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Quality by design thinking in the development of long-acting injectable PLGA/PLA-based microspheres for peptide and protein drug delivery

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ABSTRACT

Adopting the Quality by Design (QbD) approach in the drug development process has transformed from "nice-to-do" into a crucial and required part of the development, ensuring the quality of pharmaceutical products throughout their whole life cycles. This review is discussing the implementation of the QbD thinking into the production of long-acting injectable (LAI) PLGA/PLA-based microspheres for the therapeutic peptide and protein drug delivery. Various key elements of the QbD approaches are initially elaborated using Bydureon®, a commercial product of LAI PLGA/PLA-based microspheres, as a classical example. Subsequently, the factors influencing the release patterns and the stability of the peptide and protein drugs are discussed. This is followed by a summary of the state-of-the-art of manufacturing LAI PLGA/PLA-based microspheres and the related critical process parameters (CPPs). Finally, a landscape of generic product development of LAI PLGA/PLA-based microspheres is reviewed including some major challenges in the field.

1. Introduction

In the last few decades, peptide and protein drugs with high purity and great therapeutic potential have been arising owing to the rapid advancements in techniques such as DNA-recombination technology, molecular grafting approach as well as by editing naturally occurring peptide sequences (Ma, 2014; Qvit et al., 2017; Wang and Craik, 2018). Currently, there are more than 500 peptide-based drugs in pre-clinical trials, 140 in clinical trials and 60 on the market. In 2015, the global peptide therapeutic market was pegged at US$ 21.3 billion and is estimated to reach a value of US$ 46.6 billion in 2024, as the approval rate of peptide-based drugs is remarkably enhanced in current era (Kharel et al., 2018). As compared to the conventional small-molecule drugs, the peptides and proteins are extremely selective attributed to their complex structures and multiple points contact with the targets. It in turn results in a decrease in their side effects and toxicity. However, the delivery of these macromolecules *via* oral administration is inefficient. This is ascribed to their rapid enzymatic degradation in the gastrointestinal tract and limited membrane permeability (Wan and Yang, 2016). As a consequence, injectables remain as the most common means to deliver therapeutic peptides and proteins in the physiological system (Wan and Yang, 2016). Unfortunately, the requirement of frequent injections owing to the typically short plasma half-lives of the peptides and proteins leads to poor patient compliance and increased financial burden (Xia et al., 2013; Yang et al., 2019). Therefore, a flurry of scientific investigations have recently developed numerous long-acting injectables (LAIs) for peptides and proteins, which could elute the payload in a controlled manner and improve their pharmacological effects (He et al., 2015; Kharel et al., 2018; Martin-Sabroso et al., 2015; Wan et al., 2014b; Wu et al., 2018a).

Long-acting microsphere-based injectables have been over-exploited for the delivery of various peptide and protein therapeutics due to their high loading capacity and ability to provide prolonged release profiles of the biologically active macromolecules. Over the years, numerous biocompatible and biodegradable polymers, either of natural or synthetic origin, were extensively studied to accomplish ideal microspheres as LAIs of peptides and proteins. Among them, poly lactic-co-glycolic acid (PLGA) and poly lactic acid (PLA) are the most commonly employed polymers for formulating LAI microspheres because of their long safety history (Gomez-Gaete et al., 2017; Noviendri et al., 2016; Qi et al., 2014). These are Food and Drug Administration (FDA) approved polymers and commercially available in variable compositions of lactic and glycolic units, molecular weights and capping groups, which provide the prospects of tuning the release patterns of peptides

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Table 1
Commercial LAI PLGA/PLA-based microspheres containing peptides and proteins approved by FDA.

Commercial Name	Company	Active Pharmaceutical Ingredient (API)	Indications	Polymer	Administration route/ duration	Active substance	Manufacturing method	Size	Reference
Lutrate Depot®	GP Pharm	Leuprolide acetate	Advanced prostate cancer *etc.*	PLA	I.M./12 weeks	Gonadotropin-releasing hormone, (GnRH) analog	/	/	/
Lupron Depot®	Takeda	Leuprolide acetate	Advanced prostate cancer *etc.*	PLGA/PLA	I.M./4, 12, 16, 24 weeks	GnRH analog	Emulsion solvent evaporation	11.4 ± 0.5 μm or ~ 20 μm (volume median diameter, D_{50})	(Andhariya et al., 2019; Okada, 1997; Shi et al., 2020; Zhou et al., 2018)
Trelstar®	Allergan	Triptorelin pamoate	Advanced prostate cancer *etc.*	PLGA	I.M./4, 12, 24 weeks	GnRH analog	Spray drying or coacervation	≤200 μm	(Hu et al., 2012; Molavi et al., 2020)
Bydureon®	AstraZeneca	Exenatide	Type 2 diabetes mellitus	PLGA-sucrose	S.C./eekly	Glucagon-like peptide-1 (GLP-1) receptor agonist	Emulsion solvent evaporation or coacervation	~ 50 μm	(Hu et al., 2012; Lin et al., 2018; Molavi et al., 2020)
Signifor® LAR	Novartis	Pasireotide pamoate	Acromegaly *etc.*	PLGA	I.M./4 weeks	Somatostatin analog	Emulsion solvent evaporation	/	(Molavi et al., 2020)
Sandostatin® LAR	Novartis	Octreotide acetate	Acromegaly *etc.*	PLGA-glucose	I.M./4 weeks	Somatostatin analog	Emulsion solvent evaporation	1 ~ 10 μm or 65.0 μm(volume mean diameter)	(Ghassemi et al., 2012; Molavi et al., 2020)
Somatuline® Depot	Ipsen	Lanreotide acetate	Acromegaly *etc.*	PLGA	S.C./4 weeks	Somatostatin analog	Spray drying	/	(Hu et al., 2012)

I.M.: intramuscular injection; S.C.: subcutaneous injection.

and proteins (Ramazani et al., 2016). The success of PLGA/PLA-based microspheres in protein and peptide drug delivery can be evidenced by their marketed formulations as summarized in Table 1. Although the PLGA/PLA-based microspheres have offered promising outcomes as LAIs for peptide and protein drug delivery, these matrices often demonstrate various challenges associated to the continuous release patterns and stability of the bioactive molecules. The problems become more prominent in case of therapeutic proteins as compared to the peptides. This is accredited to the fact that the proteins are larger in size and possess a more complex structural configuration as compared to the peptides.

Quality by Design (QbD) thinking is a crucial element in the International Conference on Harmonization (ICH) Quality guidelines, and the QbD approach offers a perfect opportunity for the pharmaceutical community to take the manufacturing sciences into the new millennium (Rantanen and Khinast, 2015). The key elements of the QbD approach can be summarized: the work starts by constructing the quality target product profile (QTPP), which is subsequently translated into the critical quality attributes (CQAs), and finally, both the critical material attributes (CMAs) and critical process parameters (CPPs) are explored in a systematic way. Currently, QbD-based thinking inspires many pharmaceutical scientists to develop LAI PLGA/PLA-based microspheres for peptide and protein drug delivery, which would pass the rigorous clinical studies and be available on the markets.

This review article summarizes key literatures on PLGA/PLA microspheres to introduce the QbD approach for the development of LAI PLGA/PLA-based microspheres for the delivery of peptide and protein drugs. Initially, the key QbD elements are introduced considering Bydureon®, a commercial product, as an example. Various factors influencing two CQAs, *i.e.* the drug release behavior and physical/chemical integrity of the peptides and proteins are then discussed. This is followed by a summary of the existing technologies to accomplish PLGA/PLA microspheres and their CPPs influencing these two CQAs. Finally, a landscape of generic product development of LAI PLGA/PLA-based microspheres is reviewed.

2. QbD thinking in the development of LAI PLGA/PLA-based microspheres for peptide and protein drugs delivery

According to ICH Q8(R2), the QbD is “a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management”. As highlighted by the guideline: "In all cases, the product should be designed to meet patients' needs and the intended product performance". Nowadays, the QbD thinking has been widely employed in the development of various drug products (Baldinger et al., 2012; Gu and Burgess, 2015; Jog and Burgess, 2019; Kozaki et al., 2017; Kumar et al., 2014). Even though the strategies for the product development vary from company to company and from product to product, in general, the implementation of the QbD starts with the identification of the QTPP. It is a prospective summary of the drug product characteristics, which are typically achieved to ensure the desired quality, taking safety and efficacy into account based on risk assessment. The QTPP elaborates various aspects that ensure the safety and efficacy of a drug product, which is eventually included in the label. A classical example of QTPP extracted from the label of Bydureon® is summarized in Table 2.

After defining the QTPP, the CQAs are identified during the drug development process. The CQAs represent the physical, chemical, biological and microbiological properties or characteristics that lie within an appropriate limit, range, or distribution to ensure the desired product quality (ICH Q8). There can be many quality attributes that are required to be considered to formulate an optimal LAIs. However, the CQAs are those if not met, would result in severe harm to a patient. As shown in Table 2, the quality attributes of Bydureon® including encapsulation efficiency, particle size, size distribution, injectability and

Table 2
A representative QTPP of Bydureon®, a LAIs.

QTPP Element	Target	Justification
Indications	• An adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes mellitus in multiple clinical settings	• Better or same with Byetta® as API is the same
Dosage form	• Extended-release injectable suspension	• For better patient compliance and even better therapeutic effects
Administration route	• Subcutaneous injection, may be administrated by patients themselves	• To support the indication of type 2 diabetes
Production method	• Coacervation	
Strength	• 2 mg	• Decided by pharmacokinetics of obtained products
Pharmacokinetics	• Steady state exenatide concentration should be maintained during dosing interval (at least 1 week) with minimal peak to trough fluctuation to reduce side effect	• Decided by potency and adverse event associated with exenatide
Stability	• At least 24-month shelf-life under 2–8 °C or better	• As long as possible • The room temperature storage should be evaluated as protein and protein drugs stability is a tricky problem
Drug product quality attributes	• Encapsulation efficiency • Identification • Assay • Content uniformity • Particle size and distribution • Drug release (sustained release/low burst release) • Injectability • Related substances • Organic solvent residual • Sterility and bacterial endotoxin	• To ensure the efficacy and safety of the product • During research and development, other attributes may need to be tested such as encapsulation efficiency, which will affect costs • Consider patient compliance and tolerability
Administration	• Subcutaneous injection once every seven days (weekly), at any time of day and with or without meals	• Consider patient compliance, tolerability
Container closure system	• Vial, prefilled syringe, needles, even auto-injector	• Suitable container closure system can achieve the target shelf-life and ensure product integrity during shipping. More importantly, special instrument such as auto-injectors can significantly improve patient compliance

organic solvent residual are important to control with respect to its cost, patient compliance *etc*. However, the failure to meet any of these quality attributes might not directly cause severe harm to a patient. On the other hand, the CQAs of Bydureon® involve identification, assay, content uniformity, drug release, related substances, sterility and bacterial endotoxin. Among them, the drug release patterns and related substances (chemical degradation of peptides and proteins) are considered as high risk CQAs.

It is generally accepted that the CQAs of the drug products are dependent on the variation of the material and process related parameters. According to the ICH Q8a, CPPs are the process parameters whose variability has a great impact on a CQA and therefore these are critically monitored or controlled to ensure that the desired quality can be achieved. In addition to the CPPs, the physical or chemical characteristics and the quality attributes of raw materials (CMAs) are critical to ensure CQAs. The Table 3 summarizes the CPPs and CMAs that influence two CQAs of Bydureon® *i.e.* release and stability of the peptides. In order to understand the effect of material and process variation, the design of experiment (DoE) may be planned and executed to connect the CPPs and CMAs to CQAs, which is often done by starting with screening experiments using fractional factorial design (Baldinger et al., 2012; Kumar et al., 2014). To determine the optimal values for CPPs and CMAs, composite face-centered design or central cubic design can be employed (Baldinger et al., 2012; Kumar et al., 2014). In addition, by establishing the relationships between the CQAs and the CPPs/CMAs, it will be possible to define a design space for a LAI depot with the desired QTPP. The QTPP therefore forms the basis for development of the QbD-based drug product.

The peptide and protein drugs are complex and heterogeneous in nature and thus every product is unique and possesses a large number of quality attributes. Eventually, it is almost impossible to completely evaluate their impacts on the safety and efficacy of each product. In the following sections, we focused on two high risk CQAs viz. the release patterns and the stability of therapeutic peptides and proteins. The factors influencing these two CQAs in the development of LAI PLGA/PLA-based microspheres for the delivery of peptides and proteins were reviewed with a focus on the quality attributes of PLGA/PLA microspheres. This was followed by state-of-the-art of the manufacturing technologies to produce LAI PLGA/PLA microspheres for peptide and protein drugs. In addition, the CPPs that influence the two high risk CQAs were summarized and discussed. A schematic diagram of QbD thinking in the development of LAI PLGA/PLA-based microspheres for peptide and protein drug delivery is presented in Fig. 1.

3. Two key CQAs of LAI PLGA/PLA microspheres and their influencing factors

3.1. Release of peptide/protein drugs from LAI PLGA/PLA microspheres

An uncontrolled release behavior of peptides and proteins from the injectable PLGA/PLA microspheres could be life-threatening because these bioactive substances are often very potent. Their release control

Table 3
CQAs of Bydureon® and corresponding CMAs and CPPs.

CQAs	CPPs	CMAs
Release	• Stirring rate • The rate of addition of the first nonsolvent to extract the primary solvent	• Polymer type (molecular weight, end group, L:G ratio)
Stability	• Stirring rate • Temperature	• Organic solvent types (solvents and non-solvents) • Polymer concentration • Volume ratio of polymer solution to peptide solution

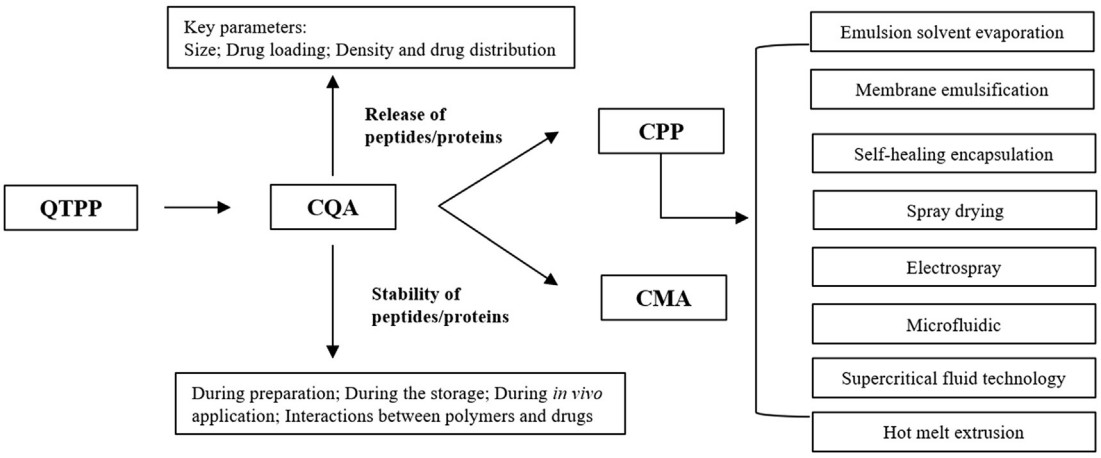

Fig. 1. Schematic diagram of QbD thinking in the development of LAI PLGA/PLA-based microspheres for peptide and protein drug delivery.

mechanisms can be classified into two modes, namely diffusion and polymer erosion (Fredenberg et al., 2011; Gaignaux et al., 2013; Zhang et al., 2018). The diffusion refers to the drug transport through the aqueous pores/channels of the polymeric matrices, while the erosion includes the drug transport with the molecular breakdown of the matrices (Martin-Sabroso et al., 2015). It is believed that the initial release pattern is commonly controlled by diffusion mechanism and erosion process predominates to regulate the drug release at later stage (Zhang et al., 2018). An ideal release profile of injectable PLGA/PLA microspheres might follow a zero-order release, maintaining a stable *in vivo* plasma concentration of the bioactive substances (Sevim and Pan, 2016). This eventually prevents the patients' exposure to the potentially toxic or sub-therapeutic concentrations of the peptides and proteins (Zhao et al., 2017). However, most of the PLGA/PLA microspheres exhibit a bi-phasic or a tri-phasic release profile (Vyslouzil et al., 2016). The matrices most frequently display tri-phasic release profiles, which consist of an initial burst release phase, followed by a lag phase with constant release rate, and a second rapid release phase attributed to polymer erosion (Li et al., 2014; Schwendeman et al., 2014; Zhang et al., 2018). The initial burst release profile might be accredited to the rapid dissolution and diffusion of the therapeutic molecules encapsulated close to the surfaces of the microspheres (Gasmi et al., 2016; Gomez-Gaete et al., 2017; Kojima et al., 2015; Liu et al., 2019a; Wan et al., 2014a; Xu et al., 2013; Zhang et al., 2016). The cessation of the initial burst release is accompanied by an onset of the lag phase of the drug release. This is attributed to the limited or no pores on the surface of the microspheres (Fredenberg et al., 2011; Schwendeman et al., 2014; Zhao et al., 2017). In the erosion phase, the polymer chains are cleaved by hydrolysis subsequently a critical PLGA molecular weight is reached (around 20 kDa) and thus the degree of macromolecular entanglement becomes insufficient to hinder substantial microsphere swelling (Gasmi et al., 2015). Interestingly, the release patterns in this phase obey apparent-zero-order kinetics.

Numerous factors influencing the release characteristics of PLGA/PLA microspheres include the molecular weight of the active substances (Shi et al., 2016), ratio of lactic and glycolic units, ending groups (Kojima et al., 2015; Makadia and Siegel, 2011; Wang et al., 2019a; Wang et al., 2016a; Xuan et al., 2013) and molecular weight of polymers (Gu et al., 2016; Kojima et al., 2015; Makadia and Siegel, 2011; Mylonaki et al., 2018; Wang et al., 2016a; Xuan et al., 2013), *etc.* Moreover, various *in vitro/in vivo* environmental factors such as pH (Andhariya et al., 2017a; Doty et al., 2017a; Doty et al., 2017b; Hirota et al., 2016; Shen et al., 2016; Tomic et al., 2016), temperature (Andhariya et al., 2017a; Shen et al., 2016; Tomic et al., 2016), buffer concentrations and their types (Doty et al., 2017a; Doty et al., 2017b; Hirota et al., 2016; Tomic et al., 2016), osmotic pressure (Tomic et al., 2016) and presence of other additives (Andhariya et al., 2017a; Doty et al., 2017b), *etc.* significantly regulate the release patterns. The material properties and their influences on the release profiles of the microspheres have been well reviewed previously (Kojima et al., 2015; Makadia and Siegel, 2011; Mylonaki et al., 2018). In the following section, the very essential quality attributes of PLGA/PLA microspheres namely the size, drug loading, density and drug distribution and their roles in modulating the drug release profiles were critically discussed. In addition, mathematical models for the drug release were reviewed.

3.1.1. Size

The diameter of the PLGA/PLA microspheres influences not only the injectability of the microspheres, which is directly correlated with the patient compliance (Ansary et al., 2016; Martin-Sabroso et al., 2015; Wang et al., 2016b), an important aspect in QTPP, but also the release behavior of the PLGA/PLA microspheres (An et al., 2016; Chen et al., 2017; Draheim et al., 2015; Joshi et al., 2013; Pervaiz et al., 2019; Ramazani et al., 2015; Wu et al., 2013; Ye et al., 2010). The size of microspheres primarily affects the diffusion-controlled release involved in the initial burst release phase and lag phase (Almeria et al., 2011; Bohr et al., 2012). The small microspheres exhibit an enhanced initial burst release as compared to large matrices due to the increased specific surface area (Lin et al., 2016; Lin et al., 2018). Furthermore, the small-size microspheres portray a rapid diffusion-controlled release, while the larger particles demonstrate a sigmoidal release pattern as the small matrices might decrease the diffusional path lengths (Chen et al., 2017; Dong et al., 2016; Han et al., 2016; Ma, 2014; Nath et al., 2013; Ramazani et al., 2015). On the other hand, the large microspheres usually evidence a faster polymer degradation rate (Lin et al., 2018), leading to a tri-phasic release pattern as polymer degradation effects are more prominent compared with small ones in which negligible effects can be observed and drug release mainly controlled by the diffusion (Busatto et al., 2018).

3.1.2. Drug loading

The drug loading efficiency is another important quality attribute of PLGA/PLA microspheres that influences the release profiles of PLGA/PLA microspheres (Gasmi et al., 2016). Typically, the microspheres with a high drug loading efficiency are pursued for the sake of reduced manufacturing cost (Ye et al., 2010). The increased drug loading could decrease the injection volume, which could potentially improve the patient compliance. It has been reported that the PLGA/PLA microspheres exhibit a distinct tri-phasic release profile when their drug loading is low. On the contrary, the release profiles become more or less bi- or mono-phasic in case of high drug loading (Gasmi et al., 2015). It was also revealed that the initial burst release of the microspheres could

be largely suppressed while their drug loading was low. The initial burst release is augmented when the drug loading is increased. This can be attributed to the fact that a reduced amount of drugs become available on the surfaces of the microspheres for initial burst release when the drug loading is low (Gu et al., 2016). An enhanced release rate for high drug loaded microspheres can be explained by the higher concentration gradient between the microspheres and the outer release medium (Falco et al., 2013). However, it is noteworthy that the potency of the peptide/protein drugs and their corresponding drug loading efficiencies play the key roles in their sustained release profiles. If the peptide/protein drug is highly potent, a low drug loading efficiency might be sufficient for the sustained pharmacokinetic profile of this bioactive molecule. However, a sufficiently high drug loading efficiency is mandatory to achieve a prolonged pharmacokinetic exposure for a peptide/protein drug with low potency. The technical complexities and challenges associated to the development of LAI PLGA/PLA microspheres may not be equal for therapeutic peptides and proteins with different potencies for injection.

3.1.3. Density and drug distribution

The densities of PLGA/PLA microspheres generally regulate their drug release profiles (An et al., 2016). Particularly, the porosity and pore formation patterns of the PLGA/PLA microspheres are likely to play a critical role in modulating their drug release characteristics (Meeus et al., 2015b; Mylonaki et al., 2018; Wang et al., 2019b). The peptide/protein drugs readily diffuse through the aqueous pores of the microspheres, due to their high water-solubility (Ford Versypt et al., 2013). Thus, the highly porous microspheres frequently exhibit an undesirable initial burst release due to the large specific surface areas for the drug diffusion (Amoyav and Benny, 2019; Hao et al., 2014; Malik et al., 2016). However, the porous structure of the microspheres is reported to be essential for the continuous and complete release of encapsulated peptide/protein drugs (Le et al., 2018). The porous microspheres generally display a shorter release profile than the dense matrices (Hao et al., 2014; Jafarifar et al., 2017; Wei et al., 2016). Analogously, the wrinkled microspheres favor a faster drug release pattern, plausibly due to their increased specific surface area (Dong et al., 2016). The distribution of the drugs in the PLGA/PLA microspheres could also play a pivotal role in influencing the release profiles (Chen et al., 2017; Qi et al., 2014). The surface-enrichment of the drug results in severe initial burst release (Li et al., 2014; Wang et al., 2019b; Zhai et al., 2015). It is evidenced that certain preparation methods favor the enrichment of the drugs on the surfaces of the PLGA/PLA microspheres (Wan et al., 2014a). Some studies also demonstrate that the release profiles of the PLGA/PLA microspheres could be modulated by tuning the distribution patterns of the peptide/protein molecules in the microspheres (Wan et al., 2014a). However, the direct characterization of the drug distribution in these microspheres is highly challenging with the currently available analytical techniques (Yang et al., 2017).

3.1.4. Mathematical models for drug release from LAI PLGA/PLA microspheres

Various mathematical models for drug release are important to elucidate the underlying physical and chemical mechanisms governing the drug release behavior, allowing for quantitative prediction of the drug release. Understanding the underlying physical and chemical mechanisms of the drug release may facilitate the formulation design and eventually decrease the amount of the required experimental studies and speed up the product development process (Fredenberg et al., 2011; Siepmann and Siepmann, 2008, 2012).

In general, the mathematical models can be roughly divided into two types: empirical/semi-empirical models and mechanistic mathematical models. The empirical/semi-empirical models are purely derived based on mathematical descriptions, without considering any real chemistry, physics of biological phenomenon affecting the drug release. As a result, no or very limited insight into the underlying drug release mechanisms could be obtained and their predictive power is relatively low (Fredenberg et al., 2011; Siepmann and Siepmann, 2008). Peppas equation, Higuchi equation, Hopfenberg model and Weibull equation are considered as empirical/semi-empirical models (Fredenberg et al., 2011; Siepmann and Siepmann, 2008).

On the other hand, the mechanistic mathematical models are based on real phenomena, such as diffusion, degradation and erosion, *etc.*, providing a mechanistic understanding of the release process (Fredenberg et al., 2011; Siepmann and Siepmann, 2008). The diffusivity of the Fick's law is the basis for the mechanistic models to describe the drug release (Fredenberg et al., 2011). The Fick's first law of diffusion and Fick's second law of diffusion are expressed in the following equations (1 and 2, respectively) (Siepmann and Siepmann, 2012).

$$F = -D\frac{\partial c}{\partial x} \tag{1}$$

$$\frac{\partial c}{\partial X} = D\left(\frac{\partial^2 c}{\partial X^2}\square\frac{\partial^2 c}{\partial y^2}\square\frac{\partial^2 c}{\partial z^2}\right) \tag{2}$$

The F denotes the rate of transfer per unit area of section (flux), c is the concentration of the diffusion species and D represents the diffusion coefficient (diffusivity). Fick's second law of diffusion is based on the Fick's first law of diffusion and mass balance considerations.

It is noted that the effective diffusion coefficient of drug release is a constant in some models, while it varies upon other parameters in others models (Fredenberg et al., 2011). For example, the drug diffusion coefficient depends strongly on the level of degradation of the polymer (Sevim and Pan, 2016). The effective diffusivity of drugs is often increased exponentially as a function of the extent of polymer degradation (Ford Versypt et al., 2013). The molecular weight of the polymer is decreased as it degrades, and the microparticles become more porous due to erosion, which could lead to a rapid increase in the effective diffusion coefficient of the drug molecules through the polymeric matrix (Busatto et al., 2018). The degradation refers to the chain scission events, which is commonly mediated through the hydrolysis process in the case of bio-erodible polymers (Sackett and Narasimhan, 2011). In addition, the drop in the internal pH due to the acidic degradation products further promotes the polymer degradation *via* ester bond cleavage (Busatto et al., 2017), which is known as "autocatalytic effect".

Many attempts have been made to develop the models correlating the degradation behavior and/or drug release behavior of PLGA/PLA microparticles. Casalini et al. described the degradation behavior of the microparticles exploiting the mass conservation equations, which considered both hydrolysis kinetics and autocatalytic behavior responsible for the bulk erosion of the polymers (Casalini et al., 2014). In their research, the diffusion of oligomers, water, and drug are assumed to follow Fick's law. An increased diffusivity is observed in an enhanced polymer hydrolysis. In addition, Sevim and Pan presented a mechanistic-based mathematical model, which can be used to calculate polyester degradation and drug release pattern (Sevim and Pan, 2016). This model considered the autocatalytic effects on the polymer degradation arising from both carboxylic acid end groups of oligomers and acidic drug molecules. Furthermore, the mathematical models based on pseudo-first-order kinetics, second-order, and 1.5th-order kinetic were reviewed by Ford Versypt and co-workers for polymer degradation, autocatalytic hydrolysis and polymer hydrolysis, respectively (Ford Versypt et al., 2013).

The effects of the size of the matrices on the development of mathematical models of polymer degradation behavior and drug release behavior are also equally important. Busatto and co-researchers (Busatto et al., 2017) found that the microsphere degradation rate was notably influenced with their particle size. The degradation products formed within smaller particles can diffuse drugs easily to the matrix

surface, while the length of the diffusion pathways is increased in the larger particles. In this case, the trapped degradation products could catalyze the degradation of the remaining polymer. The model established by them can not only predict the effects of the particle size and molecular weight on the degradation of PLGA-based microspheres, but also estimates the morphological changes of the particles caused by the autocatalytic effect. Similar results were also described by Ford Versypt *et al.* The study demonstrated that the larger microspheres experienced a faster erosion in their centers than the smaller microspheres (Ford Versypt et al., 2013). Furthermore, Busatto et al. developed a mathematical model that predicted the drug release profiles from the different-sized PLGA microspheres, considering the dissolution and diffusion of the drug molecules in the polymeric matrix and the autocatalytic effect of the polymer degradation (Busatto et al., 2018). In addition, the initial drug release rate is decreased with an increase in the size, which is consistent with Fick's law of diffusion. This could be attributed to an increase in the diffusional path length due to the reduced surface area to volume ratio of the larger microspheres (Berchane et al., 2007). Berchane and co-workers (Berchane et al., 2007) also found that an incorporation of the size distribution into the mathematical model for the highly polydisperse formulations gave a better fit to the experimental results than the representative mean diameter alone.

In addition, it has been reported that the initial effective diffusion coefficient of the drug may vary with the drug loading of the microparticles (Busatto et al., 2018). Casalini et al. firstly established a mathematical model that considered the influence of initial drug distribution throughout the matrix on drug diffusion (Casalini et al., 2014). The mathematical treatment could be simpler if the distribution of drug in the matrix is homogeneous (Siepmann and Siepmann, 2012). Ford Versypt and co-researchers also reviewed the models involving the effects of porous networks on the diffusion rates of the drug (Ford Versypt et al., 2013).

The mathematical modeling of the polymer erosion has been reviewed by Sackett and Narasimhan (Sackett and Narasimhan, 2011). However, the polymer erosion has been defined as a combination of degradation, dissolution, diffusion processes and in most cases, the degradation appears to be the controlling step of erosion (Sackett and Narasimhan, 2011). In addition, Siepmann et al. reviewed the mathematical models of diffusion-controlled drug delivery, which were based on different assumptions (Siepmann and Siepmann, 2012). Nevertheless, it should be noted that the aforementioned mathematical models are based on the *in vitro* release behavior without considering *in vivo* factors. For example, the reactive oxygen species (ROS)-induced degradation of the biodegradable polymers may be the primary effector causing an accelerated *in vivo* release (Knab et al., 2015).

3.2. Stability of peptides/proteins in PLGA/PLA microspheres

The physical instability (the alteration of the higher order structures without any covalent modification) of the peptides/proteins includes the unfolding, aggregation, precipitation and adsorption while the chemical instability (formation of new chemical entities with altered properties) refers to the hydrolysis, oxidation, deamidation, racemization, isomerization as well as acylation (Hajavi et al., 2018; Oak et al., 2012). Injectable PLGA/PLA microspheres with physically degraded or chemically mutated peptides and proteins may cause life-threatening consequences to the patients. Therefore, preserving the physical and chemical integrity of the peptide and protein drugs during the stages of microsphere preparation, their storage and *in vivo* application is critical. In this section, several essential factors modulating the stability of the peptides/proteins during the life cycle of the PLGA/PLA microspheres are discussed.

3.2.1. Factors influencing the peptides/proteins stability during preparation

The commonly used technique to encapsulate hydrophilic peptides/proteins in hydrophobic PLGA/PLA matrices is emulsification followed by solvent diffusion, evaporation, phase separation, or drying to remove the solvent. Among various protocols, the water-in-oil-in-water ($W_1/O/W_2$) double emulsion solvent evaporation method is probably the most widely exploited method (Tomar et al., 2011). In this process, the interfacial force between water and organic phase induce stresses to the peptide and protein drugs (Marquette et al., 2014a; Montalvo-Ortiz et al., 2012; Moreno et al., 2016), leading to the aggregation and denaturation of these molecules on the interfaces. This is attributed to the surface active properties of the peptide and protein molecules (Nuzzo et al., 2014). It is reported that the oil/water interfaces could serve as a hydrophobic adsorbent and the peptides and proteins with intermolecular antiparallel beta-sheets are most vulnerable to such denaturation (Tomar et al., 2011). In order to eliminate oil/water interfaces, some innovative emulsion solvent evaporation-based novel methods are attempted. For example, Hong et al. has introduced a novel method to accomplish an emulsion ($W/O/O_h/W$) to formulate LAI PLGA-based microspheres loaded with protein drug (Hong et al., 2013). The 'O_h' designates a hydrophilic oil phase such as a mixture of ethylene glycol and glycerol. It is reported that these hydrophilic oils could reduce the hydrophobicity within oil/water interfaces and eventually enhance the bioactivities and encapsulation efficiencies of protein drug (Hong et al., 2013). Moreover, a solid-in-oil-in-water (S/O/W) emulsion process has also been adopted, where the solid powders of peptides or proteins are dispersed in the PLGA/PLA oil phase to restrict the conformational change of these labile molecules (He et al., 2015; Marquette et al., 2014a; Rawat et al., 2012). The freeze-dried bulk powders of the peptides and proteins are mechanically grinded to reduce their particle size in the range of 1–10 μm and then introduced in S/O suspensions (Hong et al., 2013). Another approach to overcome the interfacial denaturation of the peptides/proteins is adding stabilizers, such as sugar, surfactant, *etc.* during preparation of the PLGA/PLA microspheres. Some commonly used stabilizers are listed in Table 4. The mechanical shear forces (*e.g.* ultrasonic, homogenization) applied to formulate the primary emulsions also induce stresses that potentially cause peptide/protein degradation during the microsphere production (Marquette et al., 2014a; Tomar et al., 2011; Zhai et al., 2015).

Table 4
The stabilizers commonly used to maintain the stability of the peptide/protein drugs during their formulations.

Stabilizers	Mechanism	Reference
Sugars	Shield protein from the organic solvent *via* preferential hydration of their surfaces and by increasing the free energy of system	(Tomar et al., 2011)
Cyclodextrin	Decrease the interfacial tension and weaken hydrophobic interaction between protein and interface	(Ma, 2014; Moreno et al., 2016)
Proteins	Form a protective layer due to the aggregate on the interface	(Wang et al., 2013; Xu et al., 2015)
Polyethylene glycol (PEG)	PEG is a macromolecular component and makes the interfacial environment crowded in nature, which can decrease the adsorption of the protein at the interface	(Rawat et al., 2012)
Surfactants	Nonionic surfactant (*e.g.* Tween 20): steric effectIonic surfactant (*e.g.* carboxymethyl cellulose (CMC): electrostatic force of repulsion	(Rawat et al., 2012)

3.2.2. Factors influencing the peptides/proteins stability during the storage

The temperature and humidity are two of the climatic factors that can significantly threaten the physical and chemical integrity of the peptides and proteins during storage (Wang and Burgess, 2013). An exposure of LAI PLGA/PLA microspheres to the elevated temperature and humidity might also influence their release behavior (Marquette et al., 2014b; Meeus et al., 2013). Thus, most of LAI PLGA/PLA-based microspheres for peptide/protein drug delivery are stored and transported *via* cold chain. The stability of the PLGA/PLA microspheres is well correlated with the glass transition temperature (T_g) of the polymer chains. It is reported that these microspheres stored at ambient temperature (below their T_g) are stable for 12 months (Wang and Burgess, 2013). It is postulated that a reduced polymer chain mobility at low storage temperature might result in negligible physical change with age (Wang and Burgess, 2013). The PLGA/PLA chains could absorb moisture at high humid condition, leading to a decrease in the T_g of the polymer chains with an increase in the chain mobility (Wang and Burgess, 2013). This plasticization effect accelerates the degradation of the polymer chains. In addition, the presence of moisture can also induce the hydrolytic degradation of the PLGA/PLA chains (Wang and Burgess, 2013).

3.2.3. Factors influencing the peptides/proteins stability during in vivo application

The peptides and proteins are exposed to extremely complex environment upon their *in vivo* applications and could be degraded following several routes. The body temperature with *in vivo* 100% RH could boost the degradation rate of peptides and proteins. The acidic inner micro-environmental pH (μpH) created due to the polymer hydrolysis could catalyze the chemical degradation of the peptides/proteins, losing their therapeutic potentials (Liu et al., 2015; Mao et al., 2012; Wang et al., 2015a; Wang et al., 2015b; Wang et al., 2015c). The low μpH could also induce the cationic peptides to create new covalent bonds with the PLGA oligomers by acylation reactions (Kharel et al., 2018), which is discussed in the Section 3.2.4. However, the effects of low μpH on the drug release is controversial. Kang et al. elaborated that the low μpH was responsible for poor release kinetics (*e.g.* a triphasic release pattern and incomplete release) (Kang et al., 2012), while Qi et al. found that the acidic pH was able to accelerate the PLGA hydrolysis and consequently could fasten the drug release rate (Qi et al., 2019). Several factors may contribute to the development of μpH, including the amount of acidic impurities, the production rate of water-soluble acids, the liberation rate of water-soluble acids out of polymer, the partition coefficient of the acids between polymer and aqueous phases, and the dissociation constant (pKa) of the acids might make it a relatively complicated process (Liu et al., 2012).

Some attempts have been made to circumvent the effects of the low μpH. The antacids such as magnesium hydroxide and calcium hydroxide solutions are conventionally added to circumvent this problem (Wang et al., 2015a). However, a weak base cannot really neutralize the acidic environment and a stronger base might impart more detrimental effects to the stability of peptides and proteins (Wang et al., 2015a; Wang et al., 2015c). In addition, the fast-releasing magnesium hydroxide and calcium hydroxide cannot follow the different biodegradation rates of PLGA/PLAs chains (Liu et al., 2015). Alternatively, Wang et al. developed poly (cyclohexane-1, 4-diyl acetone dimethylene ketal) (PCADK) blended PLGA microspheres (Wang et al., 2015a; Wang et al., 2015b; Wang et al., 2015c). The degradation products of PCADK are neutral, which could cause a negligible pH alteration during applications. The bioactive glasses were also employed as biodegradable and sustained-release antacid to neutralize the degradation products of PLGA/PLA matrices and thus stabilize the lysozyme during applications (Liu et al., 2015).

3.2.4. Factors influencing the interactions between PLGA/PLA and peptides/proteins

The hydrophobic and electrostatic interactions between the PLGA/PLA and peptides/proteins could remarkably induce the undesired modifications of the peptides/proteins, resulting in loss of their activity, immunogenicity and toxicity (Mohammadi-Samani and Taghipour, 2015; Shirangi et al., 2016; Zhang and Schwendeman, 2012). It has also been proposed that the adsorption of the positively charged peptides within the negatively charged polymer matrix due to the electrostatic interactions results in a slow release of the drug over a long period of time (Andhariya et al., 2019). The physical denaturation of peptides/proteins such as deformation and aggregation could be induced by their physical interactions with the hydrophobic surfaces of the PLGA (Kang et al., 2012).

Meanwhile, the most commonly observed chemical reaction between PLGA/PLA and peptides/proteins is acylation, which involves the nucleophilic attack of the peptide/protein residues (such as the labile N-terminus or lysine side chains) to the electrophilic carbonyl groups of the lactate or glycolate esters of PLGA to form new covalent bonds between peptides and PLGA oligomers, resulting in peptide-PLGA adducts (Balmert et al., 2015; Ghassemi et al., 2012; Liu et al., 2019c; Shirangi et al., 2015, 2016). Ghassemi et al. pointed out that the nucleophilic attack of octreotide could more readily occur on the glycolic acid units due to the steric factors as compared to the lactic acid units (Ghassemi et al., 2012). There are also possible acylation sites on the peptides/proteins like the acylation of arginine and serine residues as observed in goserelin-loaded and in leuprorelin-loaded PLGA microspheres, respectively (Guo et al., 2019; Liang et al., 2013; Shirangi et al., 2016).

The electrostatically driven sorption of the protonated amino groups ($–NH_3^+$) of the peptides to the deprotonated carboxyl groups (–COO–) of the polymers is crucial for peptide acylation (Balmert et al., 2015; Liu et al., 2019d). Besides, the low μpH caused by the accumulation of the degraded products (*i.e.* lactic and glycolic acid and their oligomers) within the microspheres could catalyze the acylation (Balmert et al., 2015; Ghassemi et al., 2012). However, according to a recent research of Liu and co-researchers, the peptide acylation became extremely serious when the μpH was raising up from acidic to neutral zone. When the acidic environment inside the microsphere was neutralized, the degradation of the polymer slowed down and the lag time of drug release was prolonged, extending the retention time of the drug molecules. These could act as one of the acylation substrates in the microspheres (Liu et al., 2019c). In addition to μpH, the effects of polymer type on acylation of octreotide and the effects of water on exenatide acylation were also investigated (Liang et al., 2013; Shirangi et al., 2015; Vaishya et al., 2015b).

There are few methods that have been attempted to avoid the acylation. First, the additives such as sodium dodecyl sulfate (SDS), dextran sulfate A (DSA), dextran sulfate B (DSB) have been employed to maintain the stability of octreotide by forming a reversible hydrophobic ion-pairing (HIP) complex. It has been reported that DSA and DSB could both effectively mask the reactive nucleophile amine of octreotide and eventually less than 7% octreotide was acylated (Vaishya et al., 2015a). Next, it has also been reported that the PEGylation (polyethylene glycol) of the peptides could significantly reduce the acylation process in PLGA microspheres. Plausibly, the conjugated PEG molecules sterically hinder the peptides adsorption to the PLGA surfaces and sustain their stability (Lim et al., 2015). Moreover, an addition of water-soluble dicationic salts (Mn^{2+} and Zn^{2+}, *etc.*) could inhibit acylation reaction probably by shielding the binding sites of peptides and PLGA chains (Qi et al., 2015; Sophocleous et al., 2009). Recently, instead of using $CaCl_2$, Liu et al. found a poorly water-soluble Ca^{2+} depot ($Ca_3(PO_4)_2$) had the potentials to achieve a long-term and sustainable inhibition effects on acylation as it can gradually produce sufficient Ca^{2+} after absorbing protons from the degrading polymer (Liu et al., 2019d). Moreover, the acylation inhibition effect of dicationic salts could be further improved

Table 5
A summary of analytical tools employed to characterize the peptide/protein-loaded PLGA/PLA-based microspheres.

Tools	Main application	Description	Reference
Optical microscope	To measure the size of microspheres. To obtain a quick overview of the shape and size of microspheres.	In general, about 300 microspheres are recorded in order to obtain the arithmetic diameter. Accurate but relatively time-consuming for the measurement of size.	(Yu et al., 2019)
Laser diffraction particle analyzer	To determine the size and size distribution of microspheres.	The size is often described as volume-average values. The term "span" is commonly used to measure the size distribution and the value is calculated as $(D_{90}-D_{10})/D_{50}$ where D_{90}, D_{50} and D_{10} are the diameters measured at the 90%, 50%, 10% of undersized microparticles, respectively. Fast, stable and accurate analysis. Agglomeration of small particles can disturb the measurement, sometimes a pre-ultrasonic process is necessary.	(Shi et al., 2020; Tomic et al., 2018; Yang et al., 2017; Zhu et al., 2019)
High performance liquid chromatography (HPLC)	To quantify peptides/proteins. Characterize chemical degradation.	Both drug loading and release amount can be obtained based on an established standard curve. Limit of quantification (LoQ) and limit of detection (LoD) are depended on many parameters, such as the type of peptides/proteins, chromatographic conditions *etc.*	(Kharel et al., 2018; Liu et al., 2019a; Liu et al., 2019b; Liu et al., 2019c, d; Tomic et al., 2018; Zhu et al., 2019)
Ultra performance liquid chromatography (UPLC)	Same as HPLC.	Higher resolution, analytical speed and sensitivity compared with tradition HPLC.	(Qi et al., 2019; Shi et al., 2020)
UV–Visible spectrophotometer	To quantify peptides/proteins.	Based on the Lambert-Beer law, the peptide/protein concentration is related to the absorbance. Therefore, similar to HPLC and UPLC, both drug loading and release amount can be obtained based on an established standard curve. A very simple and fast method to quantify peptide/proteins compared with HPLC and UPLC. The LoQ and LoD may be higher than HPLC and UPLC. The drug concentration cannot be too low.	(Dong et al., 2019; Yang et al., 2017; Yu et al., 2019)
Scanning electron microscopy (SEM)	To observe the size, surface/inner structure, and shape of microspheres.	Standard method to observe the morphology of microspheres. Can obtain some information of micro-structure such as micro-porosity of the microspheres. Pre-treatment to the samples is needed.	(Kharel et al., 2018; Le et al., 2018; Liu et al., 2019a; Liu et al., 2019b; Liu et al., 2019c; Shi et al., 2020; Wu et al., 2016; Yang et al., 2017; Zhu et al., 2019)
Atomic force microscopy (AFM)	To give a detailed information of surface of the microspheres.	The surface topography and roughness can be thoroughly observed by AFM. No additional pre-treatment process to the samples. Provide the real three-dimensional (3D) surface images. The range of imaging is too small and the speed is too low. Require intensive expertise of the operator to obtain reliable information.	(Meeus et al., 2013; Qi et al., 2019)
Synchrotron radiation X-Ray-computed microtomography (SR-μCT)	To observe the 3D internal structure of microspheres.	SR-μCT has huge advantages in both 2D and 3D visualization of internal and external geometrical structures of microspheres owing to its high resolution and contrast in comparison with tradition SEM.	(Zhu et al., 2019)
Mercury porosimeter	To measure the porosity of microspheres.	Mercury porosimeter is generally used to the measure the total intrusion volume, total pore area and porosity. The porosity is calculated as (porosity (%) = 1-bulk density/apparent (skeletal) density × 100).	(Andhariya et al., 2019; Liu et al., 2019a; Liu et al., 2019b; Shen et al., 2016)
Porosity analyzer	To determine the pore size, specific surface area and pore size distribution.	The pore size, specific surface area as well as pore size distribution can be measured by nitrogen adsorption–desorption method on a specific surface area and porosity analyzer. The total surface area is calculated either using the Brunauer–Emmett–Teller (BET) model or Barrett–Joyner–Halenda (BJH) method. However, compared with mercury porosimeter, a very low temperature (~196 ℃) is needed in the measurement.	(Meeus et al., 2015; Wei et al., 2016)
Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)	To detect the structural integrity of peptide/proteins.	SDS-PAGE has the ability to identify the change of molecular weight of peptides/proteins after encapsulation. Therefore, based on the molecular weight change after encapsulation, the structural integrity and the aggregation of peptides/proteins can be confirmed to some extent.	(Dong et al., 2019; Zhu et al., 2019)
Circular dichroism (CD) spectroscopy	To analyze the secondary and tertiary structural integrity of peptides/proteins.	A fast, simple and relatively accurate tool to investigate the conformation change of peptides/proteins after encapsulation.	(Dong et al., 2019; Liu et al., 2019c; Moreno et al., 2016; Qi et al., 2019; Varshochian et al., 2013)

(*continued on next page*)

Table 5 (*continued*)

Tools	Main application	Description	Reference
		However, only pure samples can be correctly investigated by this method. In addition, other peptides/proteins or any chiral impurities will cause interferences and thus errors in results.	
Fourier transform infrared (FT-IR)	To analyze the secondary structural integrity of peptides/proteins.	The secondary structure can be confirmed by FT-IR tool. On one hand, this tool can detect the interaction between peptides/proteins and polymer matrix. On other hand, it can also observe the structure change of peptides/proteins after encapsulation.	(Andhariya et al., 2019; Dong et al., 2019; Liu et al., 2019b; Liu et al., 2019c; Qi et al., 2019; Yang et al., 2017; Yu et al., 2019)
Raman spectroscopy	To detect the distribution of peptides/proteins insides the polymer matrix and also give information about the conformational structure of peptides/proteins.	The distribution of peptides/proteins within polymer matrix contributes to elucidating the release behavior. The conformational changes (*i.e.* the shifts in the typical spectral bands of α-helix and β-sheet) can also be determined by Raman spectra to analyze the structure stability.	(Cosse et al., 2017; Qi et al., 2019)
Confocal laser scanning microscopy (CLSM)	To detect the distribution of peptides/proteins insides the polymer matrix. To evaluate the micro-climate pH (μpH) quantitatively.	In general, protein (*e.g.* bovine serum albumin, BSA) needs to be labeled with fluorescein isothiocyanate (FITC) so that it can be observed by CLSM. Besides, by encapsulating fluorescent pH-sensitive probes, μpH, which plays an important role in the stability and release of peptides/proteins, can be measured.	(Cosse et al., 2017; Le et al., 2018; Wan et al., 2014) (Liu et al., 2019c; Wang et al., 2015)
X-ray photoelectron spectroscopy (XPS)	To detect the chemical compositions on the surface of microspheres.	XPS can be used to characterize the drug surface enrichment on the microspheres. XPS can also be employed to investigate how the surface chemically changed when subjected to heat and exposure to humidity.	(Meeus et al., 2013; Wan et al., 2014)
Time-of-flight secondary ion mass spectrometry (ToF-SIMS)	To detect the chemical compositions on the surface of microspheres.	A complementary technique to XPS.	(Meeus et al., 2013; Meeus et al., 2015)
Gel permeation chromatography (GPC)	To measure the molecular weight of polymers.	GPC is widely used to evaluate the degradation behavior of polymers during *in vitro* and *in vivo* release by measuring the molecular weight of polymers at different time periods. GPC can obtain molecular weight dispersity.	(Garner et al., 2015; Kharel et al., 2018; Liu et al., 2019c; Shi et al., 2020; Wang et al., 2017)
Multi angle static light scattering (MALS)	To measure the molecular weight of polymers.	A more accurate measurement of molecular weight of polymers compared with GPC. MALS cannot obtain molecular weight dispersity.	(Garner et al., 2015; Park et al., 2019)
Nuclear magnetic resonance (NMR)	To characterize polymer properties such as the ratio of lactic and glycolic units, end-group.	^{1}H NMR is used to determine the ratio of lactic and glycolic units while ^{13}C NMR is employed to confirm the end-group of polymers. A common technology in the reverse engineering of reference listed drug.	(Garner et al., 2015; Skidmore et al., 2019; Zhou et al., 2018)
Differential scanning calorimeter (DSC)	To characterize the solid state of the polymers.	DSC is commonly used to determine the glass transition temperature (T_g) of the polymeric microspheres.	(Liu et al., 2019a; Liu et al., 2019b; Liu et al., 2019c; Moreno et al., 2016; Qi et al., 2019; Shi et al., 2020; Tomic et al., 2018; Wu et al., 2016; Yang et al., 2017)
Powder X-ray diffraction (PXRD)	To characterize the solid state of the polymeric microspheres.	A tool that always used in combination with DSC to better confirm the solid state of polymers.	(Qi et al., 2019; Wu et al., 2016; Yang et al., 2017)
Transition Temperature Microscopy (TTM)	To determine the distribution of the drug and polymer across the surface and cross section of a single microsphere.	A novel emerging technique for automated analysis of the local temperature transitions over an area of a sample surface. Compared with traditional thermal analysis tools such as DSC, which is usually performed on bulk samples, TTM is focused on the distribution of the drug and polymer across the surface and cross section of a single microsphere. It can provide a more detailed understanding of drug microstructure and spatial distribution in microspheres.	(Yang et al., 2017)

when these were co-encapsulated with carboxymethyl chitosan (CMCS), a dication chelator. The CMCS molecules could effectively retain the salts and promote these to interact with amino groups of the peptides. However, their efficacy in inhibiting the acylation reaction depends on the properties of the individual peptides/proteins (Zhang and Schwendeman, 2012). To facilitate the reading a summary of analytical tools employed to characterize the LAI PLGA/PLA microspheres loading peptide/protein drugs is provided in Table 5, where most physical and chemical characterization methods for the microspheres and stability of the payload are included.

Table 5

4. Existing state-of-the-art of the manufacturing methods of long-acting PLGA/PLA microspheres containing peptides/proteins and related CPPs

Various methods based on the principles of emulsification and co-precipitation of the polymers and bioactive macromolecules are

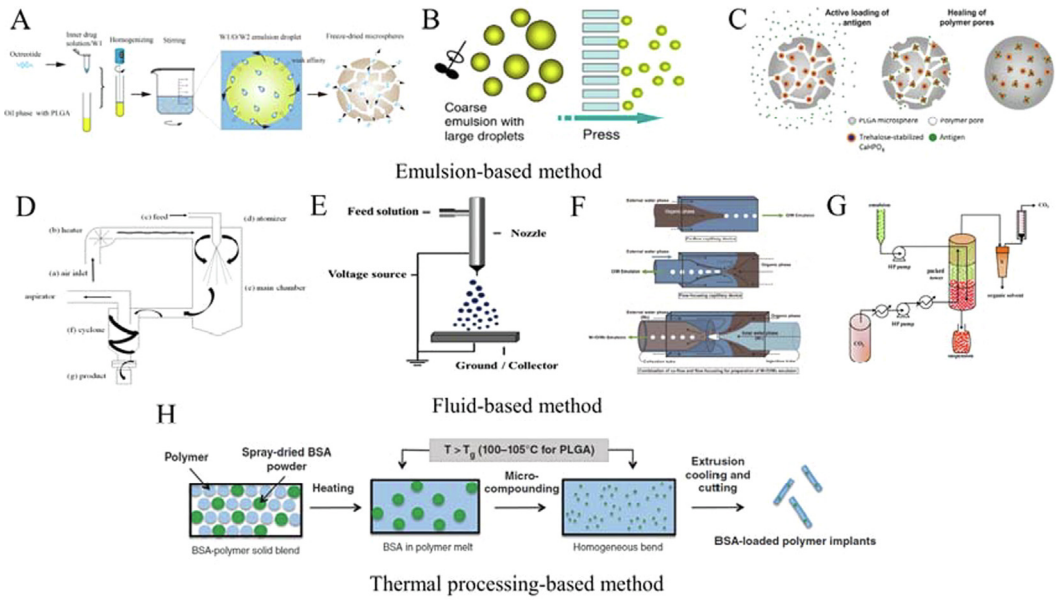

Fig. 2. State-of-the-art of the manufacturing methods of LAI PLGA/PLA-based microspheres modified from the following literatures (A: Double emulsion solvent evaporation. Reprinted from (Liu et al., 2019), Copyright 2019, with permission from Elsevier. B: Rapid membrane emulsification. Reprinted from (Ma, 2014), Copyright 2014, with permission from Elsevier. C: Self-healing encapsulation. Reprinted from (Bailey et al., 2017), Copyright 2017, with permission from John Wiley & Sons. D: Spray drying. Reprinted from (Ziaee et al., 2019), Copyright 2018, with permission from Elsevier. E: Electrospray. Reprinted from (Bohr et al., 2012), Copyright 2012, with permission from Elsevier. F: Microfluidic. Reprinted from (Ramazani et al., 2016), Copyright 2016, with permission from Elsevier. G: Supercritical emulsion extraction-continuous (SEE-C). Reprinted from (Falco et al., 2013), Copyright 2012, with permission from Elsevier. H: Hot melt extrusion. Reprinted from (Lee and Pokorski, 2018), Copyright 2018, with permission from John Wiley & Sons).

Table 6
Various preparation methods of LAI PLGA/PLA-based microspheres and their CPPs on the release and stability of peptides/proteins.

Methods	CPPs on release	CPPs on stability
Emulsion solvent evaporation	• Emulsification power • Stirring rate in the solidification process • Temperature • Pressure • Osmotic pressure gradient between the inner and outer water phase	• Emulsification power • Stirring rate in the solidification process • Temperature
Membrane emulsification	• Trans-membrane pressure • Flow velocity	• Emulsification power • Stirring rate in the solidification process
Self-healing encapsulation	• Emulsification power • Incubation temperature	• Incubation temperature
Spray drying	• Emulsification power (for the formation of W/O, S/O emulsion) • Atomization energy input • Inlet/outlet temperature • Drying flow rate • Feed rate • Feed rate ratio in 3-fluid nozzle	• Emulsification power (for the formation of W/O, S/O emulsion) • Atomization energy input • Inlet/outlet temperature
Electrospray	• Emulsification power (for the formation of W/O, S/O emulsion) • Voltage • Environmental temperature • Liquid flow rate • Flow rate ratio in co-axial electrospray	• Emulsification power (for the formation of W/O, S/O emulsion) • Environmental temperature.
Microfluidic	• Total flow rates • Flow rate ratio • Osmotic difference	• Stirring rate in solidification process
Supercritical fluid technology	• Pressure • Temperature • Emulsification power (for the emulsion formation in SEE method) • Depressurization rate	• Pressure • Temperature • Emulsification power (for the emulsion formation in SEE method)
Hot melt extrusion	• Shear force	• Temperature • Shear force • Residence Time Distribution (RTD)

exploited for the development of PLGA/PLA microspheres encapsulating therapeutic peptides and proteins (Fig. 2). The performances of these methods are significantly depended on different CPPs as enlisted in Table 6**.** In this section, the preparation methods of PLGA/PLA microspheres and their corresponding CPPs that influence the two key CQAs (*i.e.* the release and stability of the loaded peptides/proteins) are reviewed. A continuous manufacturing aspect of these methods is also introduced at the end of this section.

4.1. Emulsion solvent evaporation

The emulsion solvent evaporation protocol is probably the most widely adopted method in the research labs and also in the industries to fabricate LAI PLGA/PLA microspheres for peptide and protein drugs (Tomar et al., 2011; Yang et al., 2019; Zhang et al., 2015). This method involves three major steps namely, emulsification, solvent removal and harvesting. The peptide and protein molecules either in liquid or solid state could be encapsulated into the PLGA/PLA microspheres by following $W_1/O/W_2$ or S/O/W method, respectively. In the $W_1/O/W_2$ method, the peptides or proteins are initially dissolved in an aqueous phase (W_1), which is then emulsified in an organic phase containing PLGA/PLA under mechanical force such as ultrasonication or homogenization, yielding a primary W_1/O emulsion. This primary emulsion is subsequently mixed with an aqueous phase (W_2) containing emulsifier under mechanical force. The microspheres become solidified when the coarse $W_1/O/W_2$ emulsion is continuously stirred to remove the organic solvent. Afterwards, the free emulsifier is removed by centrifugation and washed with purified water. The PLGA/PLA microspheres are finally harvested and dried *via* lyophilizer. It has also been reported that nutsche-type filter-dryers can be used to dry and harvest PLGA/PLA microspheres in a large scale (Herbert et al., 1998).

It is important to identify CPPs of preparation method, which can significantly impact on the quality attributes of the PLGA/PLA microspheres (*i.e.* size, drug loading, density and drug distribution), and eventually influence the drug release behavior and stability of peptides/proteins (Ramazani et al., 2016). Various parameters such as ultra-sonication power, shearing rate and duration of sonication could influence the sizes of the primary emulsions (W_1) produced in emulsion solvent evaporation method. The sizes of the primary emulsions could ultimately determine the diameters of the final PLGA/PLA microspheres and regulate their release patterns (Gaignaux et al., 2012). It is notable that the heterogeneous emulsification would fabricate microspheres with non-uniform sizes (Liu et al., 2016). The primary emulsification step could also expose the peptides/proteins to the oil/water interfaces and cause the loss of peptide/protein activities (Ye et al., 2010). In addition, the stresses evolved by these CPPs could also influence the stability of the peptides/proteins (Marquette et al., 2014a). For instance, a longer ultrasonication time leads to a decrease in the drug loading as the excessive sonication can damage the structures of the peptides/proteins (Wu et al., 2016a). In the secondary emulsification step, a high emulsification power utilized to disperse preformed O/W droplets to the external water phase also demonstrates an influence on the stability of the macromolecules (Gaignaux et al., 2012). During the solidification process, the leakage of the bioactive molecules from the inner water phase to the outer aqueous phase results in a low drug loading (Bae and Park, 2015; Guo et al., 2015; Wang et al., 2016c; Wu et al., 2016a). When the polymer droplets are solidified, the leakage is significantly declined. Thus, a faster solidification of the microspheres might improve the drug loading efficiency (Ramazani et al., 2016). Furthermore, it could shorten the contact time of peptides/proteins at oil/water interfaces and could minimize the loss of therapeutic activities (Ma, 2014). The stirring rate can influence the solidification rate, the surface morphology and the size distribution of the microspheres (Wei et al., 2011). It is reported that the microspheres prepared with a low stirring rate appear spherical with smooth surface morphology and are relatively uniform in size (Wei et al., 2011). Besides, the temperature and pressure have been reported to influence the physicochemical characteristics and then the release profiles of microspheres due to their influence on the solidification rate (Li et al., 2012). The osmotic pressure gradient between the inner and outer water phase has also been proved to affect the structure and drug loading of microspheres (Cun et al., 2008).

4.2. Membrane emulsification

The conventional emulsification processes using ultrasonication, agitation, *etc.* often result in broad size distribution of the microspheres (Wei et al., 2011; Wei et al., 2016). To overcome this drawback, the membrane emulsification method classified as direct membrane emulsification and rapid membrane emulsification protocols has been developed (Ma, 2014). In the direct membrane emulsification method, the drug and polymer co-dissolved in an organic phase is pumped through a membrane with uniform pores under trans-membrane pressure together with an aqueous phase containing emulsifiers to produce O/W single emulsion droplets. The resultant O/W droplets at the membrane pore openings are detached by the shear stress of the water-phase (Ramazani et al., 2016). To produce $W_1/O/W_2$ double emulsions using this method, the W_1/O primary emulsions obtained by ultrasonic or homogenization are pumped through the pores into the continuous aqueous phase.

In a rapid membrane emulsification method, the coarse O/W or $W_1/O/W_2$ emulsions are frequently extruded through the pores of the membrane to yield uniform microspheres (Lin et al., 2016; Ma, 2014; Qi et al., 2014; Wei et al., 2011; Wei et al., 2016). The CPPs of the $W_1/O/W_2$ double emulsion evaporation method are also applicable to the membrane emulsification method and significantly influence the quality attributes. Additionally, the trans-membrane pressure could regulate the size distribution of the droplets and the particle diameters of the final PLGA/PLA microspheres (Ma, 2014; Qi et al., 2014; Wei et al., 2011). Furthermore, the flow velocity of the continuous phase could also affect the size and the size distribution of the formed emulsion droplets by generating a shear stress which can detach the formed droplets along the membrane surfaces (Kazazi-Hyseni et al., 2014).

4.3. Self-healing encapsulation

The self-healing encapsulation method has been developed to overcome the shortfalls of the traditional emulsion methods. It allows the post-encapsulation of the proteins/peptides after forming microspheres. Thus, it minimizes the potential degradation of these macromolecules induced by oil-water interfacial force and shear stress, as evidenced in the conventional emulsion protocols (Desai and Schwendeman, 2013; Huang et al., 2015; Reinhold et al., 2012). The "self-healing" is an intrinsic property of the polymer chain rearrangement at the temperatures above their T_g (Desai and Schwendeman, 2013). In brief, the porous placebo PLGA/PLA microspheres are fabricated using the traditional emulsion solvent evaporation method in the presence of porogen (*e.g.* trehalose), followed by incubation these microspheres in an aqueous protein/peptide solution under gentle agitation at a temperature below T_g of the polymers. After peptides/proteins partitioning into the microspheres, the temperature is raised to higher than T_g to induce pore closing. The passive and active peptides/proteins loadings are distinguished by the existence of additional driving force to facilitate the partitioning of the macromolecules to polymer matrices (Reinhold and Schwendeman, 2013). The calcium phosphate ($CaHPO_4$) or aluminum hydroxide ($Al(OH)_3$) are introduced into the inner aqueous phase as a peptide/protein trapping agent in the active loading protocols and these often improve encapsulation efficiency as compared to passive loading processes (Bailey et al., 2017a; Bailey et al., 2017b; Desai and Schwendeman, 2013). The pore forming and healing are critical for the self-healing process and thus various parameters related to these two processes are more likely to be the important CPPs. Among them, the surrounding temperature might be the most critical one and it could influence both the loading and the stability of peptides/proteins in the aqueous solution (Desai and Schwendeman, 2013; Shah and Schwendeman, 2014).

4.4. Spray drying

The spray drying is a continuous one-step process and has long been attempted to produce long-acting PLGA/PLA microspheres. In this method, an aqueous solution of peptides/proteins is dispersed in the PLGA/PLA organic phase to form a primary emulsion, followed by spray drying to accomplish solidified PLGA/PLA microspheres. A post-drying step might be necessary to remove the residual solvents in this process. Those CPPs, which are involved in the primary emulsion formation process in the double emulsion evaporation method may also influence the spray drying protocol in an analogous manner. In addition, various process parameters of the spray drying technique including atomization energy input, inlet/outlet temperature, drying flow rate and feed rate, *etc.*, are reported to regulate the physical properties of PLGA/PLA microspheres (*viz*. size) and thus the release behavior and the stability of the encapsulated peptides/proteins (Al Zaitone and Lamprecht, 2013; Baldinger et al., 2012; Lebrun et al., 2012; Lee and Pokorski, 2018; Meeus et al., 2015a; Shi et al., 2020; Wan and Yang, 2016; Ziaee et al., 2019). Furthermore, the PLGA molecular conformation and solvent properties have been proved to affect the particle formation and thus the drug release from PLGA microparticles (Wan et al., 2013a; Wan et al., 2018; Wan et al., 2013b).

An alternative approach to formulate the primary emulsions prior to spray drying is to disperse fine powders of peptides/proteins in PLGA/PLA organic phase (Ye et al., 2010). The obtained S/O dispersions could then be spray-dried to produce solidified PLGA/PLA microspheres (Ramazani et al., 2016). The spray-freeze drying method had also been exploited to produce growth hormone-loaded microspheres (Desai and Schwendeman, 2013; Wan and Yang, 2016), which was later withdrawn from the market in 2004, due to the "significant resources required by both companies to continue manufacturing and commercializing the product" (Pagels and Prud'homme, 2015). Recently, a 3-fluid nozzle has been proposed to afford PLGA/PLA microspheres, where the aqueous peptide/protein solution and PLGA/PLA organic phase are fed *via* two separate liquid channels into the drying chamber and subsequently atomized into fine droplets (Wan et al., 2014a; Wan and Yang, 2016). The separate liquid channels might circumvent the interfacial stresses of the aqueous and oil phases. Besides the CPPs aforementioned, the feeding rate ratio of the two liquid channels is reported to be a critical parameter for this method (Wan et al., 2014b; Wan and Yang, 2016). Other than PLGA/PLA microparticles, spray drying has shown great potential for encapsulation of peptide and protein drugs into solid lipid microparticles and hybrid polymer-lipid microparticles (Wu et al., 2019; Wu et al., 2018b).

4.5. Electrospray

In the electrospray process, the liquid sample is atomized into the fine droplets by a high voltage electrical field. The evaporation of the solvents results in the solidification of PLGA/PLA microspheres. Analogous to the spray drying protocol, the encapsulation of peptide/protein drugs into the PLGA/PLA microspheres is carried out by preparing W/O or S/O primary emulsions prior to their electrospray. It has already been proved that the solvent property could be considered as a CMA for its significant effect on the particle formation process as well as the particle characteristics and drug release profile (Bohr et al., 2012). The CPPs of the electrospray technique influencing the release and stability of peptides/proteins are similar to that of spray drying method. Those include applied electrical voltage (Hao et al., 2014; Nath et al., 2013; Yuan et al., 2015; Zhang et al., 2015), drying gas and liquid flow rate (Nath et al., 2013; Pathak et al., 2016). As the liquid flow rate increases, the diameter of the microspheres normally increases (Lee and Pokorski, 2018; Malik et al., 2016). Apart from this, a high flow rate produces particles of uniform size distribution with few fibers (Pathak et al., 2016). Maintenance of a stable spray requires balancing the solution properties with the flow rate, along with a number of other parameters (Steipel et al., 2019). For example, the voltage needs to be adjusted properly to balance the surface tension to produce "Taylor cone" (Han et al., 2016). The "Taylor cone" is an excellent "cone-jet" mode, which is disrupted by a high or low voltage, affecting the morphology of the microspheres (Pathak et al., 2016). In general, microparticles with smaller size are obtained at higher voltages (Yuan et al., 2015).

A co-axial electrospray technique has been attempted to produce core-shell structured PLGA/PLA microspheres (Yuan et al., 2015; Zhang et al., 2015). In this context, the cargo (inner peptide/protein aqueous phase) and the carrier (outer PLGA/PLA organic solution) concurrently flow through a co-axial needle and are then exposed to a high voltage electrical field. The yielded core-shell microparticles could minimize the drug loss and effectively improve encapsulation efficiency of the therapeutic peptides and proteins (Yuan et al., 2015). The flow rate ratio of the outer and inner liquid phases is one of the important CPPs affecting loading and the release patterns of peptides/proteins. A suitable flow rate ratio might result in the production of microspheres with uniform size and regular distribution (Wen et al., 2013).

4.6. Microfluidic

The microfluidic method is considered as low-cost, scalable, reproducible and tunable technique, which can facially fabricate PLGA/PLA microspheres with controlled microstructure and narrow size distribution in a one-step (Jafarifar et al., 2017). Variable microfluidic devices can be exploited to encapsulate hydrophilic macromolecules in the hydrophobic polymer matrices. The co-flow and flow-focus devices are most commonly employed to afford single emulsions. For double $W_1/O/W_2$ emulsion production, a combination of co-flow and flow-focus devices is used (Kong et al., 2013; Ramazani et al., 2016). These devices differ in their makeup based on capillary location and liquid flow direction (Ramazani et al., 2016).

In the co-flow device, polymer and drug are co-dissolved in organic solvent (inner phase) and then introduced into an inner capillary inserted at the center of a square capillary. The external water phase containing emulsifier (*viz*. PVA) is injected *via* two sides of the square capillary in the same direction as of the inner phase. The syringe pumps are used to provide a constant flow rate. Consequently, the monodispersed emulsion droplets are formed one-by-one at the junction points of the combined microfluidic channels (Ramazani et al., 2016).

The inner organic and outer water phases are introduced into the square capillary, moving in two opposite directions in the flow-focus devices. The inner organic phase is hydro-dynamically flow focused by the outer aqueous phase through the orifices. The monodispersed emulsion droplets appear when the inner organic phase enters into the orifices under the dripping or jetting condition (Ramazani et al., 2016).

To produce $W_1/O/W_2$ double emulsions using a combination device, two tapered round capillaries (*i.e.* injection and collection capillaries) with different orifice diameters are coaxially aligned inside a square capillary (Kong et al., 2013). The orifices of the injection capillaries are smaller than that of collection capillaries (Kong et al., 2013). The inner phase is pumped through the injection capillary. The middle phase flows through the region between injection capillary and square capillary in the same direction, just like the co-flow device. The outer aqueous phase passes through the region between collection capillary and square capillary in the opposite direction. Finally, the double emulsion drops are produced at the orifices of the collection capillaries (Kong et al., 2013; Wu et al., 2013). The resulting single emulsions formed by co-flow and flow-focus or double emulsions formed by combination device are stirred to evaporate the organic solvents and then separated by centrifugation and finally freeze-dried to obtain final solid microspheres (Busatto et al., 2018). This combined device is always used to produce "core-shell" PLGA microspheres, which could prolong the release profiles with a reduced initial burst release effect (Kong et al., 2013).

The microfluidic protocol to produce PLGA/PLA microspheres has mainly been used in research laboratories. In addition to the geometries (*e.g.* dimension of the capillary, diameter of collection tube) (Wu et al., 2013), surface property of the devices (Dong et al., 2016), formulation composition and solvent properties (Jafarifar et al., 2017), CPPs of this method including total flow rate (Ramazani et al., 2016; Wu et al., 2013; Wu et al., 2016b), flow rate ratio and osmotic pressure difference between inner and outer phases as they might influence the formation of emulsions and particle characteristics of PLGA/PLA microspheres (Amoyav and Benny, 2019; Jafarifar et al., 2017; Kong et al., 2013).

4.7. Supercritical fluid technology

The conventional emulsification methods used to produce long-acting PLGA/PLA microspheres require a high processing temperature to remove the organic solvents (Champeau et al., 2015). To circumvent this problem, several supercritical fluid methods such as rapid expansion of supercritical solutions (RESS), supercritical anti-solvent (SAS) and solution-enhanced dispersion by supercritical fluids (SEDS) have been proposed (Badens et al., 2018; Della Porta et al., 2013; Della Porta et al., 2011b; Lin et al., 2017; Nuchuchua et al., 2017). The supercritical carbon dioxide (SC-CO_2) is inexpensive, non-flammable, nontoxic, chemically inert and available in a large quantity with high purity and often plays variable roles in different methods (Champeau et al., 2015; Tsai and Wang, 2019).

In the RESS method, the SC-CO_2 is used to solubilize active pharmaceutical ingredient (API). The API in SC-CO_2 can penetrate into the polymer matrices, resulting in the impregnation of the drug molecules into the polymer chains. The diffusion of API is facilitated when SC-CO_2 can swell the polymer chains (Champeau et al., 2015). The microspheres can finally be obtained by a rapid depressurization of the supercritical solution (Champeau et al., 2015; Della Porta et al., 2013). The pressure and temperature during the penetration process are considered as CPPs and both of them can influence the SC-CO_2 sorption and polymer swelling (Champeau et al., 2015). Generally, an increase in the temperature improves drug loading due to the improved chain mobility ($> T_g$) and a higher amount ofSC-CO_2 can be adsorbed when the temperature is raised above the melting temperature (T_m) of the semi-crystalline polymers (Champeau et al., 2015). On the other hand, an increase in the pressure can also result in enhanced drug loading under isothermal conditions, where the solubility of the drug, CO_2 sorption and polymer swelling might be improved (Champeau et al., 2015). The depressurization rate and its corresponding temperature also show a significant influence on drug loading (Champeau et al., 2015). Meanwhile, it has been reported that the particle size can be controlled by depressurization rate of SC $-$ CO_2 from micron to nanometer (Tsai and Wang, 2019).

The SC-CO_2 is employed as anti-solvent agent to induce the polymer precipitation in its solution in SAS method (Della Porta et al., 2013; Della Porta et al., 2011b). In contrast, SC-CO_2 is used as extracting agent to induce the formation of the solid microspheres in SEDS technique. The SAS and SEDS protocols are usually utilized when API is soluble in organic solvents and thus these are not suitable for most of the peptide/protein drugs. The effects of formulation composition and process parameters including the saturation ratio of the polymer solution, concentration of the organic solution and flow rate of the solution on the properties of the microspheres were reviewed by Chen et al. (2007). Recently, the supercritical emulsion extraction (SEE) technique has been proposed for the production of the microspheres. In this method, the produced single or double emulsions are extracted from the organic solutions using SC-CO_2. The SEE method has two different operation layouts, namely batch (SEE-B) and continuous (SEE-C) (Della Porta et al., 2011a). These two SEE processes differ in their contacting mode between the liquid and supercritical phase (Della Porta et al., 2011a). In the SEE-B layout, the mixing is favored by the SC-CO_2 bubbling in the extraction vessel. On the contrary, the SEE-C process is working based on the countercurrent contact between SC-CO_2 and preformed double emulsions in a continuous operating mode (Della Porta et al., 2016; Della Porta et al., 2011a; Della Porta et al., 2011b). Consequently, a reduced processing time, higher microsphere recovery and process efficiencies can be achieved in SEE-C (Della Porta et al., 2016; Della Porta et al., 2011a; Della Porta et al., 2011b). In SEE method, the operating pressure and temperature affecting the state of SC-CO_2 are considered as important CPPs. The liquid to gas ratio is also identified as a CPP as the high flow rate of the gas stream can cause process failure (flooding) (Della Porta et al., 2013). Furthermore, Moura et al. employed SC-CO_2 in spray drying technology to produce inhalable trehalose and leucine composite particles and the CPPs were identified (Moura et al., 2019). For more details about the area of supercritical fluid technology, the reader is referred to some excellent review articles (Padrela et al., 2018; Tsai and Wang, 2019).

4.8. Hot melt extrusion (HME)

Hot melt extrusion (HME) is a manufacturing process first used in the plastics industry (Gosau and Müller, 2010), which has been introduced into the pharmaceutical industry as a well-established continuous process (Kirchberg et al., 2019). Furthermore, HME has become popular for the production of extended release formulations (Cosse et al., 2017). The physical mixture of polymer and peptide/protein powder is melted in a heated barrel with a conveying screw and forced to pass through a die to solidify (Lee and Pokorski, 2018). The twin-screw extruder is then used to impart high shear to break up particles and disperse them (Wilson et al., 2012). In addition, the resultant rod-shaped extrudates can be cut into the appropriate length and/or further processed using mini-type pulverizer and mesh sieves (Guo et al., 2017). It has been reported that a smaller particle size of the peptides/proteins leads to their less aggregation and segregation within the PLGA matrix. As a consequence, the spray drying or ball milling could be utilized to control the particle size of peptides/proteins prior to the melt extrusion (Lee and Pokorski, 2018). According to Ghalanbor et al., a burst-free release of peptides/proteins could be achieved if these were milled prior to the extrusion process (Ghalanbor et al., 2012). Rajagopal and co-researchers also found that the spray-dried forms of peptides/proteins could be processed at elevated temperature without significantly affecting their stability (Rajagopal et al., 2013).

Obviously, the peptides/proteins can be processed in the solvent-/moisture-free environment in the HME (Ghalanbor et al., 2013), thus avoiding the risks of peptide/protein instability on oil/water interfaces as well as the potential toxic solvent residues (Cosse et al., 2017; Guo et al., 2017). In addition, the processing temperature is fairly low (~80 °C) due to the low T_g of the polymer, which can minimize the risk of heat-induced protein denaturation during the extrusion process (Cosse et al., 2017). It has been reported that although this temperature may maintain the stability of peptides/proteins in its solid state, the encapsulated peptides/proteins could still show some denaturation and aggregation depending on the biochemical properties of the peptides/proteins and additives used (Lee and Pokorski, 2018). This appears to be significant when the peptides/proteins with a denaturation temperature lower than the process temperature used (Duque et al., 2018). Therefore, the temperature can be considered as a stability-related CPP. Besides, the shear force imparted by the extrusion process can potentially affect the peptide/protein integrity *via* physical and/or chemical modifications and consequently lead to their inactivation (Ghalanbor et al., 2010). McConville et al. observed an insignificant burst drug release due to the absence of drug molecules on the surfaces owing to the application of a higher shear force (McConville et al., 2015).

The residence time distribution (RTD) is another CPP. Commonly, RTD is used in the continuous manufacturing to describe process equipment design and related process dynamics. It is defined as the probability distribution of time that solid or fluid materials stay inside one or more unit operations in a continuous flow system (Gao et al.,

2012). Nowadays, RTD is an important factor in, *e.g.* HME manufacturing system as it strongly influences the product quality (Wahl et al., 2018). The RTD is typically expressed by the residence time density function (Wesholowski et al., 2018a; Wesholowski et al., 2018b). We can obtain some useful information from the onset, width, medium residence time and offset time of a typical RTD plot. The offset time of the RTD highlights the maximum exposure time to mechanical and thermal stresses, which correlates to the degradation processes (Wesholowski et al., 2018a). The stimulus response test is a common method used to measure RTD (Gao et al., 2012), accompanied by some offline (Gao et al., 2012) and inline analytical tools (Wahl et al., 2018; Wesholowski et al., 2018a). In general the residence time should be reduced as too long time would cause the degradation of the peptides/proteins when being exposed to the high temperature and shear forces (Wahl et al., 2018).

4.9. Continuous manufacturing (CM) aspect

The continuous manufacturing (CM) has been increasingly used in the pharmaceutical industry (Kleinebudde et al., 2017). There are several commercial examples, mainly in the field of syntheses of active compounds, as well as in the secondary manufacturing of traditional solid dosage forms. In fact, all the aforementioned methods for producing long-acting PLGA/PLA microspheres for peptide and protein drugs could be potentially manufactured using the CM principles. There are few examples indicating the potential (Della Porta et al., 2011b; Operti et al., 2018), the published research work on CM of long-acting PLGA/PLA microspheres is limited though. The concept of CM will revise the entire idea of the traditional batch-based operations. In general, the CM procedure deals with a sequence of time-invariant unit operations containing a continuous flow of material, which are constantly monitored and controlled by in-line process analytical technology (PAT) in order to obtain final products complied with pre-defined quality attributes (Rantanen and Khinast, 2015). The CM offers the advantages like higher flexibility, faster supply chain and reduced scale-up difficulties over the batch production. With these potential economic benefits, it will be attractive to adopt the concept of CM in the manufacturing of LAI PLGA/PLA microspheres.

Nevertheless, it may be worth mentioning that, as compared to the huge market's demands for the traditional dosage forms such as tablets, a relatively small amount of LAI PLGA/PLA microspheres might be sufficient to meet the market's requirements. Therefore, from an economic point of view, it may not be financially feasible for a shifting from an existing batch process to the CM approach. The CM demands significant time, finance, and effort from all aspects from the drug development to regulatory filings. It can be an unbearable burden for especially the generic product development with low-margin profits and/or complex production process like emulsion solvent evaporation. Although some manufacturing methods such as spray drying, microfluidics, and HME might suit better for CM, the companies still need to make a prudent assessment.

5. Landscape of generic product development of LAI PLGA/PLA-based microspheres for peptide/protein drug delivery

The first PLGA/PLA-based LAIs *i.e.* Lupron Depot was approved by FDA in January 1989. However, at present, only about 20 drugs have been formulated into injectable long-acting depots and are available on the market as compared to the thousands of oral sustained release formulations. Moreover, none of them are within generic counterparts yet (Skidmore et al., 2019), which is not because of being protected by the patents, yet simply the development of PLGA/PLA injectables as generic products as well as branded counterparts remain a significant challenge (Park et al., 2019).

The attitude of FDA towards the generic product development is quite positive and some actions have already been taken to improve generic drug submissions (Park et al., 2019). The generic LAI formulations are required to be qualitatively (Q1) and quantitatively (Q2) same as its reference-listed drug (RLD) for approval in an Abbreviated New Drug Application (ANDA) according to the 505 (j) pathway (Shi et al., 2020). Therefore, the reverse engineering of the RLD products is needed and a comprehensive characterization of PLGA is the main challenge in this context. The properties such as molecular weight, ratio of lactic and glycolic units, end groups, shape, inherent viscosity, T_g and other properties need to be characterized. The molecular weight, ratio of lactic and glycolic units and end-group of PLGA are usually characterized by gel permeation chromatography (GPC), ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR, respectively (Garner et al., 2015; Skidmore et al., 2019). Multi angle static light scattering (MALS) technique could be exploited to estimate molecular weight more accurately as compared to GPC (Park et al., 2019). However, these conventional approaches may not be suitable or sufficient if a formulation has more than one type of PLGA, especially when they have similar molecular weights, but different ratios of lactic and glycolic units (Skidmore et al., 2019). Skidmore et al. utilized a series of semi-solvents, which exhibit varying degrees of PLGA solubility depending on the ratio of lactic and glycolic units of the polymer to isolate and analyze complex PLGA formulations made of more than one type of PLGA (Skidmore et al., 2019). Besides, the conformational structure (*e.g.* linear or branched) of PLGA should be considered. Sandostatin®, a clinical product, is made of branched PLGA based on glucose (Glu-PLGA) but there are no literatures available on how to characterize the branch number of the Glu-PLGA, thus it is almost impossible to develop a generic product of Sandostatin®, which resulting in an extended product life cycle even if its patents are expired (Park et al., 2019). Furthermore, the properties of the PLGA can be altered during the manufacturing processes, and the PLGA in the finished drug product may no longer be representative of the original PLGA in terms of certain key characteristics (Skidmore et al., 2019; Zhou et al., 2018). In conclusion, the current state of the PLGA characterization techniques cannot elucidate the differences among the variable batches and suppliers and there are no compendial testing method to fully characterize PLGAs, which may lead to a failure in the Q1/Q2 sameness (Park et al., 2019).

The PLGA properties may be well captured by the Q1/Q2 requirements and the Q1/Q2 sameness could be then a good starting point for the development of generic products. However, the Q1/Q2 sameness does not make sure the sameness of the *in vitro* drug release profile and *in vivo* performance as compared to the RLD products (Garner et al., 2018). For complex generic drug products like LAIs, many parameters such as the drug distribution within the microspheres, drug crystallinity, manufacturing process, size distribution of microspheres cannot be captured by the Q1/Q2 sameness, although the effects of these parameters on the performance of the final product are profound (Garner et al., 2018). The microspheres prepared with minor change in manufacturing method may result in significant differences in their physicochemical properties, which may subsequently affect the peptide-polymer interactions, resulting in their considerable alteration in safety, stability and release characteristics.

Therefore, the microstructural (Q3) equivalence may be necessary. Numerous analytical tools such as scanning electron microscopy (SEM), differential scanning calorimeter (DSC), powder X-ray diffraction (PXRD), Fourier transform infrared (FTIR), as included in Table 5, have been utilized to measure these properties to meet Q3 equivalence. Furthermore, the *in vitro* release testing (IVRT) is utilized as a valuable tool to obtain the *in vitro* performance data to support the Q3 equivalence. At present, there is a lack of compendial *in vitro* release methods for LAI microspheres. Various IVRT methods (*e.g.* sample-and-separate, membrane dialysis, and continuous flow (USP apparatus 4)) have been used (Andhariya et al., 2017a; Shen et al., 2016). Each method has its own advantages and shortcomings (Garner et al., 2018; Shen et al., 2016). A good IVRT method should be reproducible, sensitive and

discriminating, allowing detection of the changes in the manufacturing process, polymorphic form, physical structure and other properties. Garner et al. found that the *in vitro* experimental condition (*e.g.* agitation speed, vessel-dimensions, solid beads, and media exchange volume, *etc*.) seemed to affect the drug release kinetics more than the difference in manufacturing methods (Garner et al., 2018). Therefore, the methodology parameters must be validated and harmonized before the equivalence evaluation and the establishment of an appropriate *in vitro-in vivo* correlation (IVIVC) (Garner et al., 2018).

LAIs face high dropout rates in clinical trials due to their long study durations and unique challenges in establishing bioequivalence. An approach potentially overcome this challenge is to establish IVIVC mathematical model. According to the FDA guidance, an IVIVC is a predictive mathematical model, which demonstrates the relationship between an *in vitro* property (*e.g.* rate or extent of drug release) of a dosage form and a relevant *in vivo* response (*e.g.* plasma drug concentration or amount of drug absorbed *vs* time) (Andhariya et al., 2019; Shen and Burgess, 2015; Shen et al., 2015). The FDA has categorized four main levels of IVIVC: Levels A, B, C, and multiple level C (Shen and Burgess, 2015; Shen et al., 2015). A Level A IVIVC represents a point-to-point correlation between the *in vitro* and *in vivo* input rates (*e.g.* the *in vivo* dissolution), which is considered as the most informative and also recommended by FDA. A Level A IVIVC is also the only level of IVIVC that can be used to obtain a bio-waiver (Andhariya et al., 2017b; Shen and Burgess, 2015). When a Level A IVIVC model is well established, the IVRT may potentially be used as a surrogate for bioequivalence studies that would otherwise be required for any scale-up and post-approval changes (SUPAC). The need for the animal studies and clinical trials can be significantly reduced if an IVIVC model can be established, which further reduces the financial burden and duration of generic microsphere development (Andhariya et al., 2017b). The “Guidance for industry, extended release oral dosage forms” recommends the use of a minimum of two, preferably three or more formulations with different release rates to develop a reliable IVIVC if the *in vitro* releases of the formulations are dependent on the release testing conditions (Andhariya et al., 2017b). The procedures of developing a Level A IVIVC for LAIs have been reported elsewhere (Shen and Burgess, 2015). Unfortunately, it has never been an easy task to establish an IVIVC model for LAIs, which primarily due to the complex release behavior of the PLGA/PLA microspheres, the lack of compendial IVRT methods as well as formal FDA guidelines (Andhariya et al., 2019; Andhariya et al., 2017b; Doty et al., 2017b). Moreover, the presence of the foreign body response, exposure with endogenous components and poorly defined hydrodynamics may impose difficulties on the development of rational mechanism-based IVIVCs (Hirota et al., 2016). Doty and their colleagues also pointed out that the lack of reports on *in vivo* analyses of the polymer during the drug release may result in inaccurate predictions of IVRT (Doty et al., 2017a). The *in vivo* behavior should be reflected as much as possible by an IVRT method and it may be necessary to develop IVIVCs based on discriminatory accelerated IVRT method (Shen and Burgess, 2015). Another challenge for the complex microspheres is how to deconvolute *in vivo* data and correlate these with *in vitro* release data (Shen and Burgess, 2015). It is very difficult for the establishment of IVIVC for peptide/protein loaded microspheres as a result of the complex structure, instability, and complicated release mechanisms of peptides/proteins as compared to small molecule drugs loaded microspheres (Andhariya et al., 2019).

6. Conclusions

The development of the LAI PLGA/PLA-based microspheres containing therapeutic peptides and proteins possesses a high technical complexity with a low trade barrier. Currently, there are a handful of long-acting depot formulations of protein/peptide drugs available on the market. It is believed that more long-acting peptide/protein depot injectables might offer remarkable benefits to the patients in the near future. The QbD approach enables guiding the systematic development of drug products including LAI in pharmaceutical industry. Considering the safety and efficacy of the LAI PLGA/PLA-based microspheres, the stability and release profiles of protein/peptide drugs can be regarded as two key CQAs. The related CMAs and CPPs that impact on CQAs need to be identified in order to design the process and define the product design space. In addition, the process needs to be validated to demonstrate the effectiveness of the control strategy prior to the filing. The risk assessment and management, statistical approaches and PAT would provide a foundation for these activities. Most commercial LAI PLGA/PLA-based microspheres for peptide and protein drugs on the market are produced by the principle of emulsification followed by the removal of the solvents. A few new manufacturing technologies are also being actively explored and investigated in various research labs though, and many of them possess potential of CM principles. Lastly, the attitude of FDA towards the generic product development is quite positive, the development of LAI PLGA/PLA-based microspheres as generic products has not been very successful. One of the main challenges is to obtain the comprehensive characterization of this complex delivery system in order to understand the physical and chemical mechanisms governing the release and stability of the payload in the LAI PLGA/PLA-based microspheres *in vivo*.

CRediT authorship contribution statement

Chengqian Zhang: Writing - original draft. **Liang Yang:** Writing - original draft. **Feng Wan:** Writing - review & editing. **Hriday Bera:** Writing - review & editing. **Dongmei Cun:** Writing - review & editing. **Jukka Rantanen:** Writing - review & editing. **Mingshi Yang:** Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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