Expert Opinion

Insulin encapsulation

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Many researchers all over the world are exploring various methods to find better insulin delivery routes for diabetic patients. Microencapsulation in biodegradable polymeric matrices is the source of developing new insulin formulations. This paper will attempt to explain the techniques utilized for insulin microencapsulation, their specifications and the parameters that influence the process. This review takes a closer look at recent researches and methods that have been reported in this decade.

Keywords: Biodegradable polymers, emulsification, insulin, membrane emulsification, microencapsulation, reverse micelle, sol – gel processes, spraying

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1. Introduction

Diabetes is the cause of morbidity and mortality of epidemic proportions. There are two major types of diabetes; Type I and Type II. Type I diabetes, also called insulin-dependent diabetes mellitus (IDDM), is characterized by an absolute insulin deficiency due to autoimmune destruction of the beta cells in the pancreas. It typically occurs in children and adolescents. Approximately 5 – 10% of patients with diabetes have Type I disease. The majority (90 – 95%) of patients with diabetes have Type II disease, also called non-insulin-dependent diabetes mellitus (NIDDM), which is characterized by insulin resistance (i.e., reduced sensitivity of cells to the action of insulin) and a relative deficiency of insulin. Complications of hyperglycemia include macrovascular complications (e.g., coronary artery disease, myocardial infarction, stroke, etc) and microvascular complications (e.g., nephropathy, end stage renal disease, blindness, etc).

Insulin therapy by frequent injections is required for the treatment of all patients with Type I diabetes and many patients with Type II diabetes. This treatment involves one or more doses daily of intermediate- or long-acting insulin injection, as well as an injection before each meal [1]. This approach is satisfactorily efficient, but brings distress and inconvenience to patients, induces unstable curative effects and side effects. Recently, there has been a great deal of interest in the development of new insulin delivery strategies, molecular design, device design and formulation (e.g., oral, nasal) [2-4]. The convenience and acceptability of the oral route for drug administration has meant that it has received much attention for the delivery of insulin. However, there are several limitations. These include low bioavailability because of degradation in the stomach, inactivation and digestion by proteolytic enzymes in the luminal cavity, poor permeability across the intestinal epithelium because of its high molecular weight and lack of lipophilicity [5].

Attempts to overcome these problems include the use of encapsulation to provide protection for the incorporated insulin. Most oral (as well as some nasal and pulmonary) delivery strategies for insulin are based on micro, nanoparticle carriers. These have the following advantages: their small size allows them to pass the intestinal mucosa, thus increasing their effectiveness and distributing more uniformly in the gastrointestinal tract; and their large specific area favours their capacity as a loading drug [6,7].

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Table 1. Different techniques used for insulin micro, nano-encapsulation.

Chemical processes	Ref.	Physical processes			
		Physico-chemical	Ref.	Physico-mechanical	Ref.
Emulsification (single)	[15-19]	Sol – gel encapsulation	[74,75,82,83,85-87, 90,91,94,95]	Electro-spray drying	[118]
Multiple emulsification	[18,19,21-32]	Layer-by-layer assembly	[110,111]	Spray freezing into liquid	[120]
Interfacial polymerization	[49-56]	Membrane emulsification	[102-104]	Spray drying	[113,114,116]
Polymer complexation	[57-62,129-131]	Gas antisolvent CO_2 precipitation	[123]	Surface acoustic wave atomisation	[124]
Phase inversion	[63,64]	Supercritical fluid enhanced mixing and spraying	[115]		
Reverse micellar	[46,48]	Coacervation	[107,108]		
Film shaking and membrane destabilizing/detergent dialysing	[65]				
Deposition	[66]				
Nanoprecipitation – solvent displacement	[68]				
Melt dispersion	[18]				

As an encapsulating agent, polymers are of special interest from the pharmaceutical point of view and they have sustainable release properties [6]. Both biodegradable and non-biodegradable polymers have been investigated, but non-biodegradable polymers pose problems of toxicity, difficulty in removal and also sustained release of drug cannot be achieved using these polymers [8].

According to the process and the composition used in the preparation of micro, nanoparticles, micro, nanospheres or micro, nanocapsules can be obtained. Micro, nanospheres are homogeneous systems in which the drug is dispersed within the polymer throughout the particle. Micro, nanocapsules are heterogeneous systems in which the drug is confined to a cavity surrounded by a single polymeric membrane [9,10]. In this article, the term micro, nanoparticle is a collective name for micro, nanospheres or micro, nanocapsules. The distinct advantage of nanoparticles over microparticles is that their submicron size offers a relatively higher intracellular uptake [11], while microparticles larger than 10 µm do not penetrate the intestinal mucus layer [6].

Achieving an acceptable dose volume (i.e., high drug content) while maintaining satisfactory release kinetic (i.e., minimal burst, acceptable duration) represents a very significant formulation challenge. Drug content capacity is expressed by 'encapsulation efficiency' or 'drug loading'. Encapsulation efficiency refers to the amount of drug encapsulated in the finished micro, nanoparticles product, expressed as the percentage of the total amount of drug added in the process, while drug loading expresses the percentage of drug by weight in the final formulation [9].

Many techniques have been used for insulin micro, nano-encapsulating. Some of the important processes used for insulin micro, nano-encapsulation, are summarized in **Table 1**. They are usually categorized into two groupings: chemical processes and physical processes, with the latter being further subdivided into physicochemical and physico-mechanical techniques [12]. These labels can, however, be somewhat misleading, as some processes classified as physical might involve or even rely upon a chemical reaction, and some chemical techniques rely solely on physical events.

The present mini-review outlines major new findings, primarily since 2000, and describes the techniques for insulin micro, nano-encapsulating. Myriad parameters affect the process and the product properties; this review attempts to explain those that are the most effective. Research activities in this area are increasing and hence this review is timely.

2. Chemical methods

2.1 Emulsification (single and multiple)

A single emulsion may be defined as a dispersion of two or more mutually immiscible liquids, one in the other. The liquids are typically water and oil. The dispersion may either be that of oil in water (oil-in-water; O/W) or vice versa (water-in-oil; W/O). Emulsions are stabilized by the addition of surfactant, which lowers the interfacial tension between the two phases, thereby reducing the amount of work needed to form the emulsion.

Single emulsification for insulin encapsulation basically consists of four major steps: dissolving or dispersing

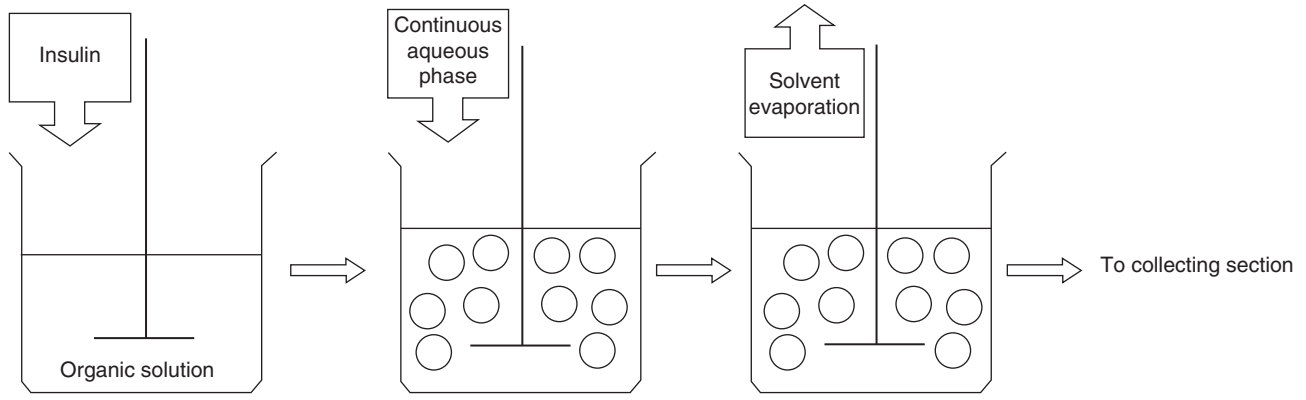

Figure 1. Schematic illustration of the emulsification (single) technique.

insulin in an organic solvent containing matrix forming polymer(s), which is then emulsified in a second continuous aqueous phase containing surfactant, removing organic solvent by evaporation which results in solid micro, nanoparticles and finally collecting them [13,14]. The emulsification step is performed by stirring [15-19] or packed bed emulsifying apparatus [15].

A schematic overview of this method is shown in **Figure 1**. Those reports in which single emulsions were used to produce insulin microparticles are summarised in **Table 2**. Since poly(D, L-lactic-co-glycolic acid) (PLGA)-based microparticles typically release high levels of insulin immediately after administration (a high burst effect), Hinds *et al.* [15] used the combination of PEGylation and microencapsulation and this offered the potential for sustained delivery of basal insulin with a single weekly dose. Takenaga *et al.* [17] used zinc compounds to stabilize the protein and to control the release from microparticles. Based on their study, the factors controlling drug release included the molecular weight of the PLGA, the lactic acid (LA)/glycolic acid (GA) ratio of the PLGA and the particle size.

Trotta *et al.* [16] and Reithmeier *et al.* [18] developed lipid microparticles as an alternative to polymeric microparticles as a parenteral controlled release device for insulin. Results of biocompatibility experiments of lipid microparticles on mice were comparable to those of polymeric microparticles. The *in vitro* insulin release was slow [16,18] and incomplete in the 13 days of monitored time period [18]. An initial burst effect of 20% of the dose was also observed [16].

Double or multiple emulsions can be made by emulsification of a single emulsion (O/W or W/O) in oil or in water. Depending to the order of incorporating oil and water phases, different emulsions e.g., W/O/W, O/W/O, W/O/W/O can be produced.

Multiple, or precisely double emulsion method is a modification of single emulsification employing a double emulsion. In this technique, a W/O/W double emulsion must be prepared, using a two-step procedure. In the first step, a primary W/O (water droplets in matrix oil) is prepared utilizing water and in an immiscible organic solution (sometimes a low hydrophilic – lipophilic balance (HLB) surfactant is incorporated in the 'oil' phase). In the second step, the primary W/O emulsion is re-emulsified in an aqueous solution of a high HLB surfactant [20].

Using a probe sonicator [19,21-23], high speed homogenization [24-30] or both [31,32] have been reported for the first step of emulsification. The second emulsification step is carried out in a low shear method so as to avoid expulsion of internal droplets to the external continuous phase. The solvent is evaporated by continuous gentle stirring or under reduced pressure. Separation of solidified micro, nanoparticles is usually done either by filtration or centrifugation. The particles may then be rinsed, with appropriate liquids, dried or lyophilized.

A summary of reports involving multiple emulsifications is given in **Table 3**. Manoharan and Singh [33] studied the effect of three different zinc salts (zinc oxide, zinc carbonate and zinc acetate) on insulin encapsulation release and stability. Their results showed that using zinc oxide can improve insulin encapsulation by about 25%. Insulin preserved its stability in formulations prepared with the addition of zinc salts. With zinc acetate, a high burst effect was observed. But all the formulations prepared with zinc salts released more than 87% of the encapsulated insulin within two weeks. Teply *et al.* [21] utilized polymeric encapsulation and complexation with micromagnets and this led to a long-term retention in the small intestine of mice, which allowed for the continual release and absorption of the drug. It was shown that insulin-loaded Eudragit® (Degussa [now Evonik], Darmstadt, Germany) microspheres retarded the release of insulin at low pH and caused an insulin slow release at pH 7.4 in the colon. *In vivo* study with these microspheres demonstrated that the polymer could protect insulin against proteolytic degradation in the gastrointestinal tract [22]. It was also demonstrated that the use of surfactants

Table 2. Single emulsion method for insulin micro, nano-encapsulation.

Particle type	Encapsulating polymer	Organic solvent	Insulin type	Surfactant	Mean particle size	Encapsulation efficiency	Further explanation	Ref.
Microsphere	PLGA	Methylene chloride	Human recombinant insulin	PVA	20 μm	84.2%	–	[19]
Microsphere	PLGA	Methylene chloride	Human crystalline zinc–insulin	PVA	65 μm	85 – 100%	Insulin is PEGylated before encapsulation	[15]
Microsphere	Lipid	Isobutyric acid	Bovine insulin	Lecithin and taurodeoxycholate	1.3 – 1.5 μm	78 – 84%	–	[16]
Microcapsule‡	Polybutane-1,4-diol succinate	Chloroform	Bovine insulin		5 – 20 μm§	N/A	Span 85 and Tween 20 were added, in separate steps, to the prepared microcapsules	[132]
Microcapsule	PLGA	Methylene chloride	Human insulin	PVA	15 – 30 μm	> 70%	Using zinc compounds ,as additives, control the release of insulin	[17]
Microsphere	Lipid	Methylene chloride or ethyl acetate or ethylmethyl ketone	Bovine insulin	PVA	21 – 58 μm	94% (max)	Using methylene chloride results in smaller microparticles and higher encapsulation efficiency	[18]*

*Reithmeier, Herrmann and Gopferich also incorporated insulin as an acidic solution in a W/O/W system.

‡Based on the original report.

§The size range of the cluster of particles.

Table 3. Double emulsion method for insulin micro, nano-encapsulation.

Particle type	Encapsulating polymer	Internal aqueous phase	Organic solvent	Insulin type	Surfactant in external aqueous phase	Mean particle size	Encapsulation efficiency	Further explanation	Ref.
Microsphere	PLGA	Insulin aqueous solution	Methylene chloride	Radio labelled insulin	PVA	3 – 5 μm	60 – 80%	Microparticles were mixed with positively charged micromagnets to prolong intestinal retention of drug	[21]
Microsphere	PLGA	Insulin in phosphate buffered saline + zinc salt	Methylene chloride	Human recombinant insulin	PVA	N/A	85% (max)		[33]
Nanosphere	PLGA	Insulin aqueous solution	Mixture of methylene chloride and acetone	Bovine insulin	PVA or Pluronic F-68®	735 nm (max)	73% (max)	Using PVA results in smaller nanoparticles and higher encapsulation efficiency	[24]
Nanosphere	Mixture of Eudragit RS® and Poly(-ε-caprolactone)	Insulin aqueous solution	Methylene chloride	Regular Human insulin	PVA	358 nm	96.3%	Poly(-ε-caprolactone) is the resorbable agent	[23]
Microsphere	PLGA	Acetic acid + insulin aqueous solution	Methylene chloride	Bovine insulin	PVA	~ 30 μm	84% (max)*	Hydroxypropyl-β-cyclodextrin was added to the internal aqueous phase to enhance absorption	[25]
Microsphere	PLA or PLGA	HCl + insulin aqueous solution	Methylene chloride	Bovine insulin	PVA	53 μm (max)	80% (max)	Using PLGA results in larger microparticles and higher encapsulation efficiency	[31]
Microsphere	Eudragit S100®	Concentrated aqueous insulin solution	Mixture of methylene chloride, ethanol and isopropyl alcohol	Porcine insulin	PVA or Polyvinyl pyrrolidine	32.1 μm	82% (max)	Polysorbate 20 was added to the internal aqueous phase to enhance regularity in particle shapes, insulin loading and stability	[22]
Microsphere	PLGA	Glycine-HCl buffer + insulin aqueous solution	Methylene chloride	Human recombinant insulin	PVA	55 μm	85.9%		[19]

*Calculated from the reported data.

‡In this report, microparticles were not collected from the liquid phase.

§The authors referred to the method as interfacial coacervation. But the preparation method coincides better to multiple emulsification.

¶Oil phase, in this case.

#Yamaguchi *et al.* reported an encapsulation efficiency of 106.7%, using glycerol.

Table 3. Double emulsion method for insulin micro, nano-encapsulation (continued).

Particle type	Encapsulating polymer	Internal aqueous phase	Organic solvent	Insulin type	Surfactant in external aqueous phase	Mean particle size	Encapsulation efficiency	Further explanation	Ref.
Microglobule‡	–	Insulin and NaCl aqueous solution	Miglyol 810 N® or fish oil¶	Biosynthetic human insulin	Arlatone F127G®	28 µm (max)	94.6% (max)	Abil EM-90® was used as the low HLB surfactant	[26]
Microsphere	PLGA	Insulin microcrystal aqueous suspension + acetic acid	Methylene chloride	Bovine insulin	PVA	6.6 µm	80.5 (max)	–	[27]
Microglobule‡	–	Buffered saline insulin solution	Miglyol 810 N®,¶	Biosynthetic human insulin	Tween 80® or Arlatone F127G®	40 µm (max)	99.9% (max)	Abil EM-90 was used as the low HLB surfactant	[28]
Microcapsule	PLGA	Insulin in a polar solvent + zinc compound	Methylene chloride	Human insulin	PVA	N/A	80%#	Zinc acetate dehydrate solution was used in external aqueous phase to prevent the deactivation of insulin Addition of the hydrophilic polar solvent is to control initial rapid release of insulin	[32]
Nanocapsule§	Block copolymer of PLA – PEG – PLA, PLA, glycerol trioleate	HCl + insulin aqueous solution	Acetone	N/A	Tween 20®	726 nm (max)	70% (max)	Dextrin T-70® was used as a steric stabilizer	[30]
Microsphere	PLGA	Aqueous insulin and acetic acid solution	Methylene chloride	Bovine insulin	PVA	17.4 µm (max)	44% (max)	Either Poloxamer 188® or polysorbate 20 was added to the internal aqueous phase Sorbitan monooleate 80 was added to the organic phase	[29]

*Calculated from the reported data.

‡In this report, microparticles were not collected from the liquid phase.

§The authors referred to the method as interfacial coacervation. But the preparation method coincides better to multiple emulsification.

¶Oil phase, in this case.

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could result both in optimising microparticle properties and improving the stability of a loaded peptide [29].

Studying **Tables 2** and **3** reveals that PLGA and polyvinyl alcohol (PVA) are the most common polymers used for micro, nano-encapsulation. It seems the encapsulation efficiency of insulin is not affected by the type of insulin. Also, compared with single emulsion, double emulsion methods favour higher loading of insulin [34].

In vitro release of insulin from microspheres can be improved by physically mixing microspheres prepared by double emulsion and single emulsion methods. 'Mixed' insulin-loaded microspheres have maintained basal insulin level in diabetic rabbits for 40 days [19].

Both in single and double emulsion-based methods, organic solvent must have high solubility capacity for insulin and for the polymer. Moreover, this solvent must easily be removed from the emulsion. Toxicological problems arising from solvent residues should also be considered. Also the solvent must be slightly soluble in water. To confirm the possibility of using the solvent, the apparent partition coefficient between the solvent and water, which is defined as total drug concentration in non-polar phase divided by total drug concentration in water, and its solubility in water must be determined [16].

The size of micro, nanoparticles in both methods depends upon the mixing speed, volume of the external aqueous phase, polymer concentration in the organic phase and the mixing method used to prepare the primary emulsion [13,27].

Low solubility of polymer in organic solvent, high solubility of organic solvent in water, high concentration of polymer, low ratio of dispersed phase to continuous phase and fast solvent removal rate are factors that result in fast solidification of micro, nanoparticles and enhance high encapsulation efficiency [35].

Both the above methods are good for a laboratory-scale operation because of their simplicity, but there are several disadvantages with these techniques. Non-uniformity of particles size distribution and adverse effect of organic solvents on insulin are the main problems. Meanwhile, because they are inherently batchwise and because of high energy consumption, the scale-up is costly [36,37].

2.2 Reverse micellar

Large amounts of a polar phase (usually water) and a non-polar phase (usually oil); can be brought into a single phase, which is macroscopically homogeneous but microscopically heterogeneous, by the addition of an appropriate surfactant or a surfactant mixture. This class of dispersions is called 'microemulsions'. The essential distinction between a normal emulsion and a microemulsion is in their stability; the former is 'kinetically stable' whereas the latter is 'thermodynamically stable'. Microemulsion formation is without any energy input, that is they do not require high shear rates for their formation. Even shaking by hand alone can be enough [38-42].

Microemulsions, like emulsions, are systems with an inner structure of submicron-sized droplets stabilized by surfactant molecules. Another major difference between them is in particle size. The size of the colloidal phase in the latter is typically in the range of 10 – 100 nm. Microemulsions are single optically isotropic, transparent or slightly opalescent, non-viscous and Newtonian solutions [40,43].

Different types of microemulsions are known as W/O and O/W (water-in-supercritical-CO_2 [w/sc CO_2] is another type of microemulsion). Water-in-oil microemulsion is formed when water is dispersed in a hydrocarbon-based continuous phase. In this type of microemulsion, thermodynamically driven surfactant self-assembly generates aggregates known as reverse or inverted micelles (RM). These are very tiny water droplets stabilized in an organic solvent with the aid of surfactants. In an inverted micelle the polar groups of the surfactants are concentrated in the interior and the lipophilic groups extended towards and into the non-polar solvent [41,43,44]. RMs are highly dynamic, undergoing continuous and spontaneous fluctuations [40]. RMs were proposed as hydrophilic reservoirs to assist in the encapsulation of hydrophilic entities in polymeric nanoparticles [45].

Insulin nanoparticles have been synthesized from a single microemulsion. At first step an insulin – phospholipid complex is prepared. Phospholipid is an ampholytic surfactant. After adding the solution of encapsulating polymer in hydrophobic organic solvent, the hydrophilic head group of phospholipid is directed toward the hydrophilic areas of insulin and the hydrophobic tail is directed towards the organic phase. This 'reverse micellar' solution is subsequently emulsified in an aqueous PVA solution and further steps are taken following the conventional emulsification method. Cui *et al.* [46] used soybean phosphatidylcholine, either poly (lactic acid) (PLA) or PLGA, and either dichloromethane or ethyl acetate as phospholipid, encapsulating polymer and organic solvent, respectively. Soybean phosphatidylcholine was employed to enhance insulin liposolubility. Mean particle size and encapsulation efficiency were reported to be around 200 nm and 90%, respectively. An initial burst effect and a subsequent delayed release were observed in simulated gastric and intestinal pH conditions [46].

To improve protein absorption, its paracellular (between the adjacent cells) and transcellular transports must be increased. Modulating the tight junction of cells and increasing the fluidity of the cell membrane enhance paracellular and transcellular transports. Permeation enhancers are those materials that can improve paracellular and transcellular transports [6,47]. Sodium cholate is one of the permeation enhancers [47].

Liu *et al.* [48] incorporated insulin into solid lipid nanoparticles by the formation of sodium cholate and soybean phosphatidylcholine mixed micelles using a reverse micelle double emulsion method. Briefly, an insulin and sodium cholate solution was added to a solution of lipid (stearic acid and palmitic acid) and soybean phosphatidylcholine in

ethyl acetate. Further steps were followed by a common emulsification method. The results of *in vitro* experiments showed good physical stability. Although no burst effect was observed, drug release was rapid in the initial stages. Nanoparticles of 110 nm and an encapsulation efficiency of 98% were reported.

Proper selection of the microemulsion components for the encapsulation formulation is a key factor in the enhancement of bioavailability of drug. The formulation should contain at least one component (usually the surfactant) that can also act as a permeability enhancer by increasing paracellular transport [6,43]. The resultant particle size in this method appears to be dependent to several parameters; solvent type, surfactant type and water: surfactant ratio [44]. A major drawback of this method is the use of large amounts of surfactants to form microemulsions. Most microemulsions contain around 40% of surfactants [40].

2.3 Interfacial polymerization

The interfacial polymerization approach has been used to prepare nanoparticles of insulin. The reaction is performed in O/W emulsion systems or in microemulsions, leading to the production of oil-containing or water-containing nanocapsules.

In an O/W emulsion system, an organic phase, consisting of oil (Miglyol 812N®, Sasol Germany GmbH, Witten, Germany), monomer (Isobutylcyanoacrylate) and insulin (or insulin aqueous solution) dissolved in an organic solvent (absolute ethanol), is injected or dropped into the aqueous solution of a high HLB surfactant (Lutrol F68®, BASF, Ludwigshafen, Germany), under strong magnetic stirring. The nanocapsules form immediately to give a milky suspension. The organic solvent is then evaporated by roto-evaporation under vacuum. The mean diameter of the nanocapsules is less than 400 nm and insulin encapsulation efficiency is 60 – 100%, based on different reports [49-54]. Release behavior of these nanocapsules was studied comprehensively both *in vivo* and *in vitro* [50-52]. A high variability was observed in the concentration of insulin crossing the intestinal barrier and no modification of glycaemia was demonstrated [51]. Formulation parameters affect the size of nanocapsules, however, and pH of insulin aqueous solution and the quality of the monomer control encapsulation efficiency [49].

Sullivan and Birkinshaw [55] reported the production of nanoparticles by polymerizing butyl cyanoacrylate in aqueous acidic medium in the presence of dextran, as a stabilizer, without irradiation or an initiator. Insulin was dissolved in the polymerization medium in the middle of polymerization reaction. An insulin encapsulation of 72% was achieved and the average particle size was around 240 nm. The *in vitro* release of insulin indicated a high concentration of drug at the particle surface.

There is only one report about performing the polymerization reaction in a microemulsion system. In this report W/O microemulsion contained water, oil mixture and surfactants were blended. Ethyl 2-cyanoacrylate was used as the monomer and after its addition to the microemulsion, under stirring, nanocapsules were produced. The mean diameter of nanoparticles was 150 nm and reported encapsulation efficiency was more than 80% [56].

High encapsulation efficiency, product fast formation and producing nanocapsules are the major advantage of this technique. The drawbacks of this technique are the requirement for high input of energy and the use of organic solvents [11]. In contrast, because reactive monomers are used, unwanted chemical reactions may occur between the drug and the monomer before or during the polymerization [9].

2.4 Polymer complexation

This is a post-fabrication encapsulation technique, that is micro, nanoparticles are prepared through different copolymerization reactions and it is just after obtaining the products that insulin is loaded into/onto them.

The different reports on this method are summarised in **Table 4**. Xiong *et al.* [57] studied both *in vitro* and *in vivo* release behaviour of insulin. Their studies showed a burst release in the first 30 min and the highest blood glucose concentration, after oral delivery of insulin to diabetic mice, was achieved after about 5 h. Leobandung *et al.* [58] investigated the stability of the loaded insulin at elevated temperature and high shear stress. The amount of insulin that could be detected after heating at 60°C for 8 h was 80%. No significant effect due to shear stress on the stability of insulin was found. Morishita *et al.* [59] administrated insulin-loaded microparticles orally to diabetic rats. A strong hypoglycaemic effect was observed following single and multiple oral administrations. It was found that blood glucose level could be kept low by only one subcutaneous insulin injection along with multiple oral insulin loaded microparticles administration. Sajeesh and Sharma [60] observed a pH-dependent release profile for their prepared nanoparticles. Their experiments proved that encapsulated insulin retained biological activity. The muco-adhesive studies conducted with isolated rat intestine indicated the adhesive nature of their nanoparticles. Microspheres prepared by Varshosaz *et al.* [61] were for nasal delivery of insulin. *In vitro* and *in vivo* experiments showed that the microspheres were absorbable from the nasal route.

Besides toxicological problems arising from organic solvent and monomer residues, the major problem with this method is the 'sluggish' insulin incorporation process. To achieve high encapsulation efficiency, micro, nanoparticles must be soaked in insulin solution as long as even 72 h.

There is another report that is also based on synthesis of block copolymers and polymer complexation, although there are also a few differences.

Jiang, Qiu and DeLuca [62] prepared a microparticle insulin delivery system by hydrogel microparticle synthesis, incorporating insulin onto them and encapsulating the microparticles by PLGA. Hydrogel was a poly (acryloyl hydroxyethyl starch) and insulin was loaded by adding

Table 4. Polymer complexation method for insulin micro, nano-encapsulation.

Particle type	Insulin type	Micro-, nano-carriers copolymeric system	Polymerization reaction for producing copolymer	Insulin incorporation method	Mean particle size	Drug loading or encapsulation efficiency	Further explanation	Ref.
Nanocapsule*	Human insulin	Poly(lactic acid)-b-Pluronic-b-poly(lactic acid) (PLA-F127-PLA)	Ring opening polymerization of the monomer L-lactide using pluronic copolymer F127 as the initiator and Sn(Oct)2 as the catalyst	Adding polymer solution drop-wise to insulin solution	N/A	0.3 g/g polymer	–	[57]
Nanosphere	Human insulin	Vitamin B12 – extran	Emulsification	Adding insulin solution to lyophilized nanoparticle	150 – 300 nm	45 – 70%	–	[133]
Microsphere	Crystalline recombinant human insulin	Crosslinked poly(methacrylic acid) and poly(ethylene glycol)	UV initiated free radical solution polymerization of methacrylic acid and poly(ethylene glycol) monomethylether monomethacrylate	Imbibing dried microparticles in insulin solution	300 µm (max)	86.4% (min)	Microparticles were produced by crushing dried copolymer and sieving	[59,129,130]
Microsphere	N/A	Hydrogel‡	Inverse suspension polymerisation	Incubating microparticles with insulin solution	N/A	70 mg/g dry microparticles	–	[131]
Nanosphere	Human insulin	Polymethacrylic acid – chitosan – polyether	Free radical polymerization of methacrylic acid, chitosan and polyether	Diffusion filling§	500 – 800 nm	86%	To improve insulin stability, it was complexed by βcyclodextrin	[60]
Microsphere	Human zinc insulin	Cross linked chitosan	Emulsification	Remote loading	20 – 40 µm	> 60%	Ascorbyl palmitate or ascorbic acid was used as the cross linking agent	[61]
Nanosphere	Bovine insulin	poly(*N*-isopropyl acryl amide-co-poly(ethylene glycol) 1000 methacrylate)	Free radical dispersion polymerization	Adding insulin solution to the nanoparticle dispersion	N/A¶	65%	–	[58]

*Vesicular nanoparticles as mentioned by the authors.

‡F127-LA2-DA means a copolymer of Pluronic F127, two lactic acid and di-acryloyl groups.

§Keeping an amount of nanoparticles in remote loading medium consisting of insulin.

¶Leobandung *et al.* considered three nanoparticle sizes to study the effect of size.

an acidic insulin solution to a number of microparticles. They were then encapsulated in PLGA using a common emulsification technique. The average particle size of 20 – 25 μm and an encapsulation efficiency of more than 87% were reported. *In vivo* experiments performed on diabetic rats demonstrated that 10 days could be an appropriate dosing schedule.

2.5 Phase inversion

Phase inversion micro, nano-encapsulation is another method that has been used to encapsulate insulin. During this technique, micro, nanospheres are fabricated by the spontaneous phase inversion of dilute polymer solutions, which are then quickly dispersed into an excess of non-solvent for the polymer. As solvent leaves the polymer solution and enters the bulk non-solvent phase, polymer instantaneously precipitates into the form of micro, nanospheres.

Carino, Jacob and Mathiowitz [63] used phase inversion nano-encapsulation (PIN) for insulin encapsulation. According to their report, Zn-insulin was dissolved in Tris – HCl and a portion of that was recrystallised by the addition of 10% ZnSO. The precipitate was added to a polymer solution of polyester in methylene chloride. A number of different polymers were used: PLA, PLGA, poly-fumaric anhydride-co-sebacic anhydride (P [FA:SA]), fumaric acid oligomers (FAO) and sebacic acid oligomers (SAO). The final mixture of Zn-insulin, polymer and methylene chloride was emulsified, by agitating and sonication, and dispersed in 1 L of petroleum ether, which resulted in the spontaneous formation of nanoparticles. In general, PIN produced particles with a diameter of less than 5 μm. (Particle size distribution has not been reported. Using the word 'nanosphere' is based on the original article.) Based on their studies, insulin maintained its biological activity at least over 6 h. It was found that the specific formulation which would be able to reduce glucose levels in normal fasted rats was Zn-insulin in FAO: PLGA in the presence of iron oxide.

Furtado *et al.* [64] fabricated zinc insulin microspheres by implementing a few modifications to the above method. In this procedure the suspension of zinc insulin and methylene chloride was sonicated to break any formed agglomerate, followed by adding P (FA:SA) and mixing. This suspension was, subsequently, poured into a bath of petroleum ether. Microspheres diameter was less than 5.9 μm. It was demonstrated that insulin preserved its bioactivity.

2.6 Other chemical methods

Huang and Wang [65] prepared insulin microparticles for pulmonary delivery by incorporating it into liposomes. Two methods were employed; the film shaking method and the membrane destabilizing/detergent dialyzing technique. In the film shaking method, lipid film was formed at the bottom of a rotary vacuum evaporator, followed by adding an aqueous citric buffer solution of insulin. After mechanical shaking, multilamellar vesicles were formed. In the membrane destabilizing/detergent dialyzing method, mixed lipid – detergent micelles were prepared, aqueous citric buffer solution of insulin was added slowly, under gentle stirring, and detergent was eliminated by dialysis below its critical micellar concentration. A higher encapsulation efficiency was obtained by the membrane destabilizing method. An encapsulation efficiency of 52% and a particle size of 1μm were reported.

Zhenqing *et al.* [66] encapsulated insulin into Sanguis Draxonis (a Chinese traditional herb) nanoparticles by the deposition technique. In this method, a solution of Sanguis Draxonis in ethanol was added slowly to a mixture of insulin, Dextran-70 and Tween-20 under gentle agitation. Nanoparticles were subsequently filtered, centrifuged and lyophilized. The mean particle size and insulin encapsulation efficiency were reported as 184 nm and 70% respectively.

With the melt dispersion technique the lipid of the matrix is melted, the insulin incorporated into the lipid melt and homogenization is carried out at a temperature above the melting point of the lipid and, therefore, the product can be regarded as an emulsion. Solid particles are expected to be formed by the subsequent cooling of the emulsion to room temperature or below [67]. Reithmeier, Herrmann and Gopferich [18] used this method and reported a particle size of 95 μm.

Insulin nanoparticles with the size of smaller than 170 nm were prepared by the nanoprecipitation method [68]. In this process, a mixture of acidic aqueous insulin solution and a solution of encapsulating polymer (PLGA) in an organic solvent (acetone) was poured into the aqueous solution of a high HLB surfactant under moderate stirring. Nanoparticles were immediately formed and acetone was removed by roto-evaporation under reduced pressure.

3. Physicochemical methods

3.1 Sol – gel processes

Alginate is a well-known naturally occurring biocompatible and biodegradable linear anionic polysaccharide extracted from brown seaweed. When sodium alginate encounters divalent cations such as Ca^{2+}, the ionotropy effect occurs between $Ca^{2+}$and Na^{+}, resulting in the formation of an elastic hydrogel. The kinetic of the gel formation is usually very fast. This unique property of sodium alginate, transformation from sol to hydrogel, causes many water molecules to be physically held inside. This is of importance for the maintenance of bioactivity by providing an aqueous environment to the entrapped substances [69]. It is also possible to obtain an alginic acid gel by lowering the environment pH value [69,70].

However, alginate particles are very porous and allow fast and easy diffusion of water and other fluids in and out of the alginate matrix [71]. This leads to low encapsulation efficiency and rapid drug release. In order to optimise the

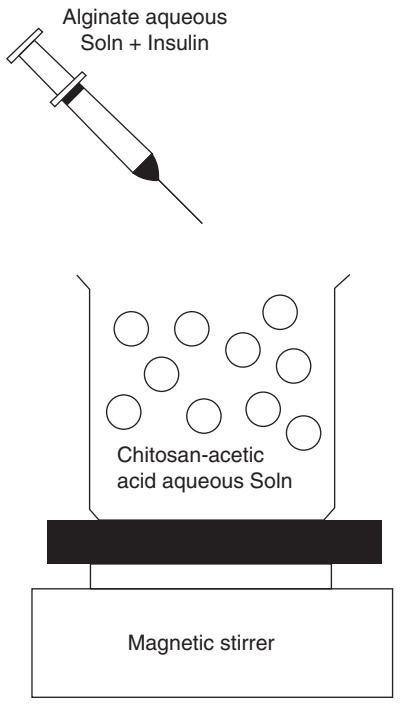

Figure 2. Schematic illustration of the external gelation.

modulation of drug release from alginate systems, its mechanical stability (its tendency to deform) must be improved and its resistance to erosion in different organic fluids must be increased. Therefore, several polymers such as chitosan, dextran sulfate, lectin, etc are used, in combination with sodium alginate.

Chitosan is a biocompatible, biodegradable, bioadhesive and non-toxic polysaccharide [72]. It can also be effective as an intestinal absorption enhancer [6,73]. Chitosan is able to establish ionic interactions with the alginate gel systems that, in turn, become more resistant and suitable [70,72].

Dextran and its derivatives are another promising candidate for the preparation of networks capable of giving a sustained release of proteins [70]. Dextran sulfate is a negatively charged polymer that shows a high affinity for proteins.

Chitosan – alginate, dextran sulfate – alginate and chitosan – dextran sulfate – alginate insulin microspheres have been conventionally prepared by external gelation. The basic set-up for microencapsulation is shown in **Figure 2**. Briefly, the alginate/insulin solution or alginate/insulin/dextran sulfate solution is extruded drop-wise through a needle into the gelation medium (calcium chloride or calcium chloride – chitosan solution) while stirring [74,75].

Martins *et al.* [74] demonstrated that chitosan – dextran sulfate – alginate insulin and dextran sulfate – alginate insulin particles provide higher retention of insulin than pure alginate matrices and than chitosan – alginate insulin particles. The insulin release from particles was investigated in simulated gastric and intestinal pH conditions. A slow insulin release in acidic medium was observed, for all of their particle types. At higher pH value, chitosan – alginate, dextran sulfate – alginate and chitosan – dextran sulfate – alginate had the highest to lowest insulin release respectively, after 24 h of assay. Mean particle size was of the order of 1 – 2 mm and encapsulation efficiency was more than 90%; no *in vivo* experiments were reported. For additional hardening of the alginate, Onal and Zihnioglu [75] used glutaraldehyde as a crosslinking agent. Glutaraldehyde treatment was performed through soaking the chitosan – alginate particles in a solution of acetate buffer, calcium chloride and glutaraldehyde, stirring, for 1 h. It was observed that glutaraldehyde treatment slowed down the release of insulin. No *in vivo* experiments were reported.

Cyclodextrins (CD) are non-reducing cyclic oligosaccharides consisting of six (α-CD), seven (β-CD) or eight (γ-CD) dextrose units. CDs have a 'doughnut' shape, with the interior of the molecules being relatively hydrophobic and the exterior being relatively hydrophilic. Because of this unique chemical structure, CDs are capable of forming complexes with many drug molecules [76]. CD-complexed drugs have higher bioavailability in the *in vivo* systems and are more stable than the drug by itself [77].

Moses *et al.* [78] prepared a complex of insulin with β-CD simply by mixing insulin solution and β-CD and further agitation. This complex was used for fabricating chitosan – alginate particles by an external gelation method. It was observed that longer complexation time led to faster release. Preliminary *in vivo* studies demonstrated a decrease in blood glucose level upon oral administration of the particles to rabbits.

Lectins are proteins of non-immune origin that recognise and bind to specific carbohydrate structural sites without modifying them. The most fully characterised groups of lectins are those of plant origin because they are frequently hydrophilic and produced in large amounts [79]. Chemical conjugation to lectins enhances the bioadhesiveness of drug delivery particles [80].

Piezoelectric ejection is a process for spraying a polymer solution through a small orifice which is vibrated by a piezoelectric transducer. This technology is widely used in ink jet printers. The advantage of piezoelectric actuation is the precise pressure control that results in monodisperse satellite free drop production and dynamic alteration of the diameter of ejected drops [81].

Kim *et al.* [82] prepared lectin – alginate insulin microspheres using external gelation. Wheat germ agglutinin (WGA) lectin was used. A piezoelectric ejection process was utilized to extrude drop-wise an alginate and insulin in NaOH aqueous solution into $CaCl_2$ solution. Lectin was conjugated to alginate microparticles subsequently by activating hydroxyl groups with carboyldiimidazole in an aprotic solvent. The average diameter of alginate microparticles was in the range of 60 – 80 μm. Microparticles were

administrated to diabetic rats orally. A greater hypoglycemic effect was observed with alginate – WGA microparticles than with pure alginate microparticles. Their results showed that alginate – WGA microparticles enhanced the intestinal absorption of insulin.

External gelation has several inconveniences, namely, the limitation in reducing microsphere diameter and the teardrop shape of the produced microparticles [69]. The ease of its scale-up is controversial. Some researchers believe that this process can easily be scaled up using multiple syringe arrays [69]. Others believe that as easy as it is to make a small batch using a syringe extruder and a stirred bath, the scaling up of the process is very difficult and the processing costs are large [71]. The structure and property of the microcapsules, such as permeability, biocompatibility and mechanical strength, can be controlled by the purity of alginate and the processing parameters [69].

Chitosan – alginate nanoparticles are prepared by ionotropic pregelation. This procedure is illustrated in **Figure 3**. In this process, calcium solution is added drop-wise and slowly to alginate/insulin solution while stirring. After pregel formation, chitosan solution is dropped into pregel over a long time.

The mean particle size and encapsulation efficiency of chitosan – alginate nanoparticles prepared by Sarmento *et al.* [83-87] was over 700 nm and around 70%, respectively. Based on their studies, insulin release was pH dependent. Also orally delivered nanoparticles in rats lowered basal serum glucose levels significantly.

Internal gelation is an alternative method to produce microspheres. In this method, an alginate/insulin solution containing an insoluble calcium salt is dispersed into oil, in the presence of a low HLB surfactant [88]. After homogenisation, gelation is induced by the further addition of a mixture of oil and an oil-soluble acid that causes calcium ion release.

Reis *et al.* [89,90] used this method to produce microspheres. A mean particle size of 4.2 µm and an encapsulation efficiency of 80% were reported [89]. Reis *et al.* [90] prepared alginate – dextran sulfate microspheres (in the original article, 'nanoparticle' was used, despite the particles size being in the micron range) with this method by using an alginate – dextran sulfate – insulin solution. The smallest obtained particle size was around 2 µm and the encapsulation efficiency was over 71%. The effect of alginate degree of gelation on the retention of protein inside microspheres was studied and an optimal calcium concentration was provided.

Silva *et al.* [91] prepared microspheres with the mean diameter ranging from 21 – 287 µm and an encapsulation efficiency of 75% by internal gelation method. It was demonstrated that an increase in surfactant concentration or increasing homogenisation speed resulted in a significant decrease in microspheres' mean size, but no effect on insulin encapsulation efficiency. However, increasing alginate concentration led to an increase in the particle size, without any significant changes in insulin encapsulation efficiency. Their microencapsulated insulin produced the same bioavailability in diabetic rats as short-acting insulin formulation and insulin preserved its integrity during the whole process.

Microparticles produced by the internal gelation method have more homogeneous but less dense matrices with larger pore sizes. Despite their homogeneity, the internal gelated matrices are more permeable, resulting in lower encapsulation efficiencies and faster release rates [92]. Considering the ease of scale-up, internal gelation technology may be a better way for large-scale production of alginate microparticles.

The use of complexation between chitosan and oppositely charged macromolecules (other than alginate) to prepare microspheres has also attracted attention. Tripolyphosphate (TPP) is a polyanion, which can interact with the cationic chitosan by electrostatic forces [93]. Insulin-loaded chitosan nanoparticles are prepared by mixing insulin with TPP solution and then adding the produced solution through a syringe needle to chitosan acidic solution under constant stirring in the presence of a high HLB surfactant.

Pan *et al.* [94] prepared chitosan – TPP – insulin nanoparticles, using the above-mentioned procedure. The particle size was in the range of 100 1000 nm and the insulin association was in the range of 50 – 92%. The effects of various particle sizes were investigated *in vivo*. It was reported that particles with a middle size range (350 nm) showed better performance *in vivo*.

In another report, insulin solution (adjusted pH to 7.2) was dropped into the chitosan solution and then TPP solution was gently added to the system, without any surfactant [95]. According the latter report, insulin nanoparticle size was in the range of 2 – 5 µm and encapsulation efficiency was less than 35%. Boonsongrit *et al.* [95] observed that microparticles maintained immune activity of human insulin.

TPP/chitosan micro, nanoparticles formed have poor mechanical strength [93,96], but the process is very simple and mild.

3.2 Membrane emulsification

In most microencapsulation techniques, it is difficult to control the size of particles and their size distribution is very broad. This disadvantage brings some limitations in applications: i) poor reproducibility of particles among batches, which will result in poor repeatability of the release behavior and efficacy of the drug among doses; and ii) low bioavailability of the drug because the accumulated locations of the particles containing drug depend on their size [97]. Furthermore, micro, nanoparticles with a narrow size distribution are necessary in the drug delivery system in order to precisely determine the dose of the drug. Therefore, it is worthwhile to develop a methodology for controlling release kinetics employing monodisperse particles.

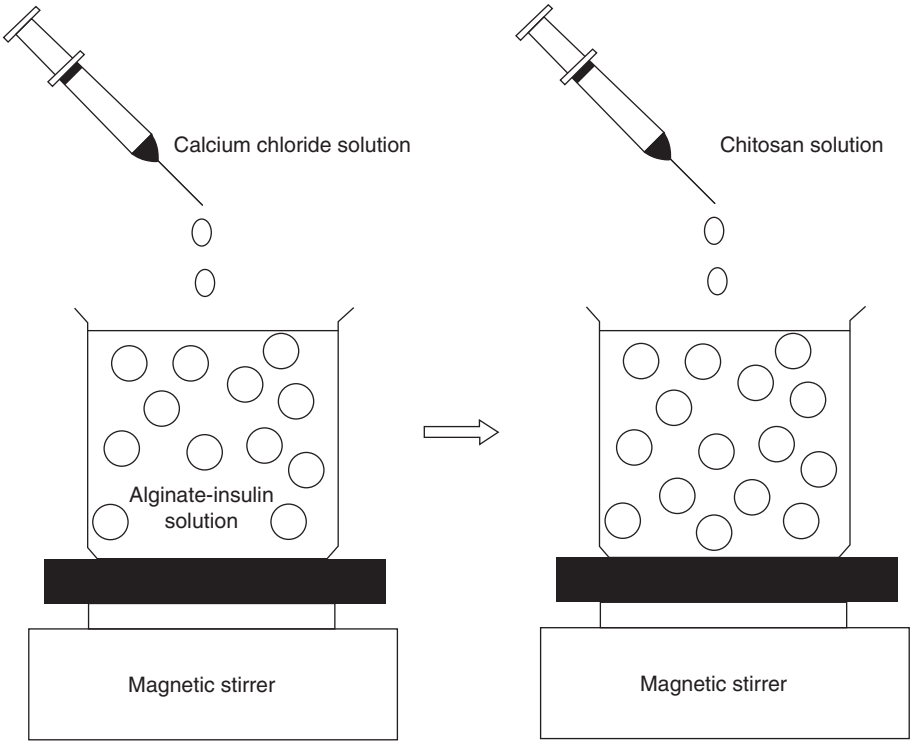

Figure 3. Schematic illustration of the external gelation for producing nanoparticles.

Membrane emulsification has been introduced as an emulsification technique based on microporous membranes with uniform pore size. The distinguishing feature is the better control of droplet size primarily by the choice of the membrane. The apparent shear stress is lower than common emulsification systems and the process is mild. Besides the possibility of using shear-sensitive ingredients, emulsions with narrow droplet size distribution can be produced. Furthermore, membrane emulsification processes allow the production of emulsions at lower energy input [98]. A notable disadvantage of membrane emulsification is the low maximum disperse phase flux through the membrane caused by the low hydraulic permeability of most of the membranes used. However, the flux of dispersed phase could be increased by using a membrane with a low hydraulic resistance [99].

Membrane emulsification operations are divided into two methods; cross-flow or direct membrane emulsification and premix membrane emulsification.

In cross-flow or direct membrane emulsification, the disperse phase is pressed through a microporous membrane while the continuous phase flows along the membrane surface. When the pressure of the disperse phase is high enough, droplets grow at pores and detach after growing up to critical size, which is determined by the balance between the forces acting on the droplet, mainly drag force and the interfacial tension force [99]. Shear stress is generated at the membrane/continuous phase interface by recirculation of the continuous phase using a low shear pump or by agitation in a stirring vessel to ensure a regular droplet detachment from the pore outlets [100]. The droplet formation in membrane emulsification is influenced by numerous process parameters, among them: wall shear rate or transmembrane pressure, wetting properties of the membranes, pore size of the membrane and wall shear stress [101]. Due to the low productivity, that is long production times, cross-flow or direct membrane emulsification is more suitable for the preparation of relatively diluted emulsions [100].

In a premix method of operation, droplets of a coarse pre-emulsion are pushed through the pores of a microporous membrane. Upon the passage of the coarse droplets through the membrane the droplets are deformed and disrupted by the flow of the emulsion through the pore structure of the membrane. This results in the formation of a fine emulsion with a narrow droplet size distribution. The primary emulsion is made by mixing the two immiscible liquids together using conventional homogenization methods. The resulting droplet size distribution is slightly wider than those obtained with cross-flow or direct membrane emulsification [99].

Liu *et al.* [102,103] prepared insulin-loaded PLA [103] and PLA/PLGA [102] microcapsules by premix method of membrane emulsification technique. In their report, an acidic solution of insulin mixed with a solution of PLA [103] or PLA/PLGA [102] and a low HLB surfactant in the mixture of methylene chloride and toluene, in a high speed mixer. This primary emulsion was subsequently permeated

through the uniform pores of a micro-porous glass (MPG) [102] (with different pore sizes) or Shirasu Porous Glass (SPG) [103] membrane (pore size: 2.8 μm) into the outer phase, aqueous PVA solution. The next steps for harvesting microparticles are similar to the end stages of the emulsification technique. Various factors were investigated that could influence the encapsulation efficiency and cumulative drug release. By optimizing the preparation process, the highest achieved insulin encapsulation efficiencies were 71 [103] and 92% [102].

In another report, a sol – gel process was combined with membrane emulsification. Wang *et al.* [104] dissolved insulin in chitosan – acetate buffer solution and used it as the dispersed phase. This dispersed phase was pressed through the pores of the membrane into a continuous phase, comprising of liquid paraffin and petroleum ether. The obtained emulsion was gelated by dropping TPP aqueous solution. Glutaraldehyde saturated toluene was dropped to crosslink the gel. The highest reported encapsulation efficiency was 77%.

The main advantage of the premix method is that a fine emulsion can be easily prepared from a low concentration coarse emulsion at high rates [100]. The process parameters are the membrane used for the production of the fine emulsion, the properties of the coarse emulsion, applied pressure, dispersed phase fraction and process conditions applied during the production of the coarse emulsion [101].

3.3 Coacervation

Coacervation is generally considered suitable for micro-encapsulation of water soluble compounds, as satisfactory loading levels and efficiencies can be achieved [105]. In one version of this method (not used for insulin encapsulation), host particles are dispersed in a solution of coating material at a high temperature and the temperature of the dispersion is step-wise decreased, while the dispersion is stirred. Due to the cooling effect, the polymer precipitates onto the surface of the host particles [34].

Apart from the above-mentioned procedure, two methods for coacervation are available: simple and complex processes. The mechanism of microcapsule formation for both processes is identical, except for the way in which the phase separation is carried out. In simple coacervation a desolvation agent is added for phase separation [12]. In this method microparticles are produced by dispersing either the solid crystal particles or an aqueous solution of the drug in an organic solution of polymer, followed by a phase separation by adding a second organic solvent in which the polymer is not soluble ('non-solvent'). The particle formation initially proceeds with an increase in size until a stable size is reached, after which the number of particles gradually increase with increasing desolvation [106]. The addition of a large volume of the non-solvent completes the extraction of the polymer solvent and hardens the microspheres [14].

Complex coacervation involves complexation between two oppositely charged polymers. It is carried out by mixing two oppositely charged polymers in a solvent. In this method core material is dispersed into a polymer solution. The second polymer (oppositely charged to the former) solution is then added to the prepared dispersion. Deposition of the shell material onto the core particles occurs when the two polymers form a complex [12].

Graves, Makoid and Jonnalagadda [107] used simple coacervation to produce insulin microcapsules with an encapsulation efficiency of more than 87%. Ethylcellulose and dichloromethane were used as polymer and as the organic solvent respectively. *N*-hexane was used as 'non-solvent'. The effect of adding cyclodextrins to insulin solution and its role for enhancing insulin thermal stability and solubility in solution were investigated.

Sarmento *et al.* [108] utilised a complex coacervation method to produce insulin nanoparticles. According to their report, nanoparticles were obtained by complexation between dextran sulfate and chitosan, performing by drop-wise addition of chitosan aqueous solution to dextran sulfate and insulin aqueous solution under magnetic stirring, followed by additional mixing. The produced particles had a mean diameter of 500 nm and an encapsulation efficiency of 85%. Meanwhile the insulin released from their nano-particles in simulated intestinal pH medium (without enzymes) kept its bioactivity for 24 h.

The coacervation process is influenced by drug : polymer ratio, pH changes, temperature changes, volume, type of non-solvent, the polymer solvent and the stirring rate [12,14]. The particles produced by this method have a wide size distribution and irregular shapes. They also highly tend to agglomerate [14].

3.4 Layer-by-Layer

Layer-by-Layer (L-b-L) is a robust and widely used method to deposit a desired number of nanometre thin layers of polyelectrolyte on a large area substrate. Core-shell particles are prepared using colloidal particle as the core material that serves as a template onto which polycation and polyanion polyelectrolyte multi-layers are made by immersing the substrate, alternatively, in positively and negatively charged polyelectrolyte solutions. Each exposure deposits a reproducible quantity of polyelectrolyte on the surface and reverses the surface charge that is critical for the depositing of the next layer. The driving force for polyelectrolyte deposition on the substrate is the electrostatic attraction and the hydrophobic nature of polymer – polymer contact. By this method, hollow capsules of organic, inorganic or hybrid particles can be obtained by dissolving the core material [12,109].

Ye *et al.* [110] prepared insulin microcapsules, using melamine formaldehyde microparticles (diameter 2.1 μm) as templates, and assembled alginate and chitosan on them followed by removal of the template through dissolving the core particles in hydrochloric acid. Microcapsules were

loaded with insulin by dispersing and incubating in insulin solution. The insulin release in simulated gastric and intestinal pH conditions was investigated. A very slow insulin release in acidic but a much faster release in neutral medium was observed.

Fan *et al.* [111] encapsulated insulin nano-aggregates (particle size of 100 – 250 nm) by L-b-L adsorption of synthetic poly (α, β-L-malic acid) and chitosan. Insulin nano-aggregates were prepared by dissolving insulin in hydrochloric acid solution, followed by adding NaCl powder or aqueous solution. Both insulin nano-aggregates and insulin nanoparticles were exposed to ultrasonication. The reported insulin encapsulation efficiency was less than 85%. Release studies revealed a very low insulin release in acidic medium, but a significant increase in insulin release was observed at pH 7.4. Also a burst effect was observed, which could be avoided by increasing the number of deposited layers.

This technique is both versatile and simple. The multi-layer film thickness can be controlled precisely by varying the total number of layers deposited, the ionic strength of polyelectrolyte solution and the solvent quality. This process may also provide exquisite control over the structure of the micro, nano-particles formed by controlling the polymer nature and the order of coating. One of the major drawbacks of this method is forming primary aggregates that were further assembled to form microscopic structures as a result of coating. It can be prevented by sonication to obtain uniform micro, nanoparticles [106,109]. The concentration of solution, molecular weight of the polyelectrolyte, time for deposition of polyelectrolyte layers and drying steps after washing do not influence the thicknesses of deposited layers appreciably but it is highly influenced by the ionic strength [109].

4. Physico-mechanical methods

4.1 Spraying techniques

With the spray drying method the drug is dissolved or suspended in the organic polymer solution and sprayed, through a nozzle, into a hot chamber. The shell material solidifies onto the core particles as the solvent evaporates such that the microparticles obtained are of polynuclear or matrix type [12]. Microparticle characteristics and size are specified by equipment design (size of nozzle), operating conditions (inlet air temperature) and the process variables (spray flow rate, atomisation pressure) [93,112]. Micro-encapsulation by spray-drying is low cost. It is a one-step process, with the possibility of being free of organic solvent and it is easy to control and scale up [112]. With this technique both water soluble and insoluble compounds can be incorporated into the spheres.

Insulin microspheres were prepared by spray-drying different feeding liquids containing insulin and PLGA. All the tested formulations were able to conserve insulin chemical and conformational stability. Particles mean diameter was in the range of 12 – 28 μm [113]. Different additives were investigated and the performance mechanism of CDs as modulator of insulin release rate was studied [114].

To improve the manufacture of insulin-encapsulated products, Whitaker *et al.* [115] mixed the dry powder insulin and PLA under supercritical CO_2 conditions before spraying it. Particle size range was between 10 – 300 μm, and biological assays of insulin confirmed that insulin kept its biological activity.

Spray-drying is, however, associated with some drawbacks; aggregation of encapsulated particles, non-encapsulated particles (if a large amount of core material is used), degradation of heat sensitive or oxidation sensitive drugs and needle-shaped crystal or fibre formation [12,14]. Meanwhile the choice of organic solvent is also important; it must evaporate quickly in the heated air in the drying phase.

To increase protein stability and to incorporate it in to the formulation process in solid form, microparticles are prepared by spray congealing process. In this technique, a heating source is attached to the spraying nozzle and droplets are formed by atomizing, which solidify upon cooling.

This technique was used for preparing lipid micro particles loaded with insulin. Maschke *et al.* [116] reported 77 – 84% of encapsulation efficiency and a mean particle size of 182 μm. It was shown that insulin stability was not affected during the preparation process. Based on the insulin release studies, a long-term (28 days) with a very small burst effect was observed.

Electrospraying (electrohydrodynamic spraying) is a method of liquid atomization by electrical forces. It is a process of simultaneous droplet generation and charging by means of an electric field. In this process, liquid flowing out from a capillary nozzle maintained at high potential is subjected to an electric field, which causes elongation and disruption of the jet of liquid into droplets.

With electrospraying, no additional mechanical energy, other than that from the electric field alone, is needed for liquid atomization. The equipment used for electrospraying is inexpensive, can operate at atmospheric conditions and the rate of particle production is easy to control by adjusting voltage and flow rate. Droplets produced by electrospraying are highly charged, that prevents their coagulation, and promotes self-dispersion. Droplets have size smaller than those available from conventional mechanical atomizers. The charge and size of the droplets can be controlled to some extent by voltage and flow rate. The size distribution of the droplets is usually narrow [117].

Monodispersed insulin doughnut-shape particles with characteristic dimensions around 100 nm were generated using this method [118]. The process involved dissolving insulin powder in a pH-adjusted (using HCl) solution of ethanol and water, followed by electrospraying the solution

and collecting the dry residue after solvent evaporation. Further experiments showed that this process did not affect the biological activity of insulin.

The main restrictions within this method are low throughput, its high sensitivity to the liquid physical properties and the electric field in the vicinity of the emitter tip and control mode of spraying [117].

Spray freezing into liquid (SFL) is a another particle engineering process developed for the production of microparticles and the encapsulation of drugs into water soluble polymeric matrix by atomizing an aqueous feed solution beneath the surface of a cryogenic liquid. The frozen particles are collected and lyophilized to obtain dry, free flowing micronized powders. Organic solvents and hydro-organic mixtures that have suitable freezing points and higher vapour pressures than water, that leads to faster sublimation rates and decreasing drying time may also be used to prepare drug and carrier dispersions. Liquid CO_2 and liquid N_2 are the preferred cryogenic liquids because of their relative inertness and physical properties such as density and viscosity, which vary significantly with pressure and temperature [119-121].

Yu *et al.* [120] have used the SFL process to produce microparticles containing insulin. In their experiment, aqueous solution of bovine insulin alone or with tyloxapol, lactose or trehalose (as cryoprotectant molecules) was sprayed into liquid nitrogen through a capillary nozzle. In order to avoid the formation of ice crystals, feed solution containing insulin must pass through the critical temperature zone very rapidly (the critical temperature zone is defined as the temperature range between crystallisation and the glass transition temperature of the solution). The mean diameter of microparticles was 5 – 7 μm and the physicochemical properties of insulin was preserved during the process [120].

The size and the porosity of the microparticles are dependent upon the velocity of the spraying, the diameter of the nozzle, the temperature and flow rate of the cryogenic liquid. Crystalline properties and encapsulation efficiency are influenced by the processing conditions [121].

4.2 Other physico-mechanical techniques

Gas anti-solvent process (GAS) is a supercritical-based process to produce submicron particles. This process is for recrystallizing solid compounds that are not soluble in supercritical fluids. The technique is especially suitable for polymers because the majority of polymers are not soluble in supercritical fluids or gases [122].

This process was used to produce insulin nanospheres. The PLA was dissolved in a methylene chloride and subsequently it was dropped into an insulin or insulin/poly (ethylene glycol) (PEG) solution in dimethyl sulfoxide. Insulin/polymer solutions were atomized through a small nozzle into a high-pressure vessel. The CO_2 was injected in co-current mode from the top of the vessel. Continuous feeding of compressed CO_2 would cause the precipitation and polymer particles were collected at the bottom of the vessel. A mean particle size of less than 685 μm and an encapsulation efficiency of less than 90% was reported [123].

Alvarez *et al.* [124] employed surface acoustic waves, which are nanometer order amplitude elastic waves that propagate along the surface of a piezoelectric substrate, to produce insulin aerosols with the droplet size of 15 – 200 nm. This procedure is rapid, single-step and straightforward.

Merisko-Liversidge *et al.* [125] prepared a nanoparticulate insulin formulation using a wet milling process. Nanoparticles were obtained after milling zinc – insulin, in the presence of a stabilizer system, F68® and sodium deoxycholate in water, at neutral pH, in a roller mill jar. The particle size was around 114 nm after 18 h of milling. To evaluate the biologic activity of the insulin nanoparticulate, blood glucose and insulin levels were monitored in a hyperglycemic rat model. The nanoparticles were effective in lowering blood glucose levels throughout the time course of the study.

5. Conclusion

Various methods of formation of insulin micron and submicron particles are reviewed here. In most micro, nanoencapsulation processes, the shell material is dissolved or suspended in an appropriate solvent in order to facilitate the formation of a uniform and complete insulin coating, followed by crosslinking of the shell material or by drying of the solvent, which leads to the formation of a continuous barrier around it. Any type of triggers can prompt the insulin release, such as pH changes, temperature and enzymatic activity. The evolution of these preparation methods has been marked by the need for less toxic reagents, good release behaviour and optimization to improve insulin stability, size distribution and encapsulation efficiency. It seems that the multiple emulsion technique is the most used technique and PLGA is the most promising polymer for insulin encapsulation. Despite considerable research efforts and impressive progress made in recent years, feasible polymer particulate systems for insulin oral delivery still remain open to research and debate.

Insulin microencapsulation needs skilful monitoring and highly controlled reaction parameters to produce functional materials. From a technologic perspective, a better understanding of controlling parameters will be greatly beneficial. The future success of biodegradable oral micro, nanoparticles will primarily depend on the commitment of immunologists, biochemists, pharmacologists, physiology specialists and industry to develop new, efficient and cost-effective strategies to orally deliver insulin.

6. Expert opinion

There are numerous preparation methods available for producing insulin micro, nanoparticles, and important

Table 5. Processes evaluation from the viewpoint of industrial application.

Method	Simplicity of procedure	Ease of scale-up	Encapsulation efficiency	Particle size	*In vivo* experiments
Emulsification	High	Low‡	High	Micron	Yes
Interfacial polymerization	Low	Low‡	High	Sub-micron	Yes
Polymer complexation*	–	–	High	Micron	Yes
Phase inversion	Medium	Low‡	N/A	Micron	No
Reverse micellar	High	Low‡	High	Sub-micron	No
Sol – gel encapsulation	High	Low or High§	High	Micron and sub-micron	Yes
Layer-by-Layer assembly	Low	Low	High	Micron and sub-micron	No
Membrane emulsification	Medium	High	High	Micron	No
Coacervation	Medium	High	High	Sub-micron	No
Spraying	High	High	N/A	Sub-micron	No

*Depending on the process for the production of polymer particulates, the process can be simple or complicated.
‡Due to high energy consumption.
§Ease of scaling up is controversial. Please refer to section 2.1.

technological advances have been achieved. Simple, safe and reproducible techniques are now available to prepare insulin-loaded micro, nanospheres and micro, nanocapsules. The most important methods for the preparation of micro, nano-particulate insulin carriers, together with their advantages and disadvantages, are summarized in **Table 5**. Efficient drug entrapment and transition to the large scale are of utmost importance to industrial applicability. Some of these processes have little industrial relevance because of extremely high cost-in-use, difficult scale-up and/or narrow applicability range. However, some of these processes stand out as being promising, sensible and likely to be scaled up in the near future. Among them, those processes that have passed *in vivo* experiments look a few steps closer.

The discovery of new drug substances for the treatment of diabetes continues at a rapid pace and is yielding promising new formulations. However, an improved therapy may not make development economically feasible. A bioengineered solution, such as islet transplantation [126-128] or artificial pancreas, eliminating the need for medication entirely, would alleviate patient compliance issues. This approach is the closest way to 'cure' diabetes; however, it is still in the formative stages. Therefore, in the treatment of diabetes, drug delivery technologies will continue to play a critical role.

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Declaration of interest

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