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RESEARCH ARTICLE

Advances in encapsulating gonadotropin-releasing hormone agonists for controlled release: a review

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) agonists are peptides consisting of nine or ten amino acid residues. GnRH agonists have been applied in the therapy of sexual hormone disorders like prostate cancer, endometriosis, uterine myoma, central precious puberty, and in-vitro fertility. Treatment is achieved by continuous hormone intake and long-term agonists administration, which is usually associated with poor patient compliance. Because GnRH agonists that are administered with the parenteral route are broken down by peptidase, their half-life is short. As a result, developing sustained release for the drug delivery system is significant. Even though some drugs have been successfully delivered with long-acting release microspheres and approved by the Food and Drug Administration (FDA), some challenges remain. This review highlighted current approaches to encapsulate GnRH agonists into delivery systems and strategies encountered during the loading process. Moreover, the following sections provide strategies to improve the release profile, and animal and human studies were summarised.

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Microspheres; encapsulation efficiency; GnRH agonist; controlled release; release studies

Introduction

Gonadotropin-releasing hormone (GnRH) plays a critical role in the reproductive system's hormonal balance and induces the synthesis and release of pituitary gonadotropin (Schneider *et al.* 2006). In male and female organisms, down-regulation of GnRH receptors and inhibition of gonadal function are pharmacologically induced by chronic GnRH agonists administration. For these reasons, GnRH agonists have been widely studied and applied in the medical field since they were discovered and produced in 1971. It has been currently applied in sexual hormone disorders like prostate cancer, endometriosis, uterine fibroids, central precious puberty, and in-vitro fertility (Shi *et al.* 2020). Compared with native GnRH, GnRH agonists have more potency, and their biological activity is 100 times stronger. Different GnRH agonists have been developed artificially by deleting, transforming and changing amino acid positions in their sequence to achieve better indications (Kumar and Sharma 2014). The list of the most therapeutically used GnRH analogues in clinical conditions is illustrated in Table 1 (Teutonico *et al.* 2012).

Nevertheless, GnRH agonists have a short half-life (around 2 h for buserelin and 3 h for leuprolide) because of the small molecular weight and cleavage of the bond between amino acid groups (Cakmak and Rosen 2015). As a result, peptides are delivered in vivo by long-acting delivery systems such as microspheres for parenteral administration. It was proved that the controlled release systems dealt with patient non-compliance, improved health-related quality of life, and decreased medical resource use (McKeage and Lyseng-Williamson 2017). More importantly, depot formulations can enhance active ingredients' stability as they are protected with polymer shells and precisely assemble at the targeted site of the organ. Hence, it allows sustained release of the drug over a certain period required by the biological system and minimises side effects such as overdose. The controlled release of GnRH agonists, therefore, gains large interest in the broad field of drug delivery (Ng *et al.* 2010). Table 2 demonstrates numerous long-acting formulations of GnRH agonists that have been approved by the FDA and commercialised in markets.

However, controlled release systems of GnRH agonists still face some challenges. First of all, GnRH agonists have a relatively small molecular weight,

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Table 1. Amino acid composition of the principal luteinizing hormone-releasing hormone LH-RH agonists. Modifications are marked in red colour.

LH-RH (Gonadorelin or GnRH)
1 2 3 4 5 6 7 8 9 10
`H-Pyr – His – Trp – Ser – Tyr – Gly – Leu – Arg – Pro – Gly – NH2
Buserelin
1 2 3 4 5 6 7 8 9
H-Pyr – His – Trp – Ser – Tyr – D-Ser(tBu) – Leu – Arg – Pro – Ethyl amide
Goserelin
1 2 3 4 5 6 7 8 9 10
H-Pyr – His – Trp – Ser – Tyr – D-Ser(tBu) – Leu – Arg – Pro – AzGly– NH2
Leuprolide (leuprorelin)
1 2 3 4 5 6 7 8 9
H-Pyr – His – Trp – Ser – Tyr – D-Leu – Leu – Arg – Pro – Ethyl amide
Triptorelin
1 2 3 4 5 6 7 8 9 10
H-Pyr– His – Trp – Ser – Tyr – D-Trp – Leu – Arg – Pro – Gly – NH2
Histrelin
1 2 3 4 5 6 7 8 9
H-Pyr– His – Trp – Ser – Tyr – D-His(1-Bn) – Leu – Arg – Pro – Ethyl amide

Table 2. List of marketed long-acting release injectable products of GnRH.

Generic name	Brand name	Company name	Formulations	Injection method	Therapeutical use
Leuprorelin, leuprolide	Lupron Depot®	Abbott	Monthly, 3,4,6-month injection	Intramuscular injection into the thigh, shoulder, and glutaeal region.	Prostate cancer Endometriosis Uterine fibroids
	Prostap SR®	Takeda UK Ltd	Monthly injection	Subcutaneous intramuscular injection	Precocious puberty Breast cancer
	Enantone®	Takeda	Monthly injection	Subcutaneous intramuscular injection	
	Lucrin Depot®	Abbvie	Monthly injection	Subcutaneously (abdomen) intramuscularly (buttocks)	
	Lectrum®	Eriochem	Monthly injection	Subcutaneous intramuscular injection	
Triptorelin	Decapeptyl SR®	Ferring Pharmaceuticals	Monthly and three-monthly injection	Intramuscular injection.	Infertility Endometriosis
	Gonapeptyl®		Monthly injection	Subcutaneous intramuscular injection	Prostate cancer, Uterine fibroids
	Diphereline®	Ipsen	Monthly injection	Intramuscular injection	
	Trelstar®	Watson pharma	Monthly, 3,6-month injection	Intramuscular injection into the glutaeal region	

consisting of only 9 or 10 amino acids. Therefore, it leads to low encapsulation efficiency due to the escape of peptides from the oil phase to a large amount of external water phase, which affects the cost of raw materials and drugs (Chaisri *et al.* 2011, Ramazani *et al.* 2016). Secondly, the microsphere's encapsulation technique involved different process parameters that can play a pivot role in encapsulation efficiency, as well as release profile.

Burst release is one of the current problems, which is caused by the quantity of the drug near the surface of the microspheres. Another problem is lag time, which is associated with the type of polymer, concentration of polymer, and structure of the microparticle (Abashzadeh *et al.* 2011, D'Souza *et al.* 2014, Mostafa *et al.* 2014, Crawford *et al.* 2015, Hirota *et al.* 2016).

In this paper, the advances in GnRH agonists sustained delivery systems were discussed in detail, including the main preparation methods and highlights of carrier materials. In addition, aiming to resolve the encapsulation and release problems associated with the microspheres, several strategies were proposed to improve the performance. Finally, pharmacodynamics and pharmacokinetic profiles were finalised to present the in-vivo testing stage of the depot system.

Encapsulation methods of GnRH agonists into delivery systems

Emulsion/solvent evaporation

The emulsion/solvent evaporation method is simple and widely used in the encapsulation of peptide drugs

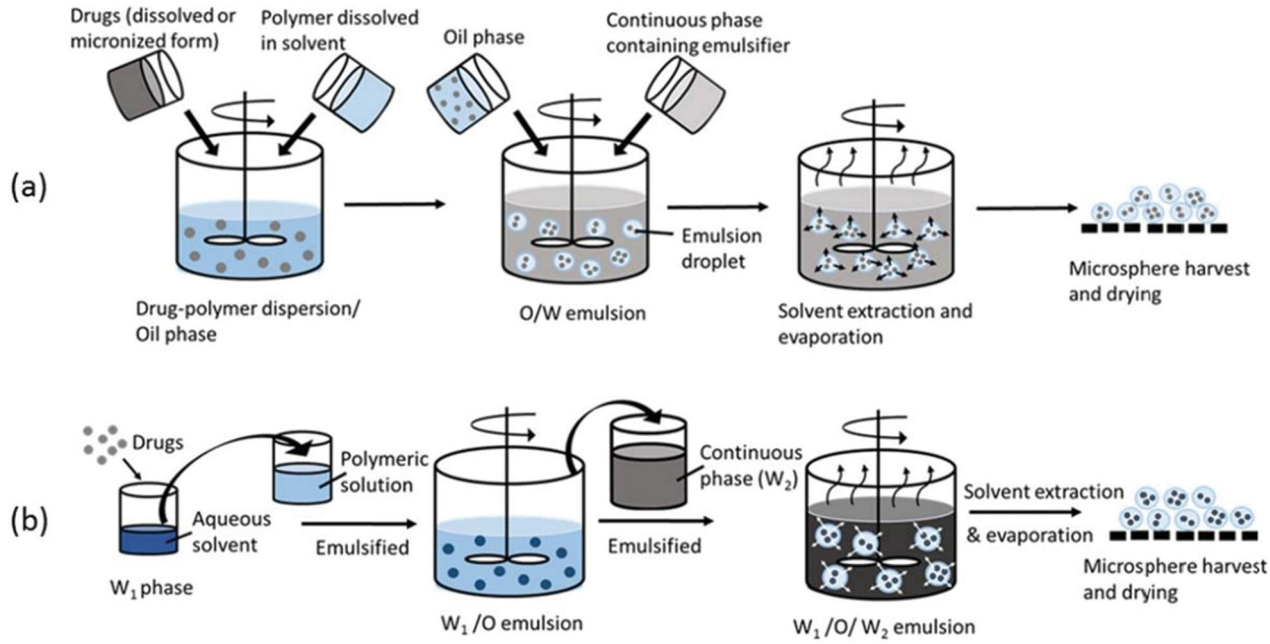

Figure 1. Schematic representation of the emulsion/solvent evaporation technique. Redrawn from (Soo-Ling *et al.* 2018).

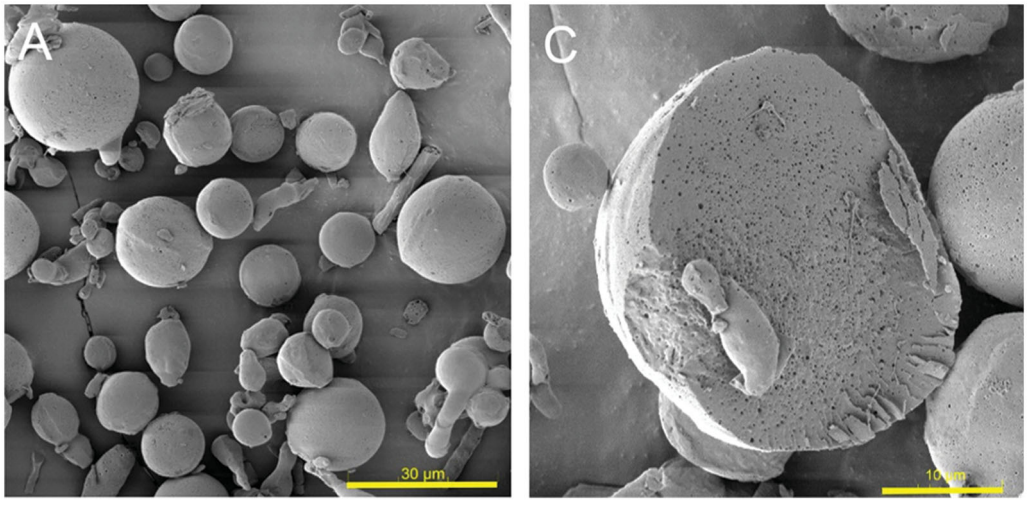

Figure 2. SEM images of microspheres prepared with emulsion/solvent evaporation method and sectioned version. Redrawn from (Zhou *et al.* 2020).

because it involves only mild process conditions such as normal temperature and constant stirring. In general, as can be seen from Figure 1, this method consists of two main stages: emulsification and solvent removal. During the primary emulsion preparation stage, active ingredients are first dissolved in the water and then added to the polymer dissolved in an appropriate solvent. Practically, dichloromethane (DCM), ethyl acetate, acetone (AC), and methanol are commonly used as organic solvents in emulsification (Schwach *et al.* 2004, Mao *et al.* 2007). After forming the dispersion-containing drug and polymer solution, it is emulsified using stirring, sonication, or homogenisation devices. Next, the primary emulsion is discharged into the large quantity of outer water phase containing stabilisers such as polyvinyl chloride, polysorbate, and other surface-active substances. Here, under mechanical agitation conditions, solvent associated with organic oil droplets is removed via evaporation. As a result, emulsion droplets are hardened and collected by centrifugation and lyophilisation (Qi *et al.* 2014, Rodríguez-Vázquez *et al.* 2015, Qi *et al.* 2019). The prepared microspheres from the emulsion/solvent evaporation method are illustrated in Figure 2, SEM images indicated that the microspheres were a fine particles with a smooth surface. The sectioned microspheres demonstrated that small pores were evenly distributed inside the microspheres. In industry,

leuprolide acetate microspheres are produced by the double emulsion $W_1/O/W_2$ method and traded under the name Lupron Depot® by the company Abbott (Shi *et al.* 2020).

Woo et al. produced leuprolide-loaded microspheres by double emulsion systems. The mean particle size of microspheres was 51.7 μm with an encapsulation efficiency of 81.2% (Woo *et al.* 2001). Another analogue of GnRH, orntide acetate was also prepared by the double emulsion method, the only difference was using the circulating water bath during the continuous phase to maintain a constant temperature. After microspheres were produced and hardened, they were passed through a membrane filter. The average particle size was 9.6 μm, with an encapsulation efficiency of 81% (Kostanski *et al.* 2000). Qi *et al.* (2019) prepared goserelin microspheres by the $W_1/O/W_2$ method: the drug was mixed with poloxamer in the water to form hydrogel as the inner aqueous phase. After double emulsion procedures, microspheres were formed by solvent evaporation and the final microspheres passed through a sieve to obtain uniform-sized particles. The mean particle size of the microspheres ranged from 52 μm to 79 μm and the highest encapsulation efficiency was 94% (Qi *et al.* 2019).

In general, emulsion/solvent evaporation technology has numerous advantages and drawbacks at the same time. First of all, this technique does not require expensive and complicated devices and it can be simple to conduct at the laboratory scale. On the other hand, the utilisation of organic solvents and a substantial amount of water can bring some difficulties during the scale up to the industry level (Blasi 2019).

Rapid membrane emulsification technique

The rapid membrane emulsification technology is used to prepare uniform and small emulsions, microspheres, and microcapsules. The working principle of membrane emulsification is illustrated in Figure 3. First of all, a certain amount of active ingredient is dissolved into the water as the internal aqueous phase (W_1), and it is mixed with the oil phase containing polymer solution to prepare primary W_1/O emulsion. Next, the primary emulsion is immediately poured into the external water phase to form a $W_1/O/W_2$ double emulsion. The coarse emulsion with larger droplets is pressed through the uniform pores of the membrane by applying optimised pressure. Large emulsion droplets can be easily broken into smaller ones of similar size by shearing action from cross-membrane pressure. This procedure is repeated 3–4 times to obtain more uniform droplets. Then, an emulsion is stirred for several hours to remove the organic solvent. Finally, droplets are washed several times with distilled water and lyophilised to obtain solidified microparticles (Wu *et al.* 2015).

The crucial factor affecting the particle size distribution is trans-membrane pressure. Under high pressure, the friction force between the particles and membrane is increased, leading to the formation of submicron droplets. Conversely, when pressure is lower, the large droplets will pass through the membrane by changing their form without being broken into smaller droplets (Qi *et al.* 2014). The most important advantage of membrane emulsification is to prepare microspheres with narrow size distribution and the particle sizes of microspheres can be controlled by adopting a

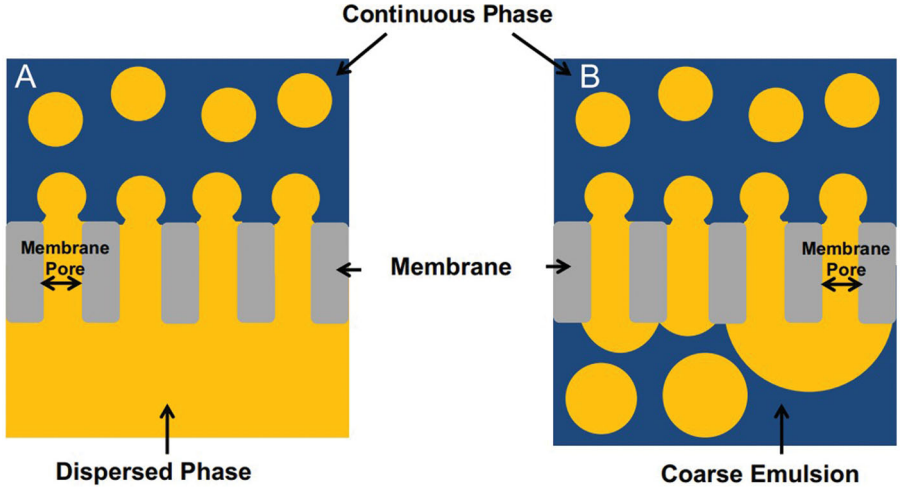

Figure 3. Schematic representation of emulsification process: (A) Direct membrane emulsification method (B) Premix membrane emulsification method. Redrawn from (Piacentini *et al.* 2014).

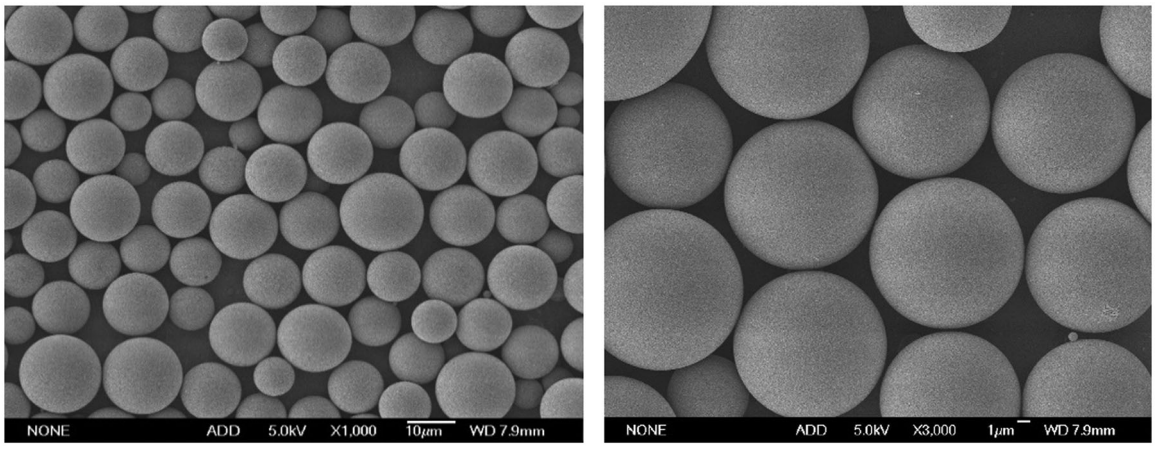

Figure 4. SEM images of microspheres prepared with premix membrane emulsification technique. Redrawn from (Wang *et al.* 2013).

membrane with the required pore diameter. Additionally, emulsification is conducted at a low shear rate, and it is suitable to encapsulate peptides to prevent loss of drug activity (Liu *et al.* 2011).

Triptorelin acetate-loaded microspheres were prepared by a rapid membrane emulsification process. Produced microspheres owned particle sizes of about ~30 μm and a SPAN value of 0.8. Figure 4 demonstrated that the rapid membrane emulsification technique produced smooth microspheres with narrow size distribution in comparison with the method. The microsphere's encapsulation efficiency reached 80.12% when the volume ratio of the internal water phase to the oil phase was 1:10 in primary emulsion (Wang *et al.* 2013).

Spray drying

Spray drying is applied to produce microencapsulated or matrix-based drug delivery systems to obtain sustained drug formulation. This technology is a continuous process that transforms feedstock solutions into dried micro-sized particles by subjecting feed to a high-temperature and gaseous medium (Al-Khattawi *et al.* 2018). According to Figure 5, spray drying consists of three main stages: atomisation, drying, and separation. Atomisation refers to converting a liquid stream into small fine particles by the appropriate device. In this stage, the prepared feedstock is delivered through a peristaltic pump to the atomiser chamber by a nozzle. Then, droplets are produced in the atomiser chamber by exposure to the interaction with a hot drying gas (higher than feed temperature) (Shi *et al.* 2020). Atomised dispersion droplets are subjected to a hot gas stream in the second stage, which mainly refers to atmospheric air. In some cases, it is required to use inert gas to obtain the stability of particles. Process conditions such as inlet temperature, drying air temperature, and device geometry simultaneously influence the drying performance and efficiency. In the last stage, dried product particles are collected using a separation device as a cyclone (Cal and Sollohub 2010).

Shi et al. prepared leuprolide-loaded microspheres using the spray-drying technique and studied the effect of the process on final microspheres characteristics. Feed concentration and nozzle properties are critical influencing factors for the uniformity of microparticles. The particle yields ranged from 30 to 60%, depending on the inlet air temperature, which was found that the high inlet temperature (T_{inlet}) could improve yield, while the lowest value of T_{inlet} around 50 °C resulted in the lowest yield of the particle. As can be seen from Figure 6, all microspheres have spherical shapes with a smooth surfaces (Shi *et al.* 2020).

Abashzadeh et al. produced chitosan microspheres loading with triptorelin acetate by spray-drying technique. Prepared microparticles mixed with opened ring polyvinyl pyrrolidone and chitosan derivatives further formed a novel physical hydrogel system for sustained delivery. In this study, the Spray-drying technique was chosen to increase the in-situ gel-forming and water absorption rate (Abashzadeh *et al.* 2011). Park *et al.* (2017) encapsulated leuprolide acetate in microspheres by a proprietary aseptic drying method. Solutions of the drug and PLA were dissolved in glacial acetic acid lyophilised in D-mannitol. Compared with the traditional technique, the microspheres prepared by aseptic spray drying owned the controllable shape, density, and morphology in comparison to the traditional method (Park *et al.* 2017).

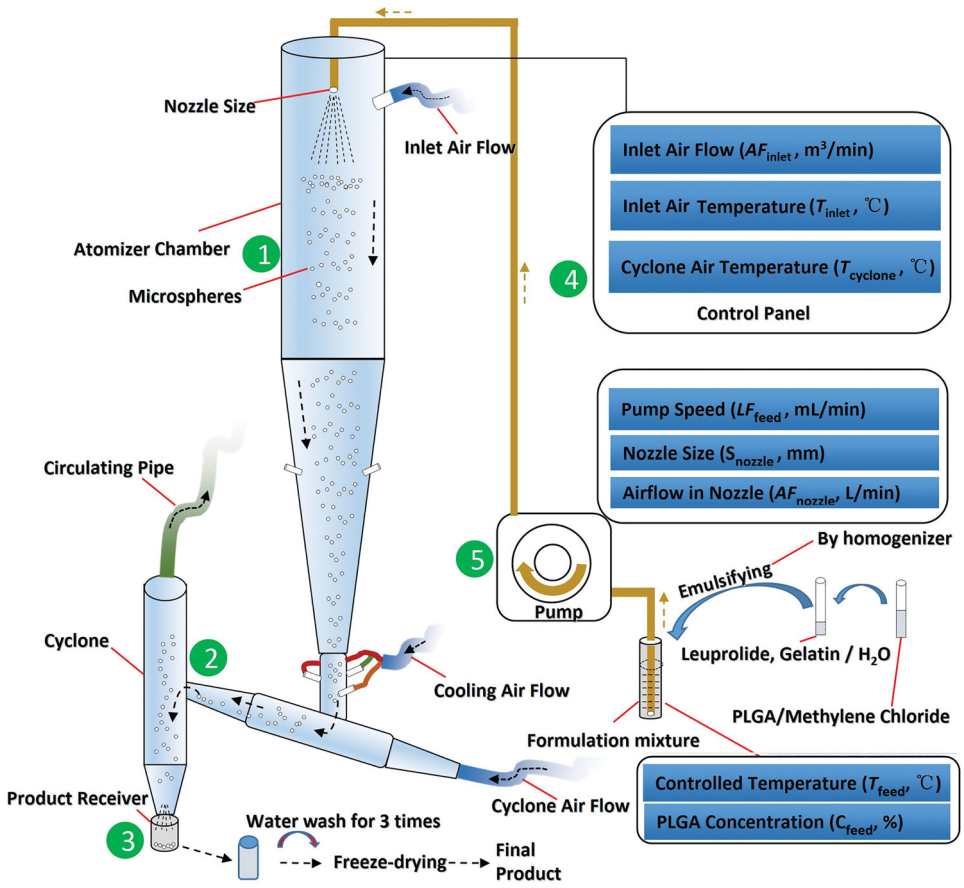

Figure 5. Schematic representation of the spray drying technique. Redrawn from (Shi *et al.* 2020).

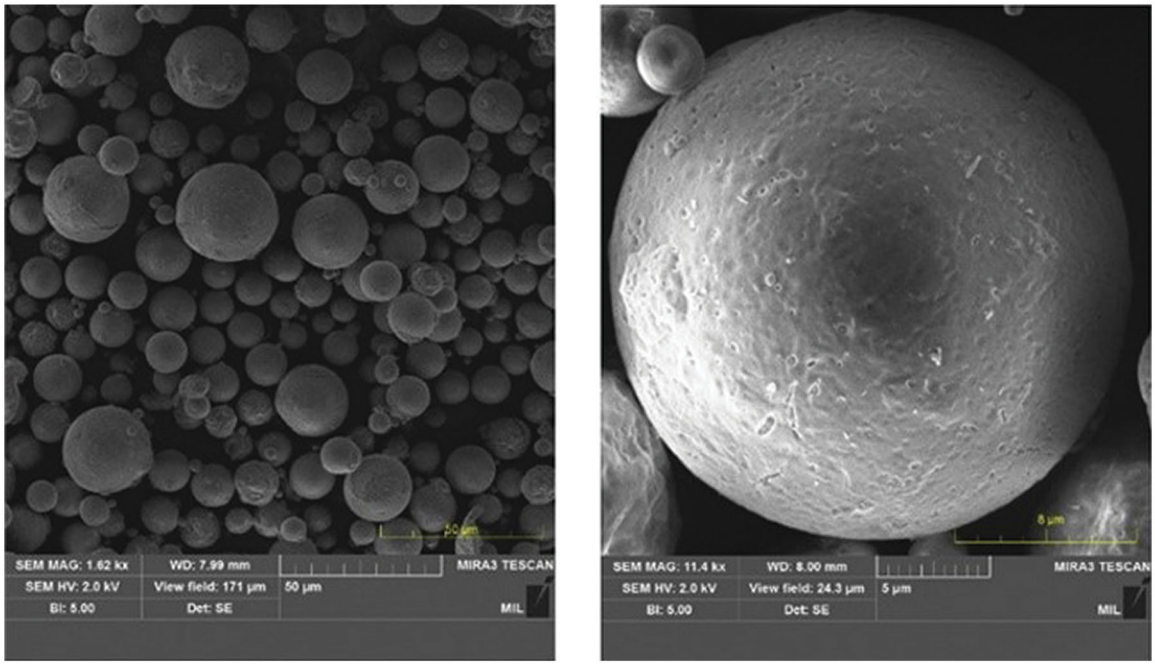

Figure 6. SEM images of microspheres prepared with spray drying technique. Redrawn from (Shi *et al.* 2020).

Lee et al. prepared leuprolide-loaded PLGA microspheres using the electrospray method. This method was a single-step procedure based on the spray drying process that did not require hot air at all. The prepared emulsion was loaded to the syringe of electrospray and under applied voltage (16.1–18.6 kV)

Table 3. Advantages and disadvantages of preparation methods.

Preparation methods	Advantage	Disadvantage	Scalability
Emulsion/solvent evaporation	Good universality Inexpensive	Low Encapsulation efficiency Broad size distribution	Dealing with organic solvent/ contaminated water Unsophisticated equipment Mild conditions
Rapid membrane emulsification	Particle size control Uniform sized particles Excellent reproducibility	Sensitive to the oil phase with high viscosity	Low energy consumption Equipment setup flexibility Batch and continuous
Spray drying	Low or no organic solvent contaminated water Control over particle size and structure Short operation time	Thermo sensitivity Wide size distribution High cost	Continuous process Large batch sizes One-step closed loop configuration

sprayed on the aluminium foil. Dried particles were collected from aluminium foil and stored in polyethylene tubes. This technology decreased the risk of denaturing active ingredients and owned the acquisition of high yield (Lee *et al.* 2020).

The most important advantages of the spray drying technique are scalability, continuity, mild process conditions, and available equipment. The produced dried particles are more chemically and physically stable, and the residual organic solvent is at a minimum. However, the final particles are relatively less uniform and surface morphology is frequently irregular. The main advantages and disadvantages of the aforementioned preparation methods and their scalability considerations for industrial production are summarised in Table 3.

Carrier materials of GnRH agonist

Chitosan

Chitosan is a natural, antimicrobial, biocompatible polymer, which has good absorbability, bioadhesive properties with immense potential in the drug delivery field. It can be decomposed further into simple molecules such as carbon dioxide, water, as well as other small molecules (Islam *et al.* 2012). There are two main advantages of chitosan in comparison with other biodegradable polymers. First of all, the degree of deacetylation (DD) determines the desirable properties of chitosan. The degree of deacetylation is defined by the molar percentage of deacetylated glucosamine units with respect to the total number of monomers that make the chitosan molecules. In general, DD has a significant impact on chitosan's physical properties such as solubility and viscosity. In vitro testing and biodegradation are also affected by this property. It has conclusively been proven that degradation is delayed at higher DD (84–90%) (Rodríguez-Vázquez *et al.* 2015). Secondly, amino and hydroxyl groups in chitosan allow them to amenable to various chemical modifications and form chitosan derivatives with definite functional groups. Moreover, derivatives show better performance than chitosan itself due to the increase in bioadhesion and permeability (Han *et al.* 2018).

Abashzadeh et al. prepared triptorelin acetate (TRP)-loaded chitosan microspheres by two different chitosan derivatives: carboxymethyl chitosan and sodium carboxymethyl chitosan, which owned and retained chitosan's advantages. From scanning electron microscopy (SEM), chitosan microspheres had a porous inner structure suitable for rapid water adsorption and fast dissolution (Abashzadeh *et al.* 2011).

Polylactic acid (PLA)

PLA is a biodegradable material that is polymerised from lactic acid. It is widely used as a wall material for microspheres production because it has several advantageous chemical and biological properties, such as thermal formability, flexibility, and non-toxicity. PLA can be redesigned by changing its molecular weight to obtain favourable biodegradable properties (Lee *et al.* 2016). Typically, PLA has low hydration and slow degradation rate and, for this reason, microparticles are used to design three-month or six-month formulations. Currently, PLA microspheres for a 3-month formulation containing leuprolide acetate are commercialised and traded under the name Lupron Depot®.

D'Souza developed a long-acting formulation by encapsulating orntide into the PLA microspheres for a 6-month release. In-vitro release study results illustrated a triphasic profile, including low initial burst, diffusion to the release medium, and erosional release. The low initial burst effect was characterised by the most active ingredients entrapped inside the polymer rather than the outer surface area or open pores. The next phase involved hydration of the PLA by body fluids and diffusion of the peptide to the release medium. During the last phase, PLA underwent drastic

and autocatalytic bulk degradation through hydrolysis which led to the cleavage of the ester bond and the yield of small fragments. The hydration data on day 180 illustrated full orntide release (D'Souza *et al.* 2014).

Park et al. produced PLA microspheres loaded with leuprolide and compared them with the marketed product Lucrin Depot®. From SEM images, microspheres had a spherical shape and smooth surface. The prepared microspheres' average particle size was 22.3 μm, comparable to the mean diameter of the marketed product. The encapsulation efficiency and drug loading of microspheres were 94.47% and 9.90% (w/v), respectively. It was illustrated around 13% of the burst release rate of leuprolide on the first day and sustained release over 84 days (Park *et al.* 2017).

Poly lactic-co-Glycolic acid (PLGA)

PLGA is a linear copolymer that can be made from three constituent monomers: d-lactic, l-lactic, and/or glycolic acids promoted by dibutyltin dimethoxide catalyst. It is one of the most used polymers in the bioengineering field for decades due to its good biodegradability and biocompatibility. The main advantage of PLGA is the regulation of the lactic and glycolic acid copolymer ratio (LA/GA) to control the degradation rate of the microsphere's matrix (Mao *et al.* 2012). Increased glycolic acid content allows a faster degradation rate of the polymer. Therefore, PLGA is generally used to produce microspheres with a one-month formulation, Currently, PLGA has been already applied in numerous marketed products such as Lupron Depot and Trelstar (Makadia and Siegel 2011, Zhou *et al.* 2018).

PLGA-based microspheres offer versatile properties suitable for the controlled GnRH delivery system. Schwach et al. prepared three types of degarelix-loaded microspheres from polymers with different lactic/glycolic acid compositions (LA/GA = 75/25, 65/35, 50/50). Duration of GnRH inhibition was 14, 21, and 36 days for 50/50, 65/35, and 75/25 PLGA microspheres, respectively. In this case, it can be seen that when the GA ratio was decreased, the degradation cycle of the microspheres was lengthened (Schwach *et al.* 2004).

Polyethylene glycol (PEG)

PEG is the hydrophilic polymer of ethylene oxide that is widely utilised in the pharmaceutical field due to its non-immunogenic and biocompatible properties. The main advantage of PEG is good solubility in both water and organic solvents due to the presence of oxyethyl groups. Therefore, PEG is usually blended with the aforementioned hydrophobic polymers to considerably facilitate drug delivery (Zhang *et al.* 2014). By functioning as a surface modifier for the hydrophobic polymer, the PEG could improve the penetration of water into the core of microspheres, thereby decreasing the acidic environment caused by the release of acidic degradation product and speeding up the diffusion of drugs in the matrix (Feng *et al.* 2015).

Mallarde et al. prepared teverelix-loaded microspheres with two different polymer types: PLGA and PLGA-PEG, then compared microspheres properties including encapsulation efficiency and drug release. Results illustrated that microspheres had similar characteristics in terms of encapsulation efficiency and particle size. The encapsulation efficiency ranged from 81% to 99% depending on the concentration of both polymers. However, there were large differences *in vitro* drug release, all microspheres prepared from PLGA-PEG had higher drug release than PLGA microspheres: the PLGA-PEG (2000/194) had the highest release of 48% in 15 days (Mallardé *et al.* 2003).

Strategies for increasing encapsulation efficiency

Encapsulating GnRH agonists in microspheres can be challenging due to several reasons. Firstly, all GnRH agonists are short hydrophilic peptides, which mainly consist of nine to ten peptides. Drugs are partitioned from organic phase droplets due to their excellent water solubility and low molecular weight. As a result, during the emulsification process, the active ingredients can escape from the inner water phase to the continuous external phase with a larger volume, resulting in low encapsulation efficiency and undesired leakage. Low encapsulation efficiency can be critical to expensive drugs and depots (Ramazani *et al.* 2016).

Effect of preparation parameters

Previous studies have established that the preparation parameters including polymer concentration and inner water phase ratio could be effectively optimised to achieve high encapsulation efficiency. Polymer concentration directly affects the viscosity of the organic phase: it increases the resistance of peptide diffusion from the organic phase to the external water phase,

thereby leading to the encapsulation of more drugs inside the microsphere. Another reason is related to the fact that at higher polymer concentration, time for polymer precipitation is reduced, so time for the peptide to diffuse out of the microspheres is also decreased. Therefore, when polymer concentration is increased in the emulsion, a rigid barrier with high viscosity is formed, resulting in elevated encapsulation efficiency (Okada 1997). Qi *et al*. (2019) supported it by increasing the concentration of PLGA from 150 mg/ml to 250 mg/ml in the oil phase; the encapsulation efficiency was raised by almost 20% and reached 94.16%. High viscosity prevented leakage of the inner water phase (Qi *et al.* 2019).

In general, the W_1/O proportion and encapsulation efficiency of the drug is complicated. When the ratio of W_1 was decreased, it could produce a stable first emulsion, which had less tendency of drug loss during the second emulsification process and resulted in high encapsulation efficiency. On the other hand, they reported loading efficiency was increased from 9% to 10% when the W_1/O ratio was reduced from 150 μL/1 ml to 100 μL/1 ml (Zhou *et al.* 2020).

Effect of using a binary organic solvent mixture

The use of a binary organic solvent mixture is another way to optimise drug loading. Solvent composition not only affects solvent removal rate but also the final characteristics of produced microspheres. It seems that one of the organic solvents can quickly partition into the continuous phase, and then hardened polymeric particles lead to the drug precipitation and incorporation into the polymer inner matrix. Hence, the peptide is inhibited from escaping to the external water phase by the layers of the polymeric wall.

Park et al. prepared TRP-loaded PLGA microspheres in a mixture of the organic solvent containing dichloromethane (DCM) and acetone (AC). By increasing the proportion of AC volume in the co-solvent system, the encapsulation efficiency was increased to 95.1% with reduced particle size. Since PLGA could be solubilised in AC and AC was miscible with the continuous phase. Consequently, due to the larger content of AC in the organic phase, it accelerated the AC diffusion into the water phase with rapid precipitation of PLGA. As a result, stable PLGA microspheres were obtained with higher encapsulation efficiency (Park *et al.* 2012).

Chen et al. dissolved peptide powder into the solution of acetic acid 3:100 (w/v) instead of water and added the obtained solution into the polymer oil phase. Next, silicon oil was added to the TRP-polymer to act non-solvent phase separation. The final suspension was poured into the N-heptane to solidify microspheres and the liquid/oil/oil (L/O/O) phase was formed. Dissolving the drug into the solution resulted in an encapsulation efficiency of 71% in comparison with the solid/oil/oil (S/O/O) method (27%) because the drug was easily entrapped in polymer droplets (Chen *et al.* 2019).

The implication of binary solvent using CH_3OH and CH_2Cl_2 as an organic phase was practiced. Laboratory experiments illustrated that higher encapsulation efficiency resulted from the binary method instead of the conventional one. The highest encapsulation efficiency (95.2%) was achieved by employing a 0.24 (w/v) ratio of methanol to methylene chloride (Ravivarapu *et al.* 2000a).

Effect of additives

The addition of surfactant provides resistance to the irreversible aggregation between the two immiscible phases and protection from separation, thereby enhancing the double emulsion's stability (Tamber *et al.* 2005). Polyvinyl alcohol (PVA) is one of the most frequently used stabilisers in the preparation of GnRH agonist-loaded microspheres. According to the previous research, encapsulation efficiency was enhanced by increasing PVA concentration in the external water phase. It could be related to the increased viscosity of the PVA solution with increasing PVA concentration, leading to the resistance to the outward diffusion of the peptide from the internal aqueous phase and the better stabilisation of the emulsion at higher PVA concentration. Past research conducted by Kakade and Hassan illustrated that adjusting PVA concentration from 0.5% to 1.0% increased encapsulation efficiency from 86.85% to 98.84% (Kakade and Dehghan 2018).

The addition of osmotic active additives to the external aqueous phase to regulate the capillary pressure between the inner and the outer aqueous phase is a common practice during the preparation of microspheres. Luan and Bodmeier observed that the addition of NaCl to the external water phase could increase the osmotic pressure, which in turn prevented inner water phase shrinkage and produced denser microspheres. As a result, encapsulation efficiency elevated from 88.7% to 99.0% (Luan and Bodmeier 2006).

Strategies for improvement of release profile

The control of initial release is a significant problem in the GnRH-loaded depot delivery system. The absence of an initial burst effect can develop delayed hormone suppression, while a high initial burst can bring high blood concentration of the drug and lead to toxicity issues. By optimising experiment conditions, it is possible to improve release behaviour and obtain the required initial release rate.

Effect of preparation parameters

Preparation parameters such as LA/GA ratio and polymer concentration play an important role in the release profile. First of all, high polymer concentration produces a solution with high viscosity, resulting in a more rigid polymer structure with fewer pores and a tortuous matrix that slows down the initial release. According to Luan and Bodmeier, the initial release of the drug decreased from 62.7 to 11.7% by increasing polymer concentration from 20% to 40% (Luan and Bodmeier 2006).

LA/GA ratio is another crucial factor for PLGA to regulate the hydrophilicity and degradation rate of a carrier matrix, which impacts the drug release duration. The previous study of the polymer composition revealed that an increase in glycolic acid ratio promotes water uptake and accelerates the degradation rate of polymers (Zhou *et al.* 2020). For instance, the rate degradation of LA/GA 50/50 copolymer is faster in comparison with LA/GA 85/15. Increasing the lactide content of PLGA (85/15) makes the polymer more hydrophobic (Panigrahi *et al.* 2021).

Effect of additives

The additives such as zinc and magnesium carbonates are used as buffer salts that pertain to a specific type of excipient for PLGA and under special formulations can have release-improving and pH-modification functions. These salts assist to achieve neutral microclimate pH all around the polymer matrix to improve controlled release. Previous studies reviewed that the faster release of the peptide from polymer was attained with $ZnCO_3$ loaded sample (Zhu and Schwendeman 2000). Figure 7 illustrated hypothesised three-stage release mechanism of leuprolide microspheres containing $ZnCO_3$. When water-soluble acid is present, the $ZnCO_3$ dissolves and produces

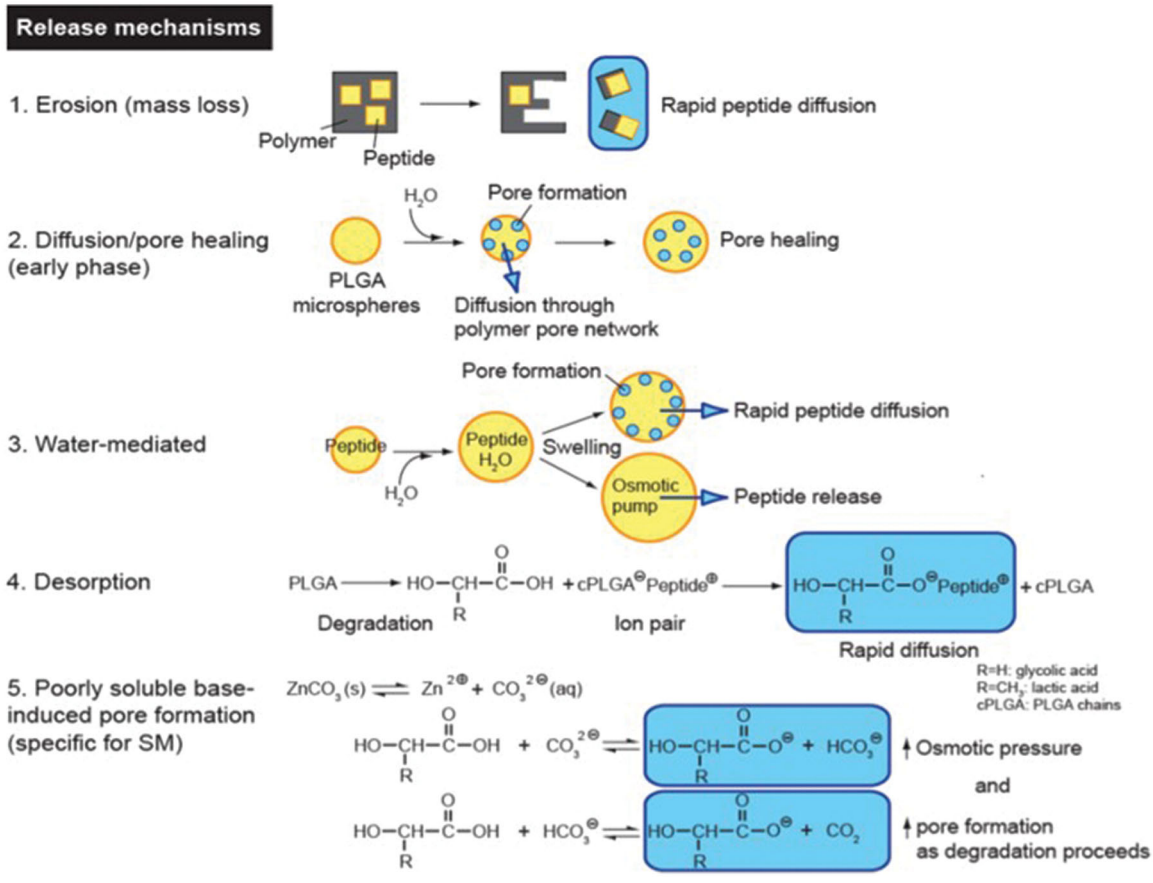

Figure 7. Description of release mechanisms of leuprolide-loaded PLGA microspheres. Redrawn from (Hirota *et al.* 2016).

water-soluble salts with carbon dioxide which elevate osmotic pressure and form pores. As a result, these pores allow the continuous release of the drug (Hirota *et al.* 2016).

In terms of a viscosity-increasing agent, Luan & Bodmeier used aluminium monostearate to increase the oil's viscosity. The high viscosity of the oil could prevent coalescence formation during emulsification. By adding aluminium monostearate into the oil, the initial burst release of the particle was also reduced. It can be related to the fact that increasing viscosity of the oil phase has a similar effect of increasing the oil amount, so less amount of peptide participated in the release medium (Luan and Bodmeier 2006).

Qi *et al*. (2019) reported that sodium acetate solution was applied as an additive to increase the osmotic pressure of the outer aqueous phase, which could decrease initial burst release and improve the release performance. As a result, the difference in pressure prevented the mass transfer to the surrounding microenvironment, as well as efficiently decreased the outward diffusion of the drug (Qi *et al.* 2019).

Pharmacokinetics (PK) and pharmacodynamics (PD) of GnRH loaded microspheres in drug delivery system

There have been currently controlled release of GnRH analogues that can be injected once every 1–6 months. For long-acting release injectable products, the internally penetrated drug is uniformly spread throughout the carrier material. Generally, the prescribed dosage of one-month, three-month, and six-month formulation of GnRH analogues in microspheres is 3.75, 11.25, and 22.5 mg, respectively. This administered amount allows high suppression of sex hormones and reduces the incidence of a hormonal breakthrough. Biodegradation of the microspheres in the body begins when the microspheres' surface is exposed to the body fluid, resulting in sustained release from the surface of the microspheres. The flux rate of the drug from the injection site to the plasma displays a biphasic profile. The initial burst effect is observable for several hours after injection in the first phase. It contributes to increasing drug concentration in plasma, serum luteinizing hormone, and testosterone level (Mostafa *et al.* 2014). After that, it is followed by a second phase, where the plasma level of agonists decreases to an adequate therapeutic level and is continuously released over a prescribed period (Anderson and Miller 1997).

Animal studies

During the animal studies, GnRH microspheres were managed to suppress the testosterone level in animals, including rats, fishes and dogs (Ravivarapu *et al.* 2000b, Witt *et al.* 2016, Kim *et al.* 2022). Table 4 summarises different model animals that were used for in vivo testing of the GnRH agonists, including injection and sampling routes. In general, a decrease in serum level and change in genital organs were measured after injection. The decline in serum testosterone following the initial flare-up of the hormone level was expected. This flare-up usually disappeared after 3 days in rats and its kinetics was presented with a pseudo-zero-order release of about 0.5 μg/L over the targeted period (Periti *et al.* 2002).

Woo et al. used Male Sprague-Dawley rats to evaluate the in-vivo mechanism of leuprolide microspheres with the 4-month formulation. The result showed that serum leuprolide concentration increased to 45 ng/ml after the first injection, and this concentration fell gradually to a plateau level of 2 ng/ml or less over 4 months. The testosterone levels of rats were suppressed to 0.5 ng/ml and remained at this rate for 120 days. These results indicated pituitary GnRH receptors were settled and regulated down by GnRH agonists (Woo *et al.* 2001). Another pharmacodynamics study of the leuprolide microspheres was conducted using male Wistar rats to compare the plasma concentration of testosterone after injecting Lucrin depot® and electrospray microspheres. The maximum testosterone level of the marketed product administered group and prepared microsphere-administered group was 107.8 and 86.2 ng/ml, respectively. The mean plasma concentration of testosterone from day 3 to day 14 was similar in both groups. Final estimates suggested that the prepared microspheres' effectiveness was consistent with the commercialised depot, and even the current formulation was enhanced by decreasing the initial burst effect (Lee *et al.* 2020). Kim et al. studied the efficiency of leuprolide-loaded PLA microspheres with a 3-months formulation on male beagle dogs. According to the PD results, microspheres have been shown to reduce testosterone levels effectively in a way that is equivalent to commercialised products. The plasma testosterone level was below 0.5 ng/ml after 28 days and this result indicated that it was lowered by maintaining a constant drug release at a concentration of 1 ng/ml which was the main goal of the research (Kim *et al.* 2022).

Abashzadeh et al. experimented with male rats to study in vivo release of the in-situ gel-forming system containing triptorelin acetate. In addition, this

Table 4. Reports of in-vivo testing conditions for GnRH analogues loaded long-acting systems.

GnRH agonist	Controlled release system	Model animal	Injection	Sampling	Time	Reference
Triptorelin acetate	The in-situ gel-forming system with chitosan microspheres	Male rats 8 weeks old 250–300 g	Subcutaneously at the back of the neck (100µl containing 186 µg TRP)	Heart	42 days	Abashzadeh *et al.* 2011
Leuprolide	PLGA microspheres	Male Wistar rats	Subcutaneously (0.1 mg/kg)	Right jugular vein	14 days	Lee *et al.* 2020
	PLGA microspheres	Male Sprague–Dawley rats ~300 g	Subcutaneously at the back of the neck	Tail vein	210 days	Woo *et al.* 2001
	PLA microspheres	Male Sprague–Dawley rats	The site on the back (100µg/kg/day)	Tail vein	90 days	Okada *et al.* 1994
	PLGA microspheres	Male Beagle Dogs	Intramuscular injection	Anterior jugular vein	150 days	Ravivarapu *et al.* 2000b
	PLA microspheres	Male Beagle Dogs (8–11 kg)	Subcutaneous Injection (0.3 mg leuprolide /kg/mL)		13 weeks	Kim *et al.* 2022
	PLGA microspheres	Fat-tailed dunnart	Subcutaneously at the back of the neck	Vagina and uterus	56 days	Witt *et al.* 2016
Orntide acetate	PLGA microspheres	Male Sprague Dawley rats 300g	Subcutaneously at the back of the neck	Tail vein	35 days	Kostanski *et al.* 2000
Goserelin	PLGA microspheres	Male Sprague Dawley rats, 180–200 g	Intramuscular injection	Retro-orbital veniplex	115 days	Qi *et al.* 2019
Alarelin acetate	PLGA microparticles	Northern pike males	Intraperitoneal injection	Sperm collection	96 hours	Knowles *et al.* 2022

experiment was designed to compare it with the currently marketed product Diphereline SR 3.75 mg. After receiving an injection of Diphereline SR, the serum testosterone level of rats was grown rapidly to 1600 ng/dl within the first 3 h and declined to the therapeutic level on the third day. The average testosterone level was continuously kept to the lowest value from day 2 to day 35. Another group injected with the in-situ gelling system illustrated a similar in-vivo profile and decreased testosterone levels up to 88% for 35 days (Abashzadeh *et al.* 2011).

Konstanski and De Luca studied the in vivo release mechanism of orntide microspheres to detect serum orntide and testosterone levels in male Sprague Dawley rats. The maximum concentration of the peptide was detected on day 6, then concentration started to decline and only 3 ng/ml was detected on day 28. Regarding testosterone suppression, complete castration was done on day 6 when serum orntide concentration elevated to 87 ng/ml (Kostanski *et al.* 2000). The pharmacokinetics of the goserelin-loaded hydrogel in PLGA microspheres was evaluated by Qi et al. and compared with a commercialised Zoladex implant. The mean plasma concentration of the PLGA microspheres in rats illustrated a double-peak pattern: the first small peak on the 12th day and the second C_{max} on the 42nd day. The first peak was formed when goserelin dispersed in polymer diffused in the early stage, while the second peak was formed when concentrated goserelin in the microspheres was released in the later stage. The double peak plasma level increased relative bioavailability and extended therapeutic effect (Qi *et al.* 2014).

The efficacy of alarelin-loaded PLGA microparticles was evaluated by Knowles et al. in male northern pike. In general, prolonged-release of hormone delivery system is extensively utilised in aquaculture to induce spermiation and to prevent the stress caused by multiple injections. Authors demonstrated that fishes injected with sustained-release formulation after 96 h had higher spermatozoon levels in comparison with the control group (Knowles *et al.* 2022).

Human studies

The delayed control system's main indication is treating diseases such as prostate cancer, endometriosis, and leiomyomata, which require long-term bioactivity of the GnRH agonists. Human studies were carried out to assess the efficacy, safety, pharmacokinetics, and pharmacodynamics of the drug. Study results illustrated that GnRH agonists were used to induce a decline in steroid hormones of gonadal which led to subsequent suppression of serum testosterone to the surgical castration level ($\leq$50 ng/dL) (Ozyigit *et al.* 2019).

Zhou et al. compared PK and PD studies of prepared leuprorelin acetate microspheres (test group) with marketed product Enantone® (reference group). The carrier material was PLA in both formulations and

microspheres were prepared with the water-in-oil emulsion method. 48 male Chinese volunteers aged from 18 to 40 years old were selected for the study and received 3.75 mg of the prepared microsphere or Enantone®. According to the PK data, the mean drug-concentration-time profile of both groups was similar. While in PD data, similar testosterone-inhibitory effects were seen in both groups, and more test group participants maintained longer castration time than those in the control group. Generally, both groups exhibited a 100% castration level after 28 days (Zhou *et al.* 2020).

Mostafa et al. studied PK and PD analysis for the 6-month depot formulation of leuprolide acetate with a 45 mg dose in prostate cancer patients. Depot formulation was prepared with the emulsion-solvent evaporation method. For this study, 300 male subjects confirmed with prostatic adenocarcinoma were involved. All patients were $\geq$18 years old with serum testosterone levels $\geq$150 ng/dl. The general clinical study lasted for 48 weeks, with the first depot formulation injected on day 1 and the second injection on day 169. The depot formulation's pharmacokinetic profile was in agreement with the previously described, and it showed two phases. The mean maximum plasma drug concentrations for the first and second doses were 6.7 and 7.4 ng/ml, respectively, and it happened nearly 2 h after injection. After that, it fell to a sustained therapeutic level, and the mean drug concentration decreased steadily from week 4 to week 24 (mean values were 1.28 and 1.14 μg h/ml). These authors demonstrated that the pharmacodynamics trend from measurements was consistent with pharmacokinetic evaluation. The initial increase in testosterone and LH level was observed after the first injection, and it reached 608 ng/dL from an average baseline of 435 ng/dL. By week 4, the hormone level declined to reach a plateau due to the pseudo-zero-order release and continued through the evaluation. On average, 93.4% of patients experienced suppression of testosterone $\leq$50 ng/dL, and a low number of patients escaped testosterone suppression before the second injection or had an acute-on-chronic response (Mostafa *et al.* 2014).

Future prospects

Long-acting GnRH analogues have been used in clinics for over 20 years, and their clinical success is unquestioned. Most probably, these microsphere products will continue to be researched and marketed for peptide delivery. The results of this review provide room for further development of a long-acting GnRH agonist system. With further optimisation, it is possible to obtain desirable yields of microspheres in terms of size, drug loading and release profile. Future work should involve parameters related to the solidification process including solidification rate and solvent evaporation pressure and their effect on encapsulation efficiency.

Conclusion

This review summarised the significant role of GnRH agonist-loaded delivery systems by focussing on developments in this field. In addition, it provided advanced projection and planning guidance to implement process conditions and parameters in the preparation of the microspheres. The main characteristics of preparation methods and applications of each type of polymer have been reviewed. Among them, PLA and PLGA are the most frequently used and commercialised polymers because of their biocompatibility and biodegradability. The main preparation method for GnRH-loaded microsphere is emulsion/solvent evaporation whereas other methods are still developing. For instance, the membrane emulsification technique has been applied to encapsulate other proteins, but the GnRH peptide is still brand-new.

At present, long-acting GnRH analogue formulations still struggle with the issues such as encapsulation efficiency and poor release behaviour. Several strategies were proposed to overcome these problems. Among them, the most effective strategies are regulation of polymer concentration, usage of binary solvents and application of additives. Despite numerous studies on encapsulating different GnRH analogues being ongoing, few of them are projected to enter clinical stages. For animal studies, the in-vivo testing of microspheres is mainly conducted using male rats by measuring their testosterone level after injection. While for human studies, in-vivo testing is conducted on prostate cancer patients and GnRH agonists can decrease the serum testosterone level to the castrate level.

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