and PE is used as hardening agent.	[37]
C	Fast release to 75% for 25 days followed by slow release to 90% for next 50 days	25	2		51	PLGA	Insulin (crystal)	Secondary structure was maintained after encapsulation	Insulin (10 mg/ml) solution in 0.01 N HCl was mixed with PLGA solution in acetonitrile (1:5 volume ratio). Oil phase 2 was mineral oil containing 3% Span 80.	[139]
C	Only initial burst was monitored	2-12	120	13.5	90	PLGA or PLA, 300~500	BSA		BSA solution was sprayed dried to form particles. PLGA acetonitrile solution was added in BSA acetonitrile suspension to form primary emulsion. Then 0.5% Span 85 paraffin was added to harden microspheres.	[3]
D	Gradual release to ~35% in 20 days	~5	52	2.9	99	PLGA, 30	BSA		The primary emulsion was formed by ultrasonic homogenizer for 10 s at 180 W. Silicon oil containing 20% DCM was used as nonsolvent and PE was used as hardening agent.	[33]
D	Gradual release to ~75% in 15 days	~20	84	0.5	90	PLGA, 0.18	Cytochrome *c*		Cytochrome *c* PEG 400 solution and Tween 80 were added into PLGA triacetin solution to form oil phase 1. Oil phase 2 was formed by adding Span 80 in miglyol 812. Then the O1/O2 emulsion was added in 1% PVA solution.	[140]
D	Gradual release to 100% in 50 days	5–10	1–10	9–10	91–101	PLGA, 20	Insulin (crystal)	Secondary structure was maintained after encapsulation	PLGA solution in DMF containing insulin crystal (1:9 weight ratio) was homogenized in corn oil containing 1% Span 83.	[141]
D	Slow release to 2–14% in 18 days and gradual release to 63% for next 55 days	6–11	123	10–15	81–100	PLGA, 500	BSA		BSA was spray freeze-dried with 2% trehalose to form particles. 3% $Mg(OH)_2$ or $ZnCO_3$ was coencapsulated. Then s/o primary emulsion was pumped in a spinning oil film and formed microspheres.	[38]
D	Gradual release to 19–41% in 28 days	4–13	18–49		46–93	PLGA, 100	Endostatin	Retain bioactivity up to day 28	Primary w/o emulsion was formed by homogenation of PLGA solution (acetonitrile/DCM, 2 ml) and protein in water (10 mg/0.2 ml). Secondary emulsion was made by stirring w/o emulsion in liquid paraffin containing 0.3% Span 80.	[142,143]
A,C	No or slow release after burst release (Higher concentration of high MW PLGA and surfactant showed slower release)		1.5–8.5		6.4–101	PLGA (6 or 35.8 KDa), 16 or 32	Insulin (crystal)		Mixture of insulin in 0.01 N HCl solution and PLGA in acetonitrile (1:5 volume ratio) was dropped into mineral oil containing 0.3 or 3% Span 80	[144]
A,B,C, D	Release profile depended on MW of PLGA and existence of zinc	A, C: 40, B, D: 4–8	33–46	1.24–1.44	93–94	PLGA 35 KDa (A, B) or 14 KDa (C, D), 100	Glucagon-like peptide-1	Retain bioactivity up to day 30	Peptide was micronized (A, C) or complexed with zinc (B, D), which was mixed with PLGA solution in acetonitrile. Mixture was dropped in cottonseed oil containing 3 mg/ml lecithin.	[145]

Table 5
Characteristics of protein-encapsulated microspheres prepared by the spray drying method.

Release profile type and characteristics	Burst release (%)	Particle size (μm)	Protein loading (%)	Protein encapsulation efficiency (%)	Polymer and concentration (mg/ml)	Protein-encapsulated	Activity of released protein	Fabrication condition or formulation highlights	References
A No release after initial burst release	~60	18	3	60	PLGA, 50	BSA		The primary emulsion (in ethyl formate) was sprayed into a vacuum vessel.	[40]
B No release after initial burst release	15	7	0.5	100	PLGA, 50	rhEPO		0.5% cyclic DL-lactide dimer was added in PLGA solution (DCM). The microspheres were formed by spray drying primary emulsion.	[46]
C Fast release to ~98% in 8 days	~30		13	78	PLGA 10	BSA		BSA solution contained 0.1% sodium oleate. The microspheres were formed by spray drying primary emulsion at 5 °C.	[39]
C Gradual release to 80% in 28 days	31		8.7	87	PLGA, 50	Vapreotide acetate		0.5% vapreotide acetate and 5% PLGA (1:1 of MW 14 KDa and 35 KDa) in acetic acid solution was spray dried.	[35]
D Gradual release to ~80% in 28 days	~20	1–15	5.9	65	PLGA, 50	Vapreotide pamoate		Vapreotide pamoate and PLGA were dissolved in acetic acid solution and then spray dried.	[45]
D Gradual release to 94% in 35 days	8	10	2.5	100	PLGA, 20	insulin		Insulin and PLGA were dissolved in glacial acetic acid solution and then spray dried.	[42]
D Gradual release to ~100% in 30 days	26		20		PLGA, 170	rhIGF-1		rhIGF-1 particles were made by spray freeze-drying. Then the s/o suspension containing 6% $ZnCO_3$ was spray freeze-dried.	[7]
D Gradual release to >90% in 35 days	<10		9		PLGA, 1700	rhVEGF	*In vivo* generate a significant angiogenic response	10 mg/ml rhVEGF and 1 mg/ml HPβCD in 5 mM sodium succinate (pH 5.0) solution were spray freeze-dried. Then the s/o suspension was spray freeze-dried.	[51]
D Gradual release to ~100 in 25 days	~1		10		PLGA, 170	rhNGF	Retain 82% activity at day 10	rhNGF and Zinc acetate (molar ratio = 1: 12) were spray freeze-dried to form particles. Then the s/o suspension containing 6% $ZnCO_3$ was spray freeze-dried.	[50]
D Nearly zero order release to ~80% in 45 days	<10	12	1.1	86	PLGA, 20	insulin		Insulin glacial acetic acid solution containing 0.1% HPβCD was dispersed in PLGA solution (DCM) to form w/o emulsion. Then the w/o emulsion was spray dried.	[41]

Table 6
Characteristics of protein-encapsulated microspheres prepared by the ultrasonic atomization method.

Release profile type and characteristics	Burst release (%)	Particle size (μm)	Protein loading (%)	Protein encapsulation efficiency (%)	Polymer and concentration (mg/ml)	Protein-encapsulated	Activity of released protein	Fabrication condition or formulation highlights	References
C In the first 30 days, BSA was slowly released to 20% followed by fast release for next 10 days. By the end of 40 days, around 70% of BSA was released. In the following 20 days, release continued to 95% or so	<1	~110		~100	PLGA, 50	BSA		The aqueous solution containing10 mg/ml BSA/100 mg/ml dextran and PLGA solution are pumped individually through the ultrasonic nozzle which was surrounded by a carrier stream containing 0.5% PVA.	[53]
C Slow release to 20% in 24 days	~5	85	3.4	71	PLGA, 50	Lysozyme[a]		PLGA solution and lysozyme solution containing 10% mannose were fed individually through the ultrasonic atomizer. Microcapsules were collected in 0.5% PVA bath containing 0.28 M NaCl.	[64]
C Gradual release to ~75% in 28 days, no further release in following days	28		9.3	93	PLGA, 100	Vapreotide pamoate		w/o emulsion was fed through the ultrasonic atomizer	[63]
C Gradual release to ~95% in 98 days	33		0.5	24	PLA, 200	BSA		w/o emulsion was fed through the ultrasonic atomizer	[63]
		36	4.1	73	PLGA, 60	BSA		Flow through ultrasonic emulsification combined with static micromixing	[56]

[a] The release profile is obtained from wet microcapsules.

Table 7
Characteristics of protein-encapsulated microspheres prepared by other methods.

Release profile type and characteristics	Burst release (%)	Particle size (μm)	Protein loading (%)	Protein encapsulation efficiency (%)	Polymer and concentration (mg/ml)	Protein-encapsulated	Activity of released protein	Fabrication condition or formulation highlights	Method	References
A Fast release to 42–75% in 4 days	38–53	26–128	<10	97–100	PLGA/PLA	hGH	Retain 100% activity after encapsulation	hGC micronized by spray drying or nanosized by zinc-induced precipitation was loaded into a pressure vessel with PLGA/PLA and exipients (Poloxamer 407 or Poloxamer 188 and/or Solutol HS15).	Supercritical fluid	[146]
B Slow release to ~28% in 3 days	<5	4.0	11.6	81	PLA, 30	BSA		w/o emulsion was formed at a ratio of BSA:PLA = 1:6 and organic phase: aqueous phase = 20:1. Then w/o emulsion was fed through electrospray system.	Electrospray	[68]
D Nearly zero order release to ~70% in 38 days	<1	20	7	77	PLGA, 100	BSA		10% Pluronic® F127 was dissolved in PLGA DCM solution. w/o emulsion was fed through electrospray system.	Electrospray	[72]
D Nearly zero order release to ~75% in 38 days	6.2 ± 1.1		3	89	PLGA/ Pluronic® F127	rhGH		Porous microsphere was made from PLGA and Pluronic® F127 mixture. Pore-closing process was performed in an ethanol vapor phase using a fluidized bed reactor.	Pore-closing	[82]
D Gradual release to ~100% in 72 days	~22	20	6.9	93	PLA, 83	BSA		BSA was dissolved in 25% Pluronic® F127 at 4 °C and waited for gelation at 37 °C. PLA solution in acetone was dispersed in the gel. Then the dispersion was cool to 4 °C and 40 ml DI water was added.	Thermoreversible gel	[11]

2.10. Microfabrication method

Recently, microfabrication techniques have been used widely to prepare microparticles. The particle replication in non-wetting templates (PRINT) method was developed for making microparticles using fluoropolymer-based templates [83–86]. Other microfabrication approach includes microcontact hot printing [87], step and flash imprint lithography [88], and the hydrogel template method [89]. A variety of particles containing different drugs, mostly low-molecular weight hydrophobic drugs, and not many protein drugs have been used in such methods. Also, the topic of microfabrication deserves a separate review, and thus, this particular approach is not considered here.

3. Characterization of microparticles

Complete characterization of microparticles requires examination of several parameters, and the following parameters are chosen for a comparative study in this review: type of release profile, burst release, particle size, protein loading amount (or capacity), protein encapsulation efficiency, polymer concentration, and protein-encapsulated for study. The summaries of comparison of many different formulations are listed in Tables 2–7, and the detailed discussion on each table is described below. The influence of operational parameters for the W/O/W approach on the particle size, protein encapsulation efficiency, protein activity, and *in vitro* release are discussed in detail, because the W/O/W method has been the most commonly used for protein microencapsulation. These characteristics also apply to other double emulsion methods and those methods which collect microparticles in the aqueous phase. If not clarified, the discussion is for the W/O/W method. The unique properties of other methods are addressed individually.

3.1. Protein release profiles

The ultimate goal of making microparticles is to release the loaded protein drug in a controlled manner to achieve the desired therapeutic effects. Although the protein release profiles from various microparticle formulations appear to be all different, they can be classified into four distinct categories (Types A, B, C, and D) as shown in Fig. 2. The categories are based on the magnitude of the initial burst release, the extent of protein release, and the steady state release kinetics following the burst release. The burst release is regarded as the initial release before reaching the steady state. Since the time to reach the steady state is quite often not clear, here the burst release is regarded as the release in the first 24 h. In Types A and B, the initial burst release is followed by little additional release. In most cases, the amount of the protein released after the initial burst release is very small. The release profile in Type C is similar to that in type A in terms of the high initial burst, but the burst release is followed by the steady state release of the remaining protein drug. If a formulation has the initial burst release of less than 30% but with more than 60% of the total release, it is categorized as Type C. Type D is considered to be the most ideal release profile, as the low initial burst release is followed by steady state release until most of the loaded protein is released.

Because of their large size and hydrophilic nature, protein drugs are not expected to be released until biodegradable polymers degrade to expose the protein drugs. Nevertheless, protein drugs are quite often released much before degradation of polymers, e.g., PLGA, occurs. Thus, protein drugs are most likely released through pores or channels formed in the microparticles (Fig. 1). In the beginning, water enters the pores on the surface to dissolve the protein for release. Usually the amount of protein release per unit time is the largest in the beginning, and thus the initial burst release is observed [90]. The initial burst release can be as high as 80% (Type A in Fig. 2), and this can be reduced significantly by closing the pores or channels. In one

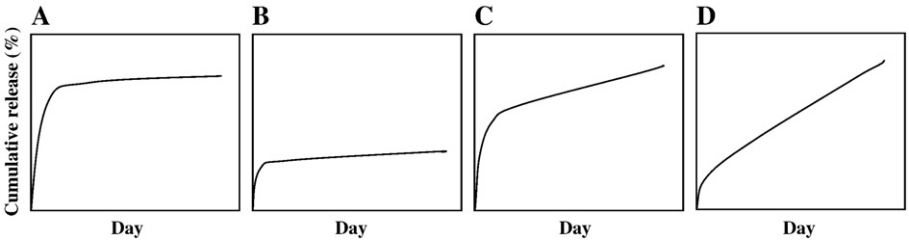

Fig. 2. Four categories of *in vitro* protein release profiles. (A) High initial burst release (>30%) followed by little additional release. (B) Low initial burst release (≤30%) followed by little additional release. (C) High initial burst release (>30%) followed by steady state release. (D) Low initial burst release (≤30%) followed by steady state release.

example, it was shown that high humidity treatment (24 h in 75% humidity) of PLGA microspheres with added Triton X-100 could decrease the initial burst release from 80% to 30% due to closing of the surface pores and channels by increased polymer chain mobility which is induced by the surfactant [91]. With polymer degradation, the osmotic pressure generated by dissolved protein, excipients, and degradation products is strong enough to break pre-closed pores and open previous isolated pores [92]. In short, the protein release is highly related with the pore status during protein release [90].

Any parameter for microsphere fabrication or formulation which can alter the dynamic transition of the pore opening and closing can effectively modify the protein release profile. For example, addition of a protein stabilizer, such as trehalose, PEG, vitamin E α-tocopheryl polyethylene glycol 100 succinate (TPGS), can facilitate the release of protein from microspheres by generating porous matrix after fast dissolution [13,93]. When phosphatidylcholine was added into a PLGA solution, both burst release and the subsequent protein release were suppressed due to its resistance to water uptake [94].

When water insoluble bases, such as $MgCO_3$, $ZnCO_3$, and $Mg(OH)_2$, are incorporated into microspheres, they build up osmotic pressure due to its neutralization effect of water-soluble acidic products out of polymer degradation. The high osmotic pressure results in a fast protein release from microspheres. Besides, the existence of osmotic pressure during microsphere preparation process can affect the internal structure of the microspheres, in turn; the protein release kinetics is influenced. Addition of salt or buffer in protein solution generates high osmotic pressure in the aqueous core, and thus the internal structure of microspheres tends to be porous and less dense. This results in a fast release of protein. If this osmotic pressure in the aqueous core is balanced by adding salt or buffer in the external aqueous bath, the microspheres will be denser and less porous. Naturally, the protein burst release is suppressed and a more progressive release profile is obtained [6,95–97]. The internal structure of microsphere can be affected by the PLGA composition (i.e., lactic acid to glycolic acid ratio), protein to organic solution ratio, and fabrication temperature. The internal structure in turn influences the protein release [1,98–100].

For the microspheres made by S/O/O method, the protein particle size is crucial to the initial burst release [101]. The encapsulation of nano-scale protein particles can decrease the initial burst release at the moderate protein loading (e.g., 5%) [3,38]. Incorporation of poloxamer (e.g., Pluronic® F68) in polymer phase during the fabrication process is reported to have decreased the initial burst and prolonged the sustained release [36]. Leach et al. studied the effect of polydispersity of microspheres on the burst release using microspheres produced by the revised S/O/O method. The results indicate that the microparticles with broader size distribution exhibit higher initial burst release [3].

3.2. Particle size

3.2.1. W/O/W method

The particle size and size distribution of microspheres prepared by the W/O/W method are mainly determined by the emulsification method used for forming a primary emulsion. In general, microspheres made by mechanical stirring or vortex mixing are larger than those by sonication [102,103]. As the stirring rate increases, the particle size decreases [13,98,100,104]. The volume of aqueous and polymer phases also affects the particle size. Because the increased volume of the internal aqueous phase generates greater resistance to mechanical breakdown during emulsification, the particle size increases accordingly [15]. Osmotic pressure is a factor in determining the particle size as well. The influx or outflux of water generated by the osmotic pressure determines the volume of the inner aqueous phase, and thus the size of solidified microspheres [95,105]. Increase in the processing temperature results in the increase in the mean particle size with a broad size distribution [99].

The presence of PVA in the external aqueous bath influences the particle size in two different ways. Increase of the PVA concentration increases the viscosity of the external aqueous phase, resulting in the increase of particle size. It is more difficult to break up aqueous solution to smaller droplets under the same mixing power, resulting in formation of larger particles [100,104–106]. At the same time, however, the presence of PVA stabilizes double emulsion droplets against coalescence, leading to smaller microspheres. In most experimental conditions, the stabilization effect dominates, and thus, smaller microspheres are formed [98,100]. The higher PVA concentration also produced smaller microspheres with a narrow size distribution for the membrane emulsification method [18].

3.2.2. S/O/W

Similar to W/O/W method, the emulsification power in generating secondary emulsion has a profound influence on the particle size and protein encapsulation efficiency. The higher the emulsification intensity is, the smaller particles with the higher encapsulation efficiency are obtained [107].

3.2.3. W/O/O

Similar to W/O/W method, the particle size and size distribution of microspheres are influenced by stirring rate, the type of nonsolvent phase volumes and viscosities, polymer concentration, and polymer molecular weight. When the viscosity of the nonsolvent is increased, the smaller and narrower microspheres are obtained. This can be explained by the increased shear force facilitating the breakdown of large coacervate droplets [34]. The stirring rate in at coacervating step also influences particle size. The higher stirring rate produces smaller coacervate droplets, so as to microspheres [108]. Raising the polymer concentration increases the coacervate viscosity and results in the production of larger coacervate droplets. Therefore, the larger and broader microspheres are obtained [33,34,108]. In addition, the volume of each phase, such as polymer phase, protein solution, the coacervating agent, all affects the particle size. Microspheres with the decreased mean size are generated when the volume of coacervating agent is increased. While the particle size increases and size distribution are broadened as the volume of protein solution increases [33].

3.3. Protein loading capacity

There have been numerous microspheres containing various protein drugs, but the protein loading capacity and protein encapsulation efficiencies have not been compared for different formulations. Tables 2–7 summarize the information categorized by the microparticle fabrication method. When the experimental parameters are not clearly described in the references, they were calculated from the information described in experimental methods. The average drug loading capacity in various microparticles summarized in the tables is $6.7 \pm 4.6\%$, and it mostly ranges from 0.5% to 20.0%.

The proteins or peptides listed in Table 2 are successfully encapsulated in the PLGA or PLA microspheres with high EE using W/O/W method. Many examples indicate that the inclusion of protein protecting agent and the balance of osmotic pressure between internal and external aqueous phases are the key to obtain the high EE. The lysozyme encapsulated microspheres showed high encapsulation efficiencies ($\geq$90%) and near zero order or triphasic release kinetics with the low burst effect ($\leq$10%). In this study, protein stabilizers were coencapsulated with the protein solution, and the osmotic pressure was balanced by addition of salt in the external aqueous phase [109,110]. When protein release is considered, most of microspheres in Table 2 do not have adequate protein release in 30 days following the initial burst release. The initial burst release is usually associated with the surface protein, and the incomplete protein release is commonly attributed to the denatured protein in the microspheres [90,111]. These results imply that maintaining intact protein structure during release is important to obtain continues protein release from the microspheres. Addition of counter base can effectively reduce the protein aggregation induced by the local acidic environment, but results in a fast protein release (this discussed below in Section 3.4.1). Therefore, the new protein stabilization method is necessary to improve the protein release from the microspheres. For example, Lee et al. applied glycol chitosan (GC) as the acidic microenvironment modifier because its degradation product (fragmented GCs, hydrolyzed by lysozyme) can form ionic complexes with lactic acid and glycolic acid [110].

The protein containing microparticles listed in Table 3 are prepared by S/O/W methods. Most of them were prepared by encapsulating the protein particles which are protected by excipient (s) during micronization, and obtained better protein release profiles (Types C and D). These results indicate the necessity to protect protein from denaturation in manufactory process. Microspheres generated by the S/O/W methods do not necessarily have higher encapsulation efficiency than W/O/W method [22]. Compared with traditional S/O/W methods, the novel method using PEG as protein micronization adjuvant encapsulated bovine superoxide dismutase with high EE of 88% and retained activity. The *in vitro* release showed a near zero-order release kinetics for 28 days at low initial burst release [132]. The trehalose-protected BSA particles-incorporated microspheres prepared by Castellanos et al. also showed superior characteristics. These microspheres have almost linear *in vitro* release profile with the moderate high BSA loading of 7.7% and encapsulation efficiency of 85% [133]. The so-called "*in-situ* S/O/W" method encapsulated luteinizing hormone-releasing hormone (LHRH) antagonist peptides with high encapsulation efficiency (81–83%) and high loading (>10%). And the *in vitro* release kinetics show good linear trends [28,29].

Table 4 lists the properties of microspheres made by W/O/O and S/O/O method. Most of them have high encapsulation efficiency (>75%). However, as compared with microparticles prepared by the W/O/W and S/O/W methods, those by the W/O/O and S/O/O methods are less successful when the release kinetics is considered. These microspheres have only 35–75% of protein release. The incomplete protein release implies that the preparation methods and formulations have not protected proteins adequately against denaturation. The SOF method, a revised S/O/O method, produced BSA-containing microspheres with a uniform size with a polydispersity of 6%. Furthermore, the high encapsulation efficiency (81–100%) under high loading (10–15%) and the low initial burst release (6–11%) could be achieved. The easy adjustment of the S/O emulsion flow rate and the rotor speed to fine-tune particle size makes this method very attractive [38].

Most microspheres made by spray freeze-drying showed a better release kinetics. The initial burst releases are small (around 10% for most proteins) and more than 80% of encapsulated proteins are released in a period of 30 days or so. However, there is limited information about particle size, loading, and encapsulation efficiency. These characteristics plus the information of microspheres made by spray drying method are all listed in Table 5. The encapsulation of insulin and vapreotide in acidic solution followed by spray drying produced microspheres with good properties but with small loading (2.5%) of insulin or high burst release (31%) of vapreotide [35,42].

In Table 6, microparticles were made using ultrasonic atomization methods. These microparticles all show reasonably good protein release profiles. For microcapsules made by Precision Particle Fabrication (PPF), the particle size is precisely controlled within 2 μm of the mean size. BSA, a model protein, was almost 100% encapsulated. However, the *in vitro* release profile showed a pulsatile pattern [53]. Due to the novelty of ultrasonic atomization method, most of the study focused on encapsulation of BSA, so there exists only limited information about the retention of protein activity. In addition, formulation studies were limited as well. Nevertheless, this method is promising by its advantages, such as relatively simple instrument setup, the ability to scale-up, and controllability of the size and morphology (microspheres or microcapsules).

Table 7 provides the characteristics of microparticles made by electrospray, pore-closing and thermoreversible-gel method. The microparticles made by these methods have good properties on particle size, encapsulation efficiency and release kinetics. Because they are newly developed methods, more studies need to be done to examine various parameters important to making protein formulations.

3.4. Encapsulation efficiency

3.4.1. W/O/W method

The loss of protein during the microparticle preparation process is attributed to two major factors. One is due the mechanical breakage of the incipient microspheres; and the other is the diffusion resulting from the concentration gradient. The mechanical breakage is difficult to avoid during fabrication of microspheres. On the other hand, the protein diffusion rate can be adjusted by changing the preparation and formulation parameters, such as the protein solubility, protein loading, inner water volume of W/O emulsion, emulsification method, addition of excipients, osmotic pressure, and polymer solidification rate. These parameters all affect the degree of protein loss directly or indirectly, and thus the encapsulation efficiency.

Many attempts have been made to add more protein to the inner aqueous phase. These attempts include choosing a high solubility protein, increasing the protein loading, and increasing the volume of protein solution. Such attempts, however, all resulted in a lower encapsulation efficiency, which in turn resulted in a large loss of these expensive proteins. The poor encapsulation efficiency becomes a limiting factor for scale-up production of microparticulate formulations. A study on encapsulation of two BSAs with different solubility showed that the higher solubility of BSA resulted in lower EE due to the higher tendency to diffuse into the external aqueous phase during microsphere formation [11]. The higher protein loading generates a higher concentration gradient between emulsion droplets and the continuous aqueous phase, resulting in the higher protein flux to the external water phase and lower EE [100]. In a study by Han et al., the EE decreased from 71% to 23% as the inner water volume was increased from 0.1 to 0.3 ml. This can be attributed to the increase in

the internal empty volume and the surface pore size [123]. Emulsification method is another factor affecting the EE. In the study on effect of homogenization method on FITC-BSA encapsulation, the microspheres generated by a mechanical homogenizer resulted in the lowest (79%) EE among the following three homogenization methods: mechanical homogenization, high pressure homogenization (W/O emulsion prepared by mechanical homogenizer was fed to a high pressure homogenizer), and ultrasonic homogenization [103].

Incorporation of surfactant usually reduces the protein EE. Addition of poloxamer 188 in BSA and lysozyme solutions [106], inclusion of vitamin E TPGS or PEG in HSA solution [13], and co-encapsulation of poloxamer 188, polysorbate 20 and sorbitan monooleate 80 in insulin solution [147] all resulted in lower EEs of the corresponding proteins. Addition of a surfactant in the polymer phase also reduces EE. Phosphatidylcholine (PC), as a phospholipid which has similar amphiphilicity as surfactants, reduced the protein EE when it was introduced into the polymer solution. The existence of PC in polymer phase might have hindered the formation of a stable interfacial BSA-PLGA film [148] and resulted in a loss of albumin to the external aqueous phase [94]. On the other hand, addition of sucrose acetate isobutyrate (SAIB) in the polymer solution effectively increased the EE of lysozyme from 62% to 98%. This is attributed to the inhibition of protein diffusion created by the hydrophobic interaction between viscous SAIB and PLGA [109].

The osmotic pressure between the inner and outer water phases affects the EE. With addition of salt into the external phase, the EE increases due to the denser polymer film generated around the microspheres and to balance of the osmotic pressure generated in the inner aqueous phase, the higher EE can be obtained [14,105,116,123]. Addition of water-miscible solvent (e.g., acetone) or water-immiscible solvent in the external aqueous phase changes the solvent removal rate, thus the polymer solidification rate is altered. As a result, the diffusion rate of water and protein from the inner aqueous phase to the external aqueous phase has been altered and the EE changes accordingly [14,104,149].

In the membrane emulsification method, formation of microspheres was accomplished by forcing the primary W/O emulsion through polymer membrane, followed by collected in the external aqueous bath. Thus, the influence of process parameters on the EE is similar to the W/O/W method. The higher inner water volume and the higher protein loading produce microspheres with lower EE. Addition of 5% NaCl in the external aqueous phase generated microspheres with the EE of 92% at the protein loading of 2.3% [18].

3.4.2. S/O/W

In S/O/W methods, protein particles are first dispersed into the polymer solution and form the primary S/O emulsion. Protein particle size plays an important role in its EE. In Takada's research, encapsulation of finer rhGH powder showed higher EE and lower burst release *in vivo* [5]. Similar to W/O/W methods, the higher protein loading results in lower protein EE due to more loss of the protein into the continuous phase [22,107]. The problem associated with this step is the homogeneity of dispersed protein particles due to their fine size. Yamaguchi et al. solved the problem of homogenously dispersed insulin in the PLGA solution in a unique way. By adding water, ethanol or glycerol in the PLGA solution forms nano-scale mini-emulsion droplets in the primary S/O emulsion, so that the insulin molecules can heterogeneously located in the PLGA microparticles, not on the surface. In addition, co-encapsulation of glycerol further suppresses the initial burst release due to annealing of the PLGA molecules to close the porous structure in the microspheres during the release process [137]. In the next step, S/O emulsion is added to a large volume of external aqueous phase. The volume ratio of external water to oil phase (W:O ratio) is critical to the protein encapsulation efficiency and protein activity. The EE and protein activity increases as the W:O ratio increases until the upper limit is reached. Beyond this critical ratio, there is no further improvement of EE and protein activity as the W:O ratio increases [24]. The emulsification power in generating secondary emulsion has a profound influence on the particle size and protein EE. The higher the emulsification intensity is, the smaller particles with the higher EE are obtained [107].

3.4.3. W/O/O method

W/O/O methods apply a coacervating agent to form primary emulsion as well as secondary emulsion. Based on the study made by Zhang et al., the volume of a coacervating agent (petroleum ether with surfactant) had negligible effect on the EE of BSA; while the type of coacervating agent (petroleum ethers with different boiling point) affects the amount of BSA encapsulated. The addition rate of a hardening agent (petroleum ether) influenced EE greatly. More protein was encapsulated with the higher addition rate. However, the microspheres generated under fast addition rate resulted in extremely high burst release (70%). The possible explanation is that the rapid solvent extraction induced the porous surface of the microparticles [108].

3.5. Protein activity

3.5.1. W/O/W method

Van der Weert and colleagues made an excellent summary on the protein instability issues during microparticle preparation, storage, and drug release in elsewhere [150]. The major loss of protein activity during the microencapsulation process is known to occur during the first emulsification step [4]. In this step, protein is exposed to huge water/organic interface, resulting in protein aggregation. The extent of protein aggregation is related to the solvent type. DCM induces more protein aggregation than ethyl acetate [25]. The method of emulsification determines the extent of protein aggregation during emulsification. The study on the effect of different emulsification methods on protein aggregation showed that sonication and vortex mixing generated slightly increased aggregation than mechanical homogenization [4]. To protect protein from denaturation during emulsification, a variety of excipients are added into the internal protein solution, such as carrier proteins (e.g., BSA and gelatin) [4,151], sugars (e.g., trehalose, maltose, lactose, and sorbitol) [25,51,151,152], and PEG [6,153]. 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 protein generated [25].

During protein release, the acidic microenvironment resulting from the PLGA hydrolysis contributes most to protein denaturation [48,154]. Proteins can be protected from the pH drop by adding counter alkaline excipients, such as zinc carbonate [48,155,156], magnesium hydroxide [93,156], or magnesium carbonate [30]. Recently, a new method of protecting proteins from acid-induced denaturation has been suggested. PEG-co-poly(L-histidine) (PEG-PHis) diblock copolymer was incorporated as a new excipient for protecting proteins during *in vitro* release process [125]. PEG-PHis diblock copolymer can form an ionic complex with negatively charged protein within a pH range of the acidic environment generated by PLGA hydrolysis. It acts as a temporary and reversible molecular shield that protects the protein from denaturation in acidic environment and improves the protein release kinetics [125].

3.5.2. S/O/W method

It is well-known that protein molecules in the solid state are more stable than those dissolved in aqueous solution. In some cases, however, the solid state protein still may not be able to preserve its conformational structure during the microparticle preparation steps. The activity of γ-chymotrypsin was lost when microspheres were prepared by an S/O/W method [23].

Solid proteins used in the S/O/W methods have been protected either by adding hydrophilic protectants or by forming protein complexes. Hydrophilic protanctants are usually co-lyophilized or co-spray-dried to form the protein-protectant microparticles. Examples of the protectants that have been used successfully to stabilize the protein include PEG [21,23–25,132,157–159], trehalose [22,25,133], mannitol [22,25], MβCD [135], ammonium acetate [5], and BSA [157]. A novel S/O/W method was developed using PEG as a protein micronization adjuvant. First, a protein-PEG mixture is lyophilized and then treated with DCM. After free PEG is dissolved in DCM, spherical, fine microparticles of the protein containing PEG are formed and ready to be dispersed into the polymer solution [132,159]. In another approach, some metals and polyelectrolytes are used to form complexes with proteins. Although protein complexes are formed to protect protein from the harsh processing condition during microparticle formation, the protein complex should be able to dissociate to release the protein. The examples of metal protein complexes are rhGH–Zn, rhNGF–Zn, and insulin–Zn complexes [48,50,160,161]. The polyelectrolyte protein complex can be amorphous precipitates, complex coacervates, gels, fibers or the soluble complexes depending on the property of the polyelectrolytes and the protein [162]. Jiang et al. prepared poly(methacrylic acid) (PMAA)–insulin complex based on the electrostatic interactions. Incorporation of PMAA–insulin complex increased the EE from 39% to 68%. The *in vitro* release profile showed a 26% initial burst release followed by sustained release for 30 days [134].

3.5.3. W/O/O

Schwendeman's group developed a new method to improve the stability of tetanus toxoid (TT) in the microencapsulation process. In this method, TT was loaded in microcores followed by standard coacervation procedure. When gelatin was chosen as the core material, the recovery of >80% of antigenicity of TT was obtained during the release [163]. When TT-containing microspheres and phosphate-adsorbed TT were administrated, the levels of specific IgG antibodies were kept high up to 25 weeks [164].

3.5.4. Spray drying and spray freeze-drying

One of the major issues associated with spray drying is the maintenance of protein activity during the high temperature process. The study on protein stability during this process, however, is very limited. As the more practical microencapsulation process, spray freeze-drying obtained a lot more attention. Many additives have been studied to be coencapsulated with protein. These additives include surfactant (ethyl stearate [45], sodium oleate [39], dioctylsulfosuccinate [39], polysorbate 20 [41], pluronic® F68 and F127 [39], and poloxamer 188 [41]), trehalose [43,44], PEG [45], BSA [43–45], chitosan [45], hydroxypropyl-β-cyclodextrin (HPβCD) [41,42], and cyclic D,L-lactide dimer [46]. Except lactide dimer, those additives are usually added in aqueous phase of W/O emulsion. The microspheres coencapsulated with additives usually show a decreased EE. The release profile shows different trends depending on the type of the additive. Due to the formation of insulin–HPβCD complex, the initial burst release of insulin and subsequent release are decreased [41,42]. The microspheres containing PEG 2000 release vaprotide in a more continuous pattern [45]. Incorporation of lactide dimer increased the encapsulation of rhEPO to almost 100%. However, there was no further release of rhEPO after the initial burst release [46].

Spray freeze-drying is also widely used to micronize protein particles. In order to obtain a proper size of protein particles with minimal loss of activity, different excipients have been added into a protein solution ready to be freeze-dried. Examples include addition of zinc acetate to growth hormone and rhNGF solution to form complexes [47,48,50,160], trehalose to rhNGF and rhVEGF solution [50,51], and polysorbate 20 to rhIGF-I and rhVEGF solution [7,51]. $ZnCO_3$ is a common additive used to co-disperse with protein particles in polymer solutions [7,47,50,51,160].

4. Future

Currently, there are no standard experimental conditions or no standard formulations that can be used for all different types of proteins. Differences in the tertiary structure, molecular weight, and charge make each protein unique, so that a specific formulation is required for each protein. Optimization of the microsphere preparation process is inherently difficult, because adjustment of one parameter usually results in complicated, often unpredictable, effects on the final microsphere properties. For example, when two different proteins are encapsulated into the same PLGA microparticles under the same condition, huge differences in the EE of the two proteins were observed [106]. Adding a protein protective agent may decrease protein denaturation for improved bioactivity, but it may adversely affect the protein release profiles [22]. Such complicated interplay of

Table 8
Examples of clinically used microparticle formulations for protein/peptide delivery.

Drug	Encapsulating compound	Name (Product)	Company	Use	Clinical status	References
Somatropin (rDNA orgin) growth hormone	PLGA	Nutropin Depot®	Alkermese & Genentech	Growth hormone deficiency	Approved	www.gene.com
Luprolide acetate	PLA	Lupron Depot®	Abbott	Endometriosis	Approved	www.lupron.com
Goserelin acetate	PLA	Zoladex®	AstraZeneca	Prostate/breast cancer, endometriosis	Approved	www.astrazeneca-us.com
Octreotide acetate	PLGA	Sandostatin® LAR	Novartis	Acromegaly, neuroendocrine cancer	Approved	www.sandostatin.com
Triptorelin pamoate	PLGA	Trelstar® Depot, Trelstar® LA	Watson Pharma	Prostate cancer	Approved	www.trelstar.com
rgp 120/HIV-1MN monovalent octameric V3 peptide vaccine	PLGA	UBI microparticulate monovalent (HIV-1 MN) branched peptide vaccine	United Biomedical	HIV	Phase I	[177,178]
GM-CSF, IL-2, tuberculin	PLA	Immunoadjuvants for autologous fixed tumor vaccine (AFTV)		Glioblastoma multiforme	Phase I	[179]
				Hepatocellular carcinoma	Phase II	[180,181]
hGH	Hydroxyethyl methacrylated dextran (dex-HEMA)	hGH dextran microsphere	Octoplus	Growth hormone deficiency	Clinical pharmacokinetic study	[182]

various formulation parameters makes development of clinically successful long-term protein delivery systems very difficult.

Protein formulations based on biodegradable microparticles require improvements in several properties. They include protein release profile, particle size, drug loading capacity, drug encapsulation efficiency, and protein bioactivity, as described above. The ultimate goal of making microparticles is to deliver protein drugs, and thus, obtaining desirable release profile is most important. Controlling the size and size distribution is important for making microparticle formulations with reproducible properties. The size and size distribution also plays a role in determining the administration route of the microparticles. The drug loading capacity needs to be controlled carefully. The higher drug loading capacity is not necessarily better or more desirable than the lower drug loading, as the degree of drug loading will affect the other properties. If, however, the same effects can be achieved with the higher loading capacity, it will lower the amount of the excipient polymer, leading to lower side effects to deal with. Another important factor to consider in drug loading is the protein EE. Since protein drugs are usually extremely expensive, achieving the EE as high as possible is critical.

For long-term drug delivery, which ranges from weeks to months, of protein drugs, one has to understand the stability of the loaded protein drug in the body, i.e., 37 °C in aqueous environment. Since most of the studies on protein formulations have used albumin as a model protein drug (if a model protein drug exists at all), the concern on bioactivity or protein stability has not been an issue. Other proteins commonly used have been lysozyme and insulin, which have bioactivities. But, the long-term stability in the *in vivo* condition has not been carefully examined. One factor that has been frequently overlooked is the ability of the microparticle preparation technique for scale-up production. For any formulation to be clinically useful, it needs to be produced in mass quantities [165]. The scale-up production in a reproducible manner is an important factor in deciding whether a microencapsulation technology can make it to the market [166]. All these factors need to be understood for successful development of long-term protein delivery systems using biodegradable polymers. Table 8 lists some examples of clinically used microparticle formulations for sustained release of macromolecules (i.e., molecular weight>1000 Da). Most of the drugs are long-acting analogues of the native proteins and peptides. It demonstrates how difficult it is to produce a sustained release formulation with a native protein. On the other hand, discovery of active derivatives will provide additional opportunities for microparticle-based protein/peptide formulations. Thus, development of stable and long-acting protein drug derivatives and study on overcoming the existing problems of microencapsulation processes should be done in parallel.

In addition, the sterilization process should be carefully considered for developing successful microparticle formulations containing protein/peptide drugs. Dry heat sterilization (160–190 °C) or steam sterilization (120–135 °C, stream pressure) may not be useful, because proteins and peptides are very unstable under elevated temperature, and PLGA, a most commonly used polymer, has low glass-transition temperature. To solve the problem, Callewaert and coworkers developed a microparticle consisting of alginate core and propylene glycol alginate-HSA shell, which showed resistance against the steam sterilization [167]. Ethylene oxide (EO) gas sterilization possibly induces toxicity after application, because of residual EO molecules in microparticles [168]. An alternative method is radiation sterilization including γ-ray irradiation that has been frequently employed to produce sterilized microparticles. It is known that to accomplish successful sterilization, the irradiation dose of γ-ray should be more than 25 kGy [169]. In many cases, γ-ray affected surface morphology of PLGA microspheres [170], decrease in MW of PLGA by random chain scission [171], radical generation [172], aggregation of protein [173], and release modulation [173,174]. Dillow et al. demonstrated an interesting sterilization method, in which the supercritical carbon dioxide is useful not only to generate microparticles but also to inactivate bacteria [175]. However, it is not applicable for any method to produce microparticle formulations. Sterilization is an essential step, but no standard protocol has been established. Microparticle formation under aseptic condition can be another solution. For example, Toguchi suggested a sterility assurance protocol for Leuprorelin-containing microspheres [176], which utilizes sterile starting materials and conduct sterility test at every processing step. Sterility assurance should be firmly established as it is one of the important safety requirements.

Recent advances in microfabrication (or nanofabrication) approaches provide new avenues of formulating protein drugs in microparticles. The advantages of the microfabrication approaches are many-folds. They can produce particles with predefined sizes with homogeneous size distribution. The fabrication methods are such that the drug loading capacity and encapsulation efficiency can be much higher than the particles obtained by the conventional methods. While some properties of the protein formulation can be improved by new approaches, some properties may not be easy to improve. The inherent material properties will not change simply by using new microparticle fabrication method. Furthermore, the inherent protein properties will not change either. The issue of protein stability, i.e., bioactivity, will have to be considered independent of the microparticle preparation. The method of making protein drugs stable for extended period of time in the body condition has to be incorporated into the microparticle preparation method. All in all the formulation of protein drugs in microparticles for long-term drug delivery is not just an engineering problem. It requires close collaboration among scientists in different disciplines, including formulation scientists specializing in excipients, polymer chemists with expertise in polymer chemistry, engineers with the ability of microfabrication, and protein chemists who can develop protein particles stable for months inside the human body.

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