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Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles

Catarina Pinto Reis, PhD,[a] Ronald J. Neufeld, PhD,[b,*]
António J. Ribeiro, PhD,[c] Francisco Veiga, PhD[a]

[a]*Laboratório Tecnologia Farmacêutica, Faculdade de Farmácia, Universidade de Coimbra, Coimbra, Portugal*
[b]*Chemical Engineering Department, Queen's University, Kingston, Ontario, Canada*
[c]*Departamento Tecnologia Farmacêutica, I.S.C.S.N., Gandra, Paredes, Portugal*

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Abstract Polymeric nanoparticles have been extensively studied as particulate carriers in the pharmaceutical and medical fields, because they show promise as drug delivery systems as a result of their controlled- and sustained-release properties, subcellular size, and biocompatibility with tissue and cells. Several methods to prepare nanoparticles have been developed during the last two decades, classified according to whether the particle formation involves a polymerization reaction or arises from a macromolecule or preformed polymer. In this review the most important preparation methods are described, especially those that make use of natural polymers. Advantages and disadvantages will be presented so as to facilitate selection of an appropriate nanoencapsulation method according to a particular application.

Key words: Drug delivery; Nanoparticles; Preparation methods

Nanoencapsulation of drugs involves forming drug-loaded particles with diameters ranging from 1 to 1000 nm. Nanoparticles are defined as solid, submicron-sized drug carriers that may or may not be biodegradable [1,2]. The term nanoparticle is a collective name for both nanospheres and nanocapsules. Nanospheres have a matrix type of structure. Drugs may be absorbed at the sphere surface or encapsulated within the particle. Nanocapsules are vesicular systems in which the drug is confined to a cavity consisting of an inner liquid core surrounded by a polymeric membrane [1]. In this case the active substances are usually dissolved in the inner core but may also be adsorbed to the capsule surface [3]. Nanoparticles are receiving considerable attention for the delivery of therapeutic drugs. The literature emphasizes the advantages of nanoparticles over microparticles [4] and liposomes [5,6]. The submicron size of nanoparticles offers a number of distinct advantages over microparticles, including relatively higher intracellular uptake compared with microparticles. In terms of intestinal uptake, apart from their particle size, nanoparticle nature and charge properties seem to influence the uptake by intestinal epithelia. Uptake of nanoparticles prepared from hydrophobic polymers seems to be higher than that of particles with more hydrophilic surfaces [7], thus more hydrophilic particles may be rapidly eliminated. Generally, nanoparticles based in hydrophobic polymers such as poly(styrene), uncharged and positively charged, provide an affinity to follicle-associated epithelia as well as to absorptive enterocytes, whereas negatively charged poly (styrene) nanoparticles show only low affinity to any type of intestinal tissues. In contrast, nanoparticles based on hydrophilic polymers, negatively charged, show a strong increase in bioadhesive properties and are absorbed by both M cells and absorptive enterocytes. A combination of both nanoparticle surface charges and increased hydrophilicity of the matrix material seem to affect the gastrointestinal uptake in a positive sense.

* Corresponding author. Chemical Engineering Department, Dupuis Hall, Queens University, Kingston, Ontario, Canada K7L3N6.
E-mail address: neufeld@chee.queensu.ca (R.J. Neufeld).

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The nanoparticle surface is also a very important consideration in targeting drug delivery. Indeed, once in the bloodstream, generally conventional nanoparticles (no surface modification) and negatively charged particles can be rapidly opsonized and massively cleared by the fixed macrophages. It is well known that the reticuloendothelial system, mainly the liver and spleen, is a major obstacle to active targeting because of its ability to recognize these systems, remove them from systemic circulation, and, consequently, avoid the effective delivery of the nanospheres to organs other than those of the reticuloendothelial system [8]. Surface modification of these nanoparticulate systems with hydrophilic polymers is the most common way to control the opsonization process and to improve the surface properties of the system, or coating modification with polymers [6] such as the attachment of poly(ethylene glycol) (PEG) chains to biodegradable polymer such as poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA). Thus the hydrophilic PEG chains allow the control of protein and peptide absorption and, in addition will allow regulation of cell behavior at the polymer surface.

Thus the polymeric composition (hydrophobicity, surface charge, and biodegradation profile) of the nanoparticles, any adjuvant substances, and the associated drug (molecular weight, charge, localization in the nanospheres by adsorption or incorporation) have a great influence on the drug absorption, biodistribution pattern, and elimination. There are now numerous preparation methods available for producing nanoparticles.

Depending on the physicochemical characteristics of a drug, it is now possible to choose the best method of preparation and the best polymer to achieve an efficient entrapment of the drug. Moreover, organic solvents assessed in this review are classified according to International Conference on Harmonization (ICH) classification according to their possible risk to human health and placed into one of three classes as follows: class 1 (solvents to be avoided), class 2 (solvents to be limited), and class 3 (solvents with low toxic potential). This classification will be crucial to evaluate the following methods and finally, to apply a specific technique to a particular pharmaceutical application.

Methods

Many methods have been developed for preparing nanoparticles; these methods can be classified into two main categories according to whether the formulation requires a polymerization reaction or is achieved directly from a macromolecule or preformed polymer [1]. The polymerization methods can be further classified into emulsion and interfacial polymerization, and there are two types of emulsion polymerization—organic and aqueous—depending on the continuous phase. Additionally, the interfacial condensation method will be further discussed later in this review. Nanoparticles can be also prepared directly from preformed synthetic or natural polymers and by desolvation of macromolecules. Recently these polymeric systems have been prepared by nebulization techniques. This review covers all these methods including their detailed procedures and technological advantages, as well as providing several examples of encapsulants that are entrapped into or adsorbed to these particles. The most important methods of nanoparticle preparation, as well as the encapsulants and the resulting approximate particle sizes, are shown in Table 1.

Nanoparticles obtained by polymerization of a monomer

Emulsion polymerization

Emulsion polymerization is one of the fastest methods for nanoparticle preparation and is readily scalable [9]. The method is classified into two categories, based on the use of an organic or aqueous continuous phase.

The continuous organic phase methodology involves the dispersion of monomer into an emulsion or inverse microemulsion, or into a material in which the monomer is not soluble (nonsolvent). Polyacrylamide nanospheres were produced by this method [10,11]. As one of the first methods for production of nanoparticles, surfactants or protective soluble polymers were used to prevent aggregation in the early stages of polymerization [12]. This procedure has become less important, because it requires toxic organic solvents, surfactants, monomers and initiator, which are subsequently eliminated from the formed particles. As a result of the nonbiodegradable nature of this polymer as well as the difficult procedure, alternative approaches are of greater interest. Later, poly(methylmethacrylate) (PMMA), poly(ethylcyanoacrylate) (PECA), and poly(butylcyanoacrylate) [13] nanoparticles were produced by dispersion via surfactants into solvents such as cyclohexane (ICH, class 2), n-pentane (ICH, class 3), and toluene (ICH, class 2) as the organic phase. Examples of drugs encapsulated with this system are triamcinolone [14], fluorescein [13], pilocarpine [15], and timolol [15].

In the aqueous continuous phase the monomer is dissolved in a continuous phase that is usually an aqueous solution, and the surfactants or emulsifiers are not needed. The polymerization process can be initiated by different mechanisms. Initiation occurs when a monomer molecule dissolved in the continuous phase collides with an initiator molecule that might be an ion or a free radical. Alternatively, the monomer molecule can be transformed into an initiating radical by high-energy radiation, including γ-radiation, or ultraviolet or strong visible light. Chain growth starts when initiated monomer ions or monomer radicals collide with other monomer molecules according to an anionic polymerization mechanism [16]. Phase separation and formation of solid particles can take place before or after termination of the polymerization reaction [17].

PMMA nanoparticles are suitable adjuvants for vaccines and are produced by a radical emulsion polymerization mechanism [18]—generally without emulsifiers [19]. The single monomer methylmethacrylate (MMA) was used to

Table 1
Examples of preparative methods, polymers, and encapsulants

Polymer	Encapsulant	Size (nm)	Reference
Nanoparticles obtained by polymerization of a monomer			
Emulsion polymerization			
Continuous aqueous phase	Influenza antigen	130	[121]
Poly(methylmethacrylate)			
Poly(methylmethacrylate) copolymers	Doxorubicin	300	[20]
Poly(methylcyanoacrylate)	Vinblastine	200–300	[122]
Poly(ethylcyanoacrylate)	Insulin	<500	[123]
Poly(butylcyanoacrylate)	Progesterone	250	[27]
Poly(isobutylcyanoacrylate)	Ampicillin	40–80	[24]
	Antibodies	120	[31]
	hGRF	140–170	[30]
Poly(hexylcyanoacrylate)	Vincamine	200	[124]
Poly(isohexylcyanoacrylate)	Ampicillin	30–80	[24]
	Doxorubicin	300	[25]
Poly(dialkylmethylidene malonate)	Primaquine	<1000	[33]
Continuous organic phase			
Polyacrylamide	Enzymes	<1000	[10]
Poly(methylcyanoacrylate)	Triamcinolone	500	[14]
	Fluorescein	800–1000	[13]
	Pilocarpine	300–600	[15]
Other polymers: Poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(styrene), poly(vinylpyridine) and poly(acroleine)			
Interfacial polymerization			
Poly(ethylcyanoacrylate)	Insulin	~151	[36]
Poly(isobutylcyanoacrylate)	Indomethacin	220240	[43,44]
	Darodipine	150	[42]
	Insulin	150–300	[40]
	Calcitonin	<1000	[11]
	Octreotide	260	[41]
Poly(isohexylcyanoacrylate)	Phthalocyanines	180	[39]
Other polymers: Polyamides, poly(phenylesters) and polyurethanes			
Interfacial polycondensation			
Polyurethane and poly(etherurethane)	α-Tocopherol	< 500	[49]
Nanoparticles obtained directly from a macromolecule or a preformed polymer			
Nanoparticles prepared with synthetic/semi-synthetic preformed polymer			
Solvent evaporation			
Poly(lactic acid)	Testosterone	<1000	[52]
	Albumin	100 or 120	[61]
	Tetanus toxoid	150	[62]
	Loperamide	~300	[53,63]
Poly(lactic acid)–poly(glycolic acid) copolymer	DNA	~100	[67]
	Cyclosporin A	~300	[66]
Other polymers: Poly(ε-caprolactone) and poly(β-Hydroxybutyrate) [60]			
Solvent displacement			
Poly(lactic acid)–poly(glycolic acid) copolymer	Indomethacin	~168	[69]
	Doxorubicin	274	[25]
	Cyclosporin A	~170	[69]
	Valproic acid	~166	[69]
	Ketoprofen	~167	[69]
	Vancomycin	~187	[69]
	Insulin	~105–170	[69]
Poly(lactic acid)	Doxorubicin	270	[25]
	Taxol	~260	[68]
	Dexamethasone	~300	[68]
	Vitamin K	~270	[68]
Poly(ε-caprolactone)	Cyclosporin A	~100–200	[75]
Other polymers: β-cyclodextrin inclusion complexes [78], PVM/MA [125], and –poly(alkylmethacrylate)			
Interfacial deposition			
Poly(lactic acid)	Indomethacin	230	[44,68,79]
Salting-out			
Poly(lactic acid)	Savoxepin	<1000	[126]
Other polymers: Poly(alkylmethacrylate) and ethylcellulose			
Emulsion/solvent diffusion			
Poly(lactic acid)–poly(glycolic acid) copolymer	p-THPP	117–118	[127]
	Doxorubicin	<1000	[82]
Poly(lactic acid)	p-THPP	125	[127]
	DNA	<300	[83]

(*continued on next page*)

Table 1 (*continued*)

Other polymer: Poly(ε-caprolactone)			
Nanoparticles produced from natural macromolecules			
Serum albumin	Doxorubicin	200–1500	[89]
Gelatin	Mitomycin C	280	[92]
Polysaccharides	Oligonucleotides	<1000	[128,129]
	Doxorubicin	<1000	[25]
	Insulin	<1000	[96]
	Tetanus and diphtheria toxoid	<1000	[6]
	Calcitonin	<1000	[102]
Desolvation of macromolecules*			
Gelatin	DNA	<1000	[116]
	Cytostatics		[117]
Other polymers: casein, albumin and ethylcellulose			
Nebulization techniques			
Poly(lactic acid)	Insulin	400–600	[130]

hGRF, human growth hormone–releasing factor; p-THPP, mesotetra(hydroxyphenyl)porphyrin.

* Induced by heat, pH changes, salts, organic solvents, complexing with macromolecules, sonication, or chemical cross-linking.

produce PMMA nanospheres until 1986, when the copolymerization of MMA, hydroxypropylmethacrylate, methacrylic acid, and ethylene glycol dimethacrylate was described to increase the hydrophilicity of the particles with the intention of modifying the distribution of the particles within the body [3]. Various drugs or tracers were encapsulated into nanospheres with high entrapment efficiencies (eg, doxorubicin) [20]. The preparation of PMMA nanospheres is simple and drugs can be successfully entrapped, but two drawbacks have to be kept in mind. First, polymerization requires a chemical or physical initiation, and second, PMMA nanospheres are not biodegradable [21].

As a result, poly(alkylcyanoacrylate) (PACA) nanoparticles seem to be a good alternative, in that PACA is readily biodegradable [22]. Polymerization occurs at room temperature and requires neither γ-irradiation nor a chemical initiator [23], thus avoiding heat and high-energy radiation. The polymerization mechanism is an anionic process. Hydrophilic drugs are encapsulated with good efficiency, such as that for ampicillin [24] and doxorubicin [25]. The percentage of drug incorporated or adsorbed decreases generally with the increasing amount of drug in the polymerization medium [26]. Sparingly water-soluble molecules such as progesterone [27], triamcinolone diacetate [28], can also be loaded in PACA nanospheres but must be dissolved in a solvent such as ether (ICH, class 3) or in a liquid surfactant before being added to the aqueous polymerization medium. Insulin [29], growth hormone–releasing factors (GRFs) [30], and monoclonal antibodies [31] can also be incorporated into PACA nanospheres.

As well, poly(dialkylmethylidene malonate) nanospheres were prepared at pH 6 [32]. The main difference is that the cyano group is replaced by an alkyloxycarbonyl group that is less reactive in the presence of the hydroxide ion initiator. Poly(diethylmethylidene malonate) nanospheres loaded with primaquine have also been prepared at pH 7.4 [33]. The entrapment efficiency rose to 100% when the drug was added to the polymerization medium.

Interfacial polymerization

Poly(alkylcyanoacrylate) nanoparticles

One of the advantages of these polymers is their very rapid polymerization—occurring during seconds—initiated by ions present in the medium [34]. Cyanoacrylate monomer and drug were dissolved in a mixture of an oil and absolute ethanol [35]. This mixture was then slowly extruded through a needle into a well-stirred aqueous solution, with or without some ethanol (ICH, class 3) or acetone (ICH, class 3) containing surfactant. Nanocapsules are formed spontaneously by polymerization of cyanoacrylate after contact with initiating ions present in the water. The resulting colloidal suspension can be concentrated by evaporation under vacuum. PECA [36], poly(isobutylcyanoacrylate) [37,38], and poly(isohexylcyanoacrylate) [39] were used in production of nanoparticles by this process. Examples of drugs encapsulated are insulin [36,40], calcitonin [11], octreotide [41], darodipine [42], indomethacin [43,44], and photoactivatable cytotoxic compounds used in photodynamic tumor therapy like phthalocyanines in an injectable vehicle [39]. To encapsulate highly water-soluble drugs, another method has been proposed [45,46]. Besides the monomer, potentially toxic compounds were not used, thus no purification procedure was necessary. The final product was a suspension of nanocapsules in Migliol, which is an acceptable excipient for oral administration. Encapsulation efficiencies reached 50% and 30% for the larger and the smaller capsules, respectively [47].

An advantage of interfacial polymerization techniques is high-efficiency drug encapsulation (eg, insulin with 95%) [34]. In addition, the advantage of obtaining nanocapsules by this method is that the polymer is formed in situ, allowing the polymer membrane to follow the contours of the inner phase of an oil/water or water/oil emulsion [34]. In this case, the main disadvantage is the use of organic solvents required for the external phase. Washing of solvents and replacement by water represents a time-consuming and difficult procedure [3].

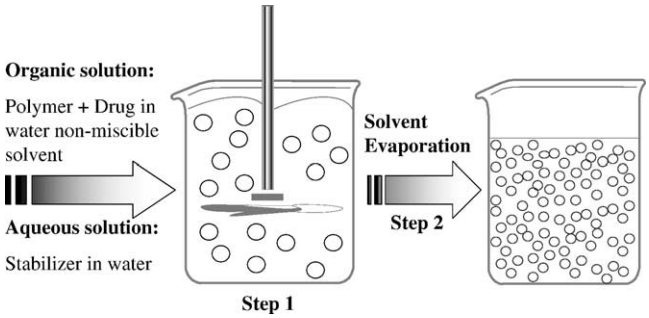

Fig 1. Schematic representation of the emulsification-evaporation technique.

Interfacial polycondensation

Polymeric nanoparticles can be also prepared by the interfacial polycondensation of the lipophilic monomer, such as phtaloyldichloride and the hydrophilic monomer, diethylenetriamine, in the presence and absence of the surfactant [48]. These nanoparticles were smaller than 500 nm. A modified interfacial polycondensation method was also developed. In this case, polyurethane polymer and poly (ether urethane) copolymers were chosen and successfully applied as drug carriers for α-tocopherol [49]. Polyurethane- and poly(ether urethane)–based nanocapsules were synthesized by interfacial reaction between two monomers.

Nanoparticles obtained from preformed polymers

With the exception of alkylcyanoacrylates and poly (dialkylmethylidene malonate), most of the monomers suitable for a micellar polymerization process in an aqueous phase lead to slowly biodegradable or nonbiodegradable polymers. In addition, residual molecules in the polymerization medium (monomer, oligomer, surfactant, etc.) can be more or less toxic, requiring meticulous purification of the colloidal material. To avoid these limitations, methods using preformed polymers instead of monomers have been proposed.

Synthetic preformed polymers

Emulsification/solvent evaporation

Emulsification-solvent evaporation involves two steps. The first step requires emulsification of the polymer solution into an aqueous phase (see Figure 1). During the second step polymer solvent is evaporated, inducing polymer precipitation as nanospheres.

A polymer organic solution containing the dissolved drug is dispersed into nanodroplets, using a dispersing agent and high-energy homogenization [50], in a nonsolvent or suspension medium such as chloroform (ICH, class 2) or ethyl acetate (ICH, class 3). The polymer precipitates in the form of nanospheres in which the drug is finely dispersed in the polymer matrix network. The solvent is subsequently evaporated by increasing the temperature under pressure or by continuous stirring [6]. The size can be controlled by adjusting the stir rate, type and amount of dispersing agent, viscosity of organic and aqueous phases, and temperature [50]. Even though different types of emulsions may be used, oil/water emulsions are of interest because they use water as the nonsolvent; this simplifies and thus improves process economics, because it eliminates the need for recycling, facilitating the washing step and minimizing agglomeration [51]. However, this method can only be applied to liposoluble drugs, and limitations are imposed by the scale-up of the high energy requirements in homogenization [6]. Frequently used polymers are PLA [52,53], PLGA [54], ethylcellulose (EC) [55], cellulose acetate phthalate [3], poly(ε-caprolactone) (PCL) [56-58], and poly(β-hydroxybutyrate) (PHB) [59,60]. Drugs or model drugs encapsulated were albumin [61], texanus toxoid [62], testosterone [52], loperamide [53,63], prazinquantel [64], cyclosporin A [65,66], nucleic acid [67], and indomethacin [55].

Solvent displacement and interfacial deposition

Solvent displacement and interfacial deposition are similar methods based on spontaneous emulsification of the organic internal phase containing the dissolved polymer into the aqueous external phase (see Figure 2). However, solvent displacement forms nanospheres or nanocapsules, whereas interfacial deposition forms only nanocapsules.

Solvent displacement involves the precipitation of a preformed polymer from an organic solution and the diffusion of the organic solvent in the aqueous medium in the presence or absence of a surfactant [68-71]. The polymer, generally PLA, is dissolved in a water-miscible solvent of intermediate polarity, leading to the precipitation of nanospheres. This phase is injected into a stirred aqueous solution containing a stabilizer as a surfactant. Polymer deposition on the interface between the water and the organic solvent, caused by fast diffusion of the solvent, leads to the instantaneous formation of a colloidal suspension [72]. To facilitate the formation of colloidal polymer particles during the first step of the procedure, phase separation is performed with a totally miscible solvent that is also a nonsolvent of the

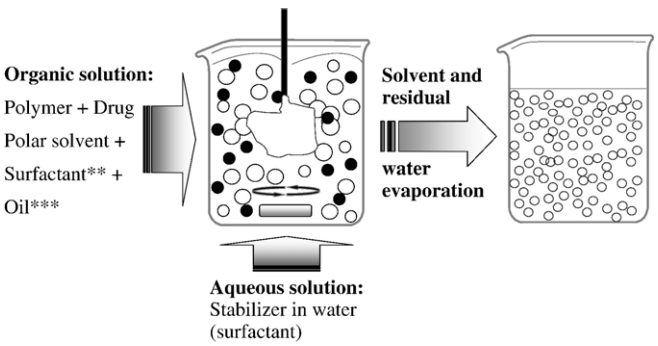

Fig 2. Schematic representation of the solvent displacement technique. **Surfactant is optional. ***In interfacial deposition method, a fifth compound was introduced only on preparation of nanocapsules.

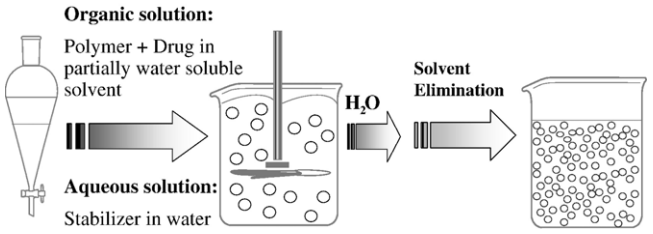

Fig 3. Schematic illustration of the ESD technique.

polymer [16]. The solvent displacement technique allows the preparation of nanocapsules when a small volume of nontoxic oil is incorporated in the organic phase [72]. Considering the oil-based central cavities of the nanocapsules, high loading efficiencies are generally reported for lipophilic drugs when nanocapsules are prepared [72]. The usefulness of this simple technique [72] is limited to water-miscible solvents, in which the diffusion rate is enough to produce spontaneous emulsification. Then, even though some water-miscible solvents produce a certain instability when mixed in water, spontaneous emulsification is not observed if the coalescence rate of the formed droplets is sufficiently high [73]. Although, acetone/dichloromethane (ICH, class 2) are used to dissolve and increase the entrapment of drugs, the dichloromethane increases the mean particle size [74] and is considered toxic. This method is basically applicable to lipophilic drugs because of the miscibility of the solvent with the aqueous phase [69], and it is not an efficient means to encapsulate water-soluble drugs.

In fact, it seems difficult to choose a drug/polymer/solvent/nonsolvent system in which particles would be formed and the drug efficiently entrapped, because the solvent and the nonsolvent of the polymer must be mutually miscible. The progressive addition of the polymer solution to the nonsolvent generally leads to the formation of nanospheres close to 200 nm in size. Nanoparticles seem to be formed by a mechanism comparable to the "diffusion and standing" process found in spontaneous emulsification. This phenomenon has been explained by local variations of the interfacial tension between the two immiscible liquids due to the mutual diffusion of the third liquid. This method has been applied to various polymeric materials such as PLA [25], PLGA [69], PCL [75], and poly(methyl vinyl ether-co-maleic anhydride) (PVM/MA) [76,77]. This technique was well adapted for the incorporation of cyclosporin A, because entrapment efficiencies as high as 98% were obtained [47]. Highly loaded nanoparticulate systems based on amphiphilic β-cyclodextrins to facilitate the parenteral administration of the poorly soluble antifungal drugs bifonazole and clotrimazole were prepared according to the solvent displacement method [78].

Interfacial deposition is a process used for the production of nanocapsules; however, this is not a polymerization technique but an emulsification/solidification technique. In interfacial deposition, a fifth compound is introduced, of oil nature, miscible with the solvent of the polymer but immiscible with the mixture. The polymer deposits on the interface between the finely dispersed oil droplets and the aqueous phase, forming nanocapsules [1]. An aqueous solution is used as the dispersing medium. The main difference is that polymers such as PLA are dissolved together with the drug in a solvent mixture (eg, benzyl benzoate, acetone, and phospholipids) [24]. This mixture is injected slowly into a stirred aqueous medium, resulting in the deposition of the polymer in the form of nanoparticles of about 230 nm in size [44,68,79]. Polymer deposition occurs at the interface between water and benzoyl nanodroplets, forming nanocapsules with a shell-like wall [44].

Emulsification/solvent diffusion

Emulsification/solvent diffusion (ESD) was proposed in the literature based on the use of organic solvents, and then it was adapted to the following salting-out procedure. The encapsulating polymer is dissolved in a partially water-soluble solvent such as propylene carbonate (ICH not given) and saturated with water to ensure the initial thermodynamic equilibrium of both liquids. In fact, to produce the precipitation of the polymer and the consequent formation of nanoparticles, it is necessary to promote the diffusion of the solvent of the dispersed phase by dilution with an excess of water when the organic solvent is partly miscible with water or with another organic solvent in the opposite case. Subsequently, the polymer-water saturated solvent phase is emulsified in an aqueous solution containing stabilizer, leading to solvent diffusion to the external phase and the formation of nanospheres or nanocapsules, according to the oil-to-polymer ratio. Finally, the solvent is eliminated by evaporation or filtration, according to its boiling point. The procedure is illustrated in Figure 3.

This technique presents several advantages, such as high encapsulation efficiencies (generally >70%), no need for homogenization, high batch-to-batch reproducibility, ease of scale-up, simplicity, and narrow size distribution. Disadvantages are the high volumes of water to be eliminated from the suspension and the leakage of water-soluble drug into the saturated-aqueous external phase during emulsification, reducing encapsulation efficiency. As with some of the other techniques, this one is efficient in encapsulating lipophilic drugs

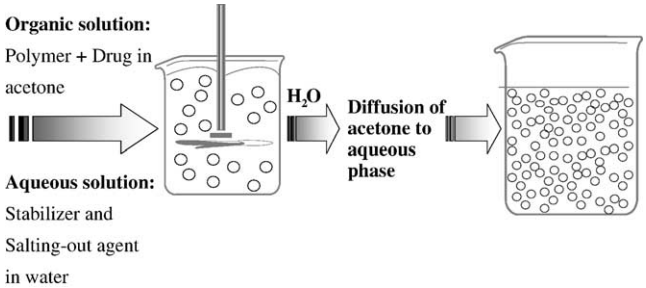

Fig 4. Schematic of the salting-out technique.

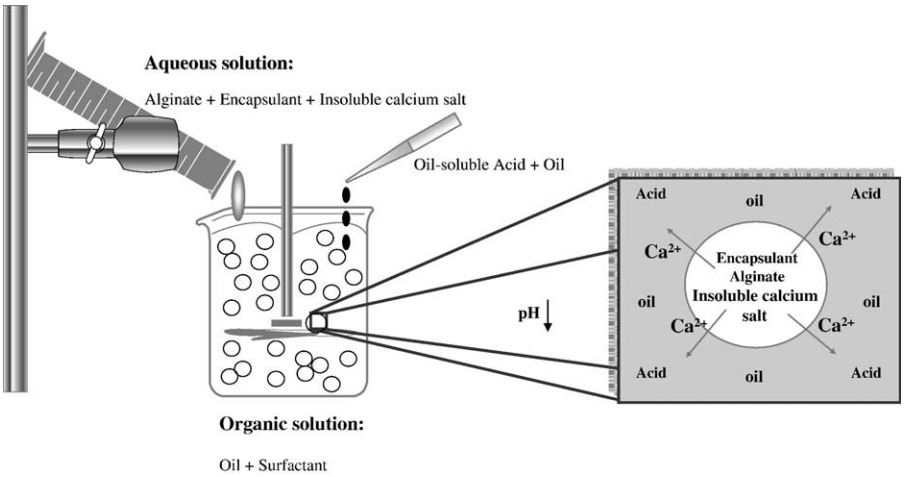

Fig 5. Schematic representation of the emulsification–internal gelation technique using alginate.

[72]. Several drug-loaded nanoparticles were produced by the ESD technique, including mesotetra(hydroxyphenyl) porphyrin-loaded PLGA (p-THPP) nanoparticles [80,81], doxorubicin-loaded PLGA nanoparticles [82], plasmid DNA-loaded PLA nanoparticles [83], coumarin-loaded PLA nanoparticles [84], indocyanine [85], cyclosporin (Cy-A)-loaded gelatin and cyclosporin (Cy-A)-loaded sodium glycolate nanoparticles [86].

Salting out with synthetic polymers

Salting-out is based on the separation of a water-miscible solvent from aqueous solution via a salting-out effect. The salting-out procedure can be considered as a modification of the emulsification/solvent diffusion. Polymer and drug are initially dissolved in a solvent such as acetone, which is subsequently emulsified into an aqueous gel containing the salting-out agent (electrolytes, such as magnesium chloride, calcium chloride, and magnesium acetate, or non-electrolytes such as sucrose) and a colloidal stabilizer such as polyvinylpyrrolidone or hydroxyethylcellulose. This oil/water emulsion is diluted with a sufficient volume of water or aqueous solution to enhance the diffusion of acetone into the aqueous phase, thus inducing the formation of nanospheres. The selection of the salting-out agent is important, because it can play an important role in the encapsulation efficiency of the drug. Both the solvent and the salting-out agent are then eliminated by cross-flow filtration [72].

This technique used in the preparation of PLA, poly-(methacrylic) acid, and EC nanospheres leads to high efficiency and is easily scaled up [72]. The main advantage of salting out is that it minimizes stress to protein encapsulants [7]. Salting out does not require an increase of temperature and, therefore, may be useful when heat-sensitive substances have to be processed [87]. The greatest disadvantages are exclusive application to lipophilic drugs and the extensive nanoparticle washing steps [1]. The preparative steps of this procedure are described in Figure 4.

Production of nanoparticles from natural macromolecules

Albumin nanoparticles produced in an external-oily emulsion

Two main methods are used in the preparation of albumin microspheres, characterized by the method of stabilization; thermal treatment at elevated temperatures (95°–170°C) or chemical treatment in vegetable oil, iso-octane emulsions, or aqueous medium. Other techniques involve slight modification of either of the two methods [88]. In this case albumin nanospheres were formed by homogenizing the oil phase containing the albumin droplets and thermally stabilized by heating at 175° to 180°C for 10 minutes [88]. This mixture was cooled and diluted with ethyl ether to reduce the viscosity of the oil phase to permit separation by centrifugation.

Heat treatment of albumin is applicable only to drug molecules that are not heat sensitive. For this reason, nanoparticles were produced emulsifying serum albumin aqueous solution in cottonseed oil at 25°C [89], then denaturing the albumin by resuspending the particles in ether containing the cross-linking agents 2,3-butadiene or formaldehyde. The particles were stirred, isolated by centrifugation, and dried by lyophilization. Particles released the drug doxorubicin much faster than particles formed by heat treatment [89], but the purification step remains the main problem with the elimination of the cottonseed oil [3]. A technique was proposed based on the desolvation of natural macromolecules, which simplifies the purification step [90].

As a modification of this method [91], an aqueous solution of albumin was emulsified in chloroform containing hydroxypropylcellulose and EC as stabilizers. The emulsified macromolecule is subsequently cross-linked with glutaraldehyde and washed. Because of the need for chlorinated solvents, this technique does not offer much advantage over the other techniques [90].

Gelatin nanoparticles produced in an external-oily emulsion

Emulsified gelatin solution droplets were hardened by cooling the emulsion below the gelation point in an ice bath,

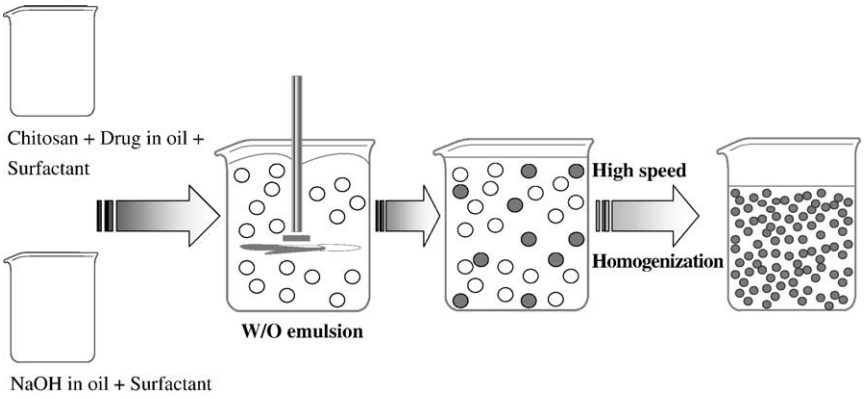

Fig 6. Schematic representation of chitosan nanoparticles preparation by the emulsification technique.

resulting in gelation of the gelatin droplets. Gelled nanodroplets were filtered, washed, and cross-linked with formaldehyde [92]. The particle size ranged between 100 and 600 nm with a mean of 280 nm [23]. This technique is applicable to heat-sensitive drugs; however, a number of drugs can be covalently bound to the gelatin by formaldehyde treatment, which constitutes a disadvantage [23]. Additionally, cross-linking increases significantly the size of the particles. Furthermore, a significant disadvantage of the cross-linking agent relates to its toxicity, and this point must be carefully considered. In this context, it would be of interest to study carefully the influence of different formulation and process parameters in this process according to the application.

Another interesting system for drug delivery systems could be nanoparticulate carriers from bioacceptable macromolecules. For this reason, vegetable protein fractions termed gliadins have been chosen from wheat gluten, to efficiently encapsulate lipophilic substances such as α-tocopherol [93]. Gliadins possess the ability to interact with epidermal keratin as a result of their richness in praline; this property leads to a desired controlled release of drug.

Alginate nanoparticles

Sodium alginate is a water-soluble polymer that gels in the presence of multivalent cations such as calcium [94]. Alginate particles are usually produced by dropwise extrusion of sodium alginate solution into calcium chloride solution. Alginate particle size depends on the size of the initial extruded droplet. The smallest particles produced had a minimum size of 1 to 5 μm, obtained by air atomization [95]. The preparation of alginate nanoparticles was first achieved in a diluted aqueous sodium alginate solution in which gelation was induced by the addition of a low concentration of calcium. This leads to the formation of invisible clusters of calcium alginate gels. In an additional advance, alginate particles have been produced by using a modified emulsification/internal gelation method [96] as illustrated in Figure 5. The preparation of alginate nanoparticles via this method does not require specialized equipment and can be performed at ambient temperature. The main difficulty of this method is the nanoparticle washing step to eliminate the residual oil droplets, but new strategies have been devised.

Chitosan nanoparticles

Chitosan nanoparticles have been developed to encapsulated proteins such as bovine serum albumin, tetanus and diphtheria toxoid [6], vaccines [97], anticancer agents [98], insulin [99], and nucleic acids [100,101]. Chitosan considerably enhanced the absorption of peptides such as insulin and calcitonin across the nasal epithelium [102].

The methods proposed to prepare chitosan nanoparticles are based on the spontaneous formation of complexes between chitosan and polyanions [103] or the gelation of a chitosan solution dispersed in an oil emulsion [104]. Various methods for producing chitosan nanoparticles are described in the literature [105,106].

Chitosan nanoparticles obtained by formation of a spontaneous complex between chitosan and polyanions such as tripolyphosphate [103] have small diameters (200–500 nm) and show a quasi spherical shape under transmission electron microscopy. Chitosan nanoparticles produced by a promoting gelation in an emulsification-based method as illustrated in Figure 6, results in a diameter of 400 nm. Compared with the previously described method, this technique has a major disadvantage of involving organic solvents during the isolation of the particles; these are difficult to remove and may cause toxicity [107].

Agarose nanoparticles

Agarose nanoparticles were developed for the administration of therapeutic proteins and peptides [108]. Agarose aqueous solution forms thermally reversible hydrogels while being cooled below the gelling temperature (31°–36°C). Thermal gelation results from the formation of helicoidal structures responsible for a three-dimensional network in which large amounts of water can be entrapped. The hydrogel, being hydrophilic, inert, and biocompatible, forms a suitable matrix for proteins and peptides that can be entrapped in the gel during formation [107].

Agarose nanoparticles were produced using an emulsion-based technology as illustrated in Figure 7. This methodology requires the preparation of an agarose

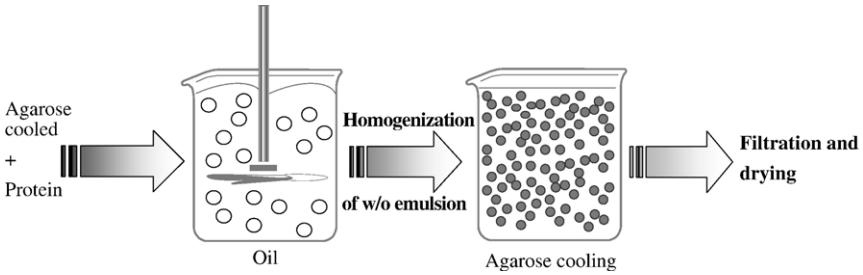

Fig 7. Schematic illustration of agarose nanoparticles preparation by the emulsification technique.

solution in corn oil emulsion at 40°C [109]. Peptides and proteins to be encapsulated are initially added to the agarose solution. The small size of the dispersed aqueous nanodroplets is achieved by homogenization. Gelation of agarose is then induced by diluting the emulsion with cold corn oil under agitation at 5°C. The liquid nanodroplets then gel to protein-containing agarose hydrogel nanoparticles.

Nanoparticles produced by desolvation of macromolecules

Another technology applicable to a wide range of polymers is based on desolvation by charge and pH changes, or by addition of a desolvating agent (ethanol or concentrated inorganic salt solutions). The main advantage is that this process does not require an increase in temperature and, therefore, may be useful when heat-sensitive drugs are used [110].

Nanoparticles were prepared using the process of reversible swelling of macromolecules [90,111] using gelatin [111], human serum albumin [111], bovine serum albumin [112], and casein [111] as the macromolecular materials. This process offers the advantage of producing nanoparticles directly in aqueous suspension, but the use of potentially toxic compounds such as glutaraldehyde and desolvating agents requires subsequent purification [1]. Variations in nanoparticle production by the desolvation process were described [113], but unfortunately the yield is comparatively low. In the case of gelatin, different methods such as the two-step desolvation method [114,115] have been applied to produce nanoparticles. Recent reports outline the important use of gelatin as drug delivery systems for DNA [116] and cytostatics [117].

New techniques based on supercritical or compressed fluids

Some of the techniques described above are complex, and the products may often be characterized by high residual solvent content, low drug loading, drug degradation or denaturation, ineffective drug release, or unsuitable physical and morphological properties [118]. Techniques based on supercritical or compressed fluid can be an interesting tool for preparation of nanoparticulate and microparticulate products [119]. In this technique, the drug and the polymer are solubilized in a supercritical fluid, and the solution is expanded through a nozzle. The supercritical fluid is evaporated in the spraying process, and the solute particles eventually precipitate. This technique is clean, because the precipitated solute is free of solvent. It also provides advantages such as suitable technological and biopharmaceutical properties and high quality. It has been demonstrated for numerous applications involving protein drug delivery systems. Protein drugs such as insulin were encapsulated in poly(ethylene glycol)/poly(l-lactide) (PEG/PLA) nanoparticles by this technique [130]. However, this new process requires a high initial capital investment for equipment, and elevated operating pressures requiring high-pressure equipment. In addition, compressed supercritical fluids require elaborate recycling measures to reduce energy costs. Finally, it is very difficult to dissolve strong polar substances in supercritical CO_2. In fact, supercritical CO_2 has solvating properties characteristic of both fluorocarbons and hydrocarbons. However, the use of cosolvents and/or surfactants to form microemulsions makes it possible to dissolve polar and ionic species.

Stability upon storage of the nanoparticles

Nanoparticles are intended to be administered as pharmaceutical dosage forms in humans. Among other requirements, they must be free of impurities. The necessity for and degree of purification are dependent on the final purpose of the formulation developed. The most commonly reported procedures are gel filtration, ultracentrifugation, dialysis, and, recently, cross-flow filtration. Another requirement relates to the sterilization of the formulation. The choice of the sterilizing treatment depends on the physical susceptibility of the system. Finally, nanoparticles should be easily stored and administered. Nanoparticles constitute a relatively stable physical system because of their colloidal nature. Many variables can affect the stability of nanoparticles. Generally, a colloidal suspension is stable and does not tend to separate as a result of slow deposition due to the mixing tendencies of diffusion and convection. However, some agglomeration can occur. To prevent a complete precipitation, it is necessary to incorporate some additives. Chemical integrity of drug is also a fundamental aspect of the overall stability evaluation of the nanoparticles. Some parameters are crucial for the stability, such

Table 2
Polymeric nanoparticles: general advantages and drawbacks of the preparation methods

Method	Simplicity of procedure	Need for purification	Facility scaling-up	EE (%)	Safety of compounds
Polymerization of monomers					
Emulsion polymerization					
Organic	Low	High	NR	Low	Low
Aqueous	High	High	High	High	Medium
Interfacial polymerization	Low	High	Medium	High	Low
Preformed polymers					
Synthetic					
Emulsification/solvent evaporation	High	Low	Low	Medium	Medium
Solvent displacement and interfacial deposition	High	NR	NR	High	Medium
Salting out	High	High	High	High	Low
Emulsion/solvent diffusion	Medium	Medium	High	High	Medium
Natural					
Albumin	NR	High	NR	Medium	Low
Gelatin	NR	High	NR	Medium	Low
Polysaccharides					
Alginate	High	Medium	High	High	High
Chitosan	High	Medium	High	High	High
Agarose	Medium	High	NR	NR	High
Desolvation	NR	High	NR	Low	Low

EE, encapsulation efficiency; NR, no reference available.

as the duration of contact with the aqueous environment when the drug is water soluble, the surrounding pH when drug degradation is pH dependent, and light exposure when the drug is light sensitive. Stability studies are thus important and can be performed according to the drug and to the polymer properties.

There are some methods to increase the stability of the nanoparticles. Lyophilization (freeze-drying) seems to be a highly stabilizing process. It is generally applied to enhance the physicochemical stability of the nanoparticles to achieve a pharmaceutically acceptable product, especially in cases in which the storage conditions are unfavorable. This technique involves the freezing of the suspension and subsequent elimination of its water content by sublimation under reduced pressure. After complete desiccation, nanoparticles are obtained in the form of a dry powder that is easy to handle and store. In most cases the freeze-dried particles are readily dispersible in aqueous solutions. In some systems the ease of redispersion depended on the manufacturing process. Ultrasonication was applied by some authors to ensure complete redispersion of nanoparticles.

Freezing is the most aggressive step of the freeze-drying operation for colloidal operations. It is thus important to improve the nanoparticle resistance by addition of a cryoprotectant to avoid alteration of the suspension [120]. Sometimes cryoprotectants like glucose, trealose, mannitol, and sorbitol were added to ensure redispersibilty or to allow vitrification of the suspension during the cooling and to avoid crystallization of the liquid suspension. It is also important to be aware of the presence of pharmaceutical excipients, usually used for purposes of isotonicity (eg, glucose) or stabilization (eg, dextran and surfactants). Such excipients are indeed cryoprotectants that facilitate the aqueous reconstitution of the freeze-dried product.

Finally, nanoparticles can be stored in sealed vials at room temperature, or in a laboratory desiccator, or even in the refrigerator, especially for temperature-sensitive drugs.

Summary and conclusions

The most important methods for the preparation of nanoparticulate drug carriers, together with their advantages and disadvantages, are summarized in Table 2.

The evolution of nanoparticle preparation methods has been marked by three aspects: need for less toxic reagents, simplification of the procedure to allow economic scale-up, and optimization to improve yield and entrapment efficiency. Efficient drug entrapment and transition to large scale are of utmost importance to industrial applicability.

There are now numerous preparation methods available for producing nanoparticles, and important technological advances have been achieved. Simple, safe, and reproducible techniques are now available to prepare drug-loaded nanospheres and nanocapsules. Depending on the physicochemical characteristics of a drug, it is now possible to choose the best method of preparation and the best polymer to achieve an efficient entrapment of the drug. The chosen method should minimize loss of the drug or its pharmacological activity. In this respect, the development of a technique that allows the incorporation of biomolecules without affecting their activity constitutes a fundamental goal for nanotechnology.

Nevertheless, there are several problems that remain to be solved. The process is not suitable to all drugs. In addition, the postpreparative steps, such as purification and preservation, particularly important for nanocapsules, and residual solvent analysis must be extensively investigated. Other difficulties remain, such as the formation of an incomplete or discontinuous film, inadequate stability of certain active components, nonreproducible or predictable release charac-

teristics, and certain limitations that make the final product economically unfeasible.

Despite these technological challenges, nanoparticles have shown great promise for the development of drug administration.

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