Preparation Methods of Solid Lipid Nanoparticles for Hydrophilic Peptides and Proteins

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ABSTRACT: OBJECTIVE In recent years, many new pharmaceutically active proteins and peptides have been developed due to the progress of biotechnological techniques and genetic engineering. However, many of them require special formulation technologies to overcome drug-associated problems such as hydrophilic and instability. **METHODS** This review described that solid nanoparticles (SLN) have been sought as an alternative carrier for hydrophilic proteins and peptides under optimized preparation methods to incorporate the drug. **RESULTS** Formulation in SLN confers improved proteins stability, avoids proteolytic degradation, as well as sustained release of the incorporated molecules. **CONCLUSION** Thus, many important peptides and proteins have been incorporated into SLN and are currently under investigation.
KEY WORDS: solid lipid nanoparticles(SLN); preparation methods; hydrophilic proteins

亲水性蛋白和多肽固体脂质纳米粒的制备方法

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摘要：目的 近年来，随着生物技术和基因工程的进步，许多新的具有药用活性的蛋白和多肽得到了发展。然而，由于蛋白和多肽的亲水性和不稳定性，需要选择特殊的载体和制备工艺，以克服药物本身的缺陷。**方法** 本综述描述了在优化的制备方法下，固体脂质纳米粒(SLN)作为亲水性的蛋白和多肽药物的可选择载体，用来包封亲水性蛋白和多肽。**结果** SLN 作为载体可以改善蛋白的稳定性，避免其水解，并能够实现药物分子的持续释放。**结论** 因此，许多重要的多肽和蛋白已被包封进 SLN 或正在研究当中。
关键词：固体脂质纳米粒；制备方法；亲水性蛋白
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Recently, a growing number of new pharmaceutically active peptides and proteins have been developed due to the progress of biotechnology and genetic engineering. These new therapeutic biomolecules are usually characterized by large size, instability, hydrophilic, limited ability to across cell membranes, and rapid enzymatic degradation. Therefore, they often show low bioavailability and have short plasma half-life[1-3], thus necessitating administered frequently or parenteral routes, such as intravenous (i.v.) injections, to sustain therapeutic effects. It is obvious that there is an urgent need for suitable delivery systems capable of controlled-release and reducing the frequency of administration, thus lessening the strain on patients.

Solid lipid nanoparticles (SLN) are used to control the release of drug carrier system, and the average particle size of the colloidal system is 10-1 000 nm, aqueous SLN dispersions are composed of natural solid lipids or the synthetic lipids example of lecithin, monostearin, *etc*[4], which is solid both at body and room temperature, being stabilized by a suitable surfactant. Drug is entrapped or inlayed in the lipids core to form the solid colloidal drug system. Since their first description by Muller et al[5], SLN have attracted increasing attention as an efficient and non-toxic alternative lipophilic colloidal drug carrier system prepared by biocompatible solid lipids matrix and for efficient drug transport to the biomembranes while avoiding unwanted phagocytic mechanisms and adverse degradation of protein drugs. Compared to the existing conventional carriers (e.g. microemulsions, liposomes and polymeric nanoparticles), SLN combining the advantages of other colloidal carriers,

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but at the same time avoiding their major disadvantages. They offer the possibility of controlled drug release, good tolerability, drug targeting and produce protection of incorporated active ingredients against degradation[6]. Despite of the encouraging potential applications of SLN, formulation of hydrophilic peptides and proteins involving these carriers still remains challenges. Such potential challenges come from the inherent unwanted properties of proteins (e.g. easy denaturation during preparation course and short half-life)[7] and the particular difficulties in incorporating and dispersing of hydrophilic drugs into the lipophilic cores of SLN with high drug loading efficiency. These obstacles hinder the clinical application of SLN *in vivo*.

Such disadvantages have provided the impetus for the development of some particular preparation methods of SLN loaded with peptides and proteins. Thus, the therapeutically relevant peptides, protein antigens and model proteins have been incorporated into or adsorbed onto SLN.

1 Preparation of SLN containing hydrophilic peptides and proteins

There are various methods that have been used in the preparation of SLN, including high pressure homogenization, melt micro-emulsification, solvent diffusion method and emulsification-evaporation technique. However, since the proteins and peptides are unstable and easily lose activity under conditions of high pressure and hyperthermia, most of the common methods are not available[8].

The SLN production is based on solidified emulsion (dispersed phase) technologies. Therefore, due to their hydrophilic nature most proteins are expected to be poorly microencapsulated into the hydrophobic matrix of SLN, tending to partition in the water phase around the particles rather than be encapsulated by the hydrophobic matrix. In this case, methods such as developing a special structure which contains aqueous core or using some special surfactant will be used during the preparation of SLN to enhance the encapsulation efficiency (EE) and loading capacity (LC). So far some proteins such as bovine serum albumin[9], lysozyme[10], cyclosporine A[11-12] and insulin[13] have been successfully incorporated into SLN.

Next, several methods have been reviewed to be used to incorporate proteins in solid lipid particles.

1.1 General ingredients of SLN[14]

General ingredients include solid lipid(s), emulsifier(s) and water. The term lipid is used here in a broader sense, includes natural and synthetic sources such as triglycerides, fatty acids and waxes. A clear advantage of SLN is the fact that the lipid matrix is made from physiological lipids which decreases the danger of acute and chronic toxicity. The emulsifiers have been used to stabilize the lipid dispersion and the choice of them depends on the administration route. It has been found that the combination of emulsifiers might prevent particle agglomeration more efficiently.

1.2 Microemulsion-based SLN

The first attempts to encapsulate peptide drugs in SLN were those of Morel et al[15], who used the warm microemulsion-based technique to incorporate thymopentin. The pentapeptide thymopentin was englobed in SLN prepared from warm microemulsions following two different methods: from O/W microem ulsion by forming the more lipophilic ion-pair with hexadecylphosphate, and from W/O/W microemulsion by dissolving the pentapeptide in the aqueous internal phase[15]. But the required high temperature (60-70 ℃) will result in the destabilization of the labile drug. In addition, high concentrations of surfactants and co-surfactants would lead to low encapsulation efficiency, though it has to be remarked critically that they are necessary for formulating purposes. Therefore, this method was not ideal that the incorporation of the hydrophilic drug was 5.2% and 1.7% respectively.

1.3 High pressure homogenization (HPH)

Among the present encapsulation methods, the HPH has been generally recognized as a reliable and powerful technique for the preparation of SLN[16]. High pressure homogenizers push a liquid with high pressure (10-200 MPa) through a narrow gap (in the range of a few microns). The fluid accelerates on a very short distance to very high velocity (over 1 000 $km \cdot h^{-1}$). Very high shear stress and cavitations' forces disrupt the particles down to the submicron range[17]. Homogenization may be performed either at elevated temperature (hot homogenization) or below room temperature (cold homogenization). In general, higher temperatures result in lower particle sizes due to the decreased viscosity of the inner phase. However, the high temperatures used in the process could affect the stability of the drug, and the higher emulsifier

concentration was also believed to cause the burst drug release.

Lysozyme was incorporated as a model drug using HPH by the hot and the cold dispersion techniques to characterise the physicochemical parameters[10]. Previous to nanoparticle preparation, lysozyme was solubilised, until saturation, into the melted lipid phase. Production was carried out by a cold homogenisation process. The entrapment efficiency was dependent on the initial solubility of the peptide in the lipid phase of the final preparation. Lipid composition, surfactants and homogenisation conditions (temperature, pressure, number of homogenisation cycles) were found to be crucial for lysozyme encapsulation. Although the encapsulation efficiency was only 59% and loading capacity was low, lysozyme remained intact and active throughout the harsh encapsulation conditions. It was shown that some proteins were able to endure the harsh procedures of formulation by high pressure homogenisation, making possible the use of SLN as antigen carriers for vaccine delivery.

1.4 Solvent emulsion–evaporation

The solvent evaporation method is a widespread procedure for the preparation of SLN, being firstly used by Sjostrom and Bergenstahl[18]. Currently, much of the work carried out on incorporating instable and hydrophilic proteins into SLN, is based on this method. Used W/O/W multiple-emulsion and solvent-displacement techniques, which avoids any thermal or pressure stress[19], is investigated with the aim to explore their potential as oral delivery systems[20].

In this method, drugs were dissolved in the aqueous solvents with different pH according to the different physicochemical characteristics of different drugs. Insulin was chosen as hydrophilic, water unstable protein with very low oral bioavailability to be dissolved in the acidic inner aqueous phase, and emulsified with partially water-miscible solvents organic solvent, in which the lipids were previously dissolved. The primary W/O emulsion obtained was then gently poured, under vigorous mixing, into solvent-saturated outer aqueous phase containing the hydrophilic surfactant. Finally, the organic solvent was removed by evaporation, getting a W/O/W emulsion[21]. This method was able to protect insulin against enzymatic degradation and improve its oral bioavailability.

However, the average size is usually not controlled by formulation parameters and the instability of water-in-oil pre-emulsion would induce lower drug encapsulation efficiency.

1.5 Solvent emulsion–diffusion

HU F Q [22] and his co-workers optimized traditional method into the novel solvent diffusion method in aqueous system for the preparation of gonadorelin. Gonadorelin and monostearin were dissolved in acetone and ethanol at 50℃ in water bath, the resultant organic solution was poured into an acidic aqueous phase containing 1% PVA under mechanical agitation.

This method had two advantages: the one is no need for emulsifiers; and the other is simple preparation procedure. Since the emulsification diffusion procedure was carried out in the hydrophilic system, the proteins and peptides were easily to be encapsulated by the SLN with stable properties. Up to 69.4% of gonadorelin can be incorporated. *In vitro* release of gonadorelin from SLN is slow. These results demonstrate the principle suitability of SLN as a prolonged release formulation for hydrophilic peptide drugs.

1.6 Reverse micelle-double emulsion method loaded with drug- mixed micelles

SLN loaded with insulin-mixed micelles (Ins-MMs) were prepared by a novel reverse micelle-double emulsion method, in which sodium cholate (SC) and soybean phosphatidylcholine (SPC) were employed to improve the liposolubility of insulin, drug entrapment efficiency, drug loading efficacy and sustained release behavior without “burst effect”[23].

That paper showed that a hydrophilic peptide, such as insulin, could be successfully formulated into SLN by formation of SC and SPC mixed micelles. Particles with a small size (<110 nm) and high entrapment efficiency (>98%) could be obtained by the reverse micelle–double emulsion method. Results of *in vitro* experiments showed good physical stability and sustained drug release behavior. Thus, SLN loaded with insulin based on mixed micelles appear to be a good candidate for invasive insulin delivery.

1.7 A technique of combining hydrophobic ion pairing (HIP) and non-aqueous oil-in-oil (O/O) emulsion-evaporation

Leuprolide (LR) was selected as the model drug, while sodium stearate (SA-Na) was used as the negative charged ion pairing material[24]. Firstly, LR HIP complex were formed. SLN loading LR HIP complex was prepared by O/O emulsion-evaporation method. LR HIP complex were completely dissolved in ethanol at 70 ℃. The resultant organic solution was dispersed in liquid paraffin containing Span-80 by an ultrasonic probe. Subsequently, the O/O emulsion was vacuumed at room temperature, while stirred magnetically, to evaporate ethanol. SLN precipitate was then centrifuge separated from the dispersion. The liquid paraffin was cleaned up by washing the precipitates with petroleum ether.

Compared with the conventional method of solvent diffusion in an aqueous system, the efficiency of LR drug entrapment with SLN increased from 28.0% to 74.6%, the leakage of drug was restricted, and the stability of biomacromolecule drugs during encapsulation was maintained. It is suggested that it could potentially be exploited as an oral delivery system for leuprolide.

1.8 Potential of modified SLN based on existing methods

As mentioned above, tradition SLN loading hydrophilic peptides and proteins was hard to overcome some drawbacks such as the comparative low cellular uptake ability and rapid clearance *in vivo*. With the development of new materials or improvement of existing materials to prepare SLN, attempts have been made to improve SLN by surface modification with hydrophilic, flexible and non-ionic polymers. Over the past years, a broad number of polymers have been examined for drug delivery applications like synthetic polymers (PLGA[8], PEG[20]), natural polymers (dextran[25], lectin[13], chitosan[4], albumin and gelatin, *etc*.) have also been applied in order to avoid the toxicological problems associated with the use of synthetic polymers. Another promising example of modified SLN was based on amphiphilic supramolecular macrocyclic skeletons, focusing initially on cyclodextrin-based SLN, but more recently on calix-arene-based SLN[9]. These progresses could significantly alter the pharmacokinetics and the biological distribution of SLN[26], in order to enhance the drug stability, increase the permeability of the cell membranes and allow sustained drug release at the targeted site during a prolonged circulation time.

It is noteworthy that, these approaches were mainly focused on the improvements of existing methods thanks to innovation coming out from the emulsification methods. SLN of surface modification were mainly prepared using the emulsion/solvent evaporation technique (multiple-emulsion)[8, 20] or the precipitation solvent diffusion technique. In the first approach, polymers were dissolved in an organic solvent immiscible to water (such as dichloromethane, chloroform and ethylacetate) and emulsified in a surfactant-containing aqueous phase, proceeded by solvent evaporation. The second technique followed the precipitation of a polymer after dissolving it in an organic solvent miscible to water (such as acetone and ethanol) and dispersed in aqueous phase generally containing a colloid stabilizer [26].

2 Conclusion and perspective

This review summarizes several methods of SLN as an effective carrier for hydrophilic peptides and proteins. Although many incorporation methods avoid the use of organic solvents, it may be concluded that some proteins are able to endure the harsh formulation procedures, making possible protein formulation using SLN. SLN-based formulations of pharmaceutical proteins, as important as lysozyme, insulin, gonadorelin and leuprolide are currently under investigation. The lipid matrix improves protein stability, avoiding proteolytic degradation after administration, and releasing the protein in a controlled manner. However the versatility of SLN as protein delivery systems has been demonstrated, because protein encapsulation efficiency depends not only on the lipid mixture employed or the technique used, but mostly on the protein molecule, confirming that every protein must be considered as a special case.

In the future, evolutions will probably come from the introduction of several local or systemic therapeutic applications, such as immunisation with protein antigens, infectious disease treatment, chronic diseases and cancer therapy. With most of these protein-SLNs may easily be obtained by applying the existing methods with only a few adjustments.

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