New Approach for the Preparation of Nanoparticles by an Emulsification-Diffusion Method

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RESEARCH PAPERS

New Approach for the Preparation of Nanoparticles by an Emulsification-Diffusion Method

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Summary

A preparation method for nanoparticles based on an emulsification of a benzyl alcohol solution of a polymer in a hydrocolloid-stabilized aqueous solution followed by a dilution of the emulsion with water, was developed. Several process parameters were varied, including the concentration of poly(vinyl alcohol) or gelatin used as stabilizing colloids in the external phase, the concentration of nanoparticle polymer in the internal phase and other process variables such as the external/internal phase ratio and the viscosity of the external phase. By increasing the percentage of poly(vinyl alcohol) to 27.5% in the external phase, it was possible to produce nanoparticles as small as 70 nm in diameter. Poly(vinyl alcohol) could be replaced by gelatin and the smallest nanoparticles obtained under these conditions had an average size of 700 nm. It was demonstrated that the nanoparticles can be loaded with the cytostatic drug chlorambucil (8.52% m/m) with an entrapment efficiency reaching 60%.

1 Introduction

The nanoparticle preparation methods can be classified into two main categories: polymerization of an emulsified monomer and dispersion of a preformed polymer (1). The latter has been mainly employed for well-established biodegradable polymers such as poly(D,L-lactic acid) (PLA) and poly(D,L-lactic-co-glycolic acid) (PLGA) (2,3). Most of the techniques involving the dispersion of a preformed polymer require the use of more or less toxic organic solvents. These solvents are generally acetone for the precipitation method (2), and chlorinated solvents for the emulsion-evaporation procedure (4). Subsequently, the nanoparticles have to be extensively purified to provide practically solvent-free parenteral formulations due to the relatively high toxicity of most of the solvents used (1). Recently, an original technique for producing biodegradable nanoparticles has been proposed (5). This technique is based on the emulsification of an acetone solution of a polymer in an aqueous gel containing large amounts of a salting-out agent. This procedure, known as the salting-out process, has proved suitable for the production of important amounts of highly drug-loaded nanoparticles which can be easily redispersed after freeze-drying without any surfactant (6). However, the use of acetone and of large amounts of salts may raise some concern about recycling of the salts and about compatibility with bioactive compounds.

The present work deals with the development of a novel method using benzyl alcohol as organic solvent and avoiding high salt concentrations. Benzyl alcohol is a widely used solvent because of its acceptability in parenteral formulations (antimicrobial) and its solubilizing properties (7). In this study, the nanoparticle size was optimized in view of drug-targeting and loading tests using chlorambucil as cytostatic model drug were performed.

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2 Materials and Methods

2.1 Materials

PLA (Medisorb® 100DL) and PLGA 85:15 (Medisorb® 8515DL) with a D,L-lactic acid and glycolic acid molar ratio of 85:15, were obtained from Medisorb Technologies International L.P. (Cincinnati, OH, USA). Poly(ε-caprolactone) (PCL) Tone 300 and the methacrylic acid copolymer Eudragit® S100 were supplied by Union Carbide (Geneva, Switzerland) and Röhm Pharma (Weiterstadt, Germany), respectively. As stabilizing hydrocolloids, poly(vinyl alcohol) having a molecular weight of 26 000 (PVAL) (Mowiol® 4–88, Hoechst, Frankfurt am Main, Germany) and gelatin (Gelatina Alba Golddruck, Siegfried, Zofingen, Switzerland) were chosen. Benzyl alcohol was purchased from Merck (Darmstadt, Germany). Chlorambucil (Sigma, Buchs, Switzerland) was selected as a hydrophobic model drug. All other chemicals were of analytical grade and used without further purification.

2.2 Methods

2.2.1 Nanoparticle preparation

An aqueous gel (or buffered gel) of a stabilizing hydrocolloid (typically 40 g) was added to a solution of polymer (typically 14.3% m/m) in benzyl alcohol (21 g) under mechanical

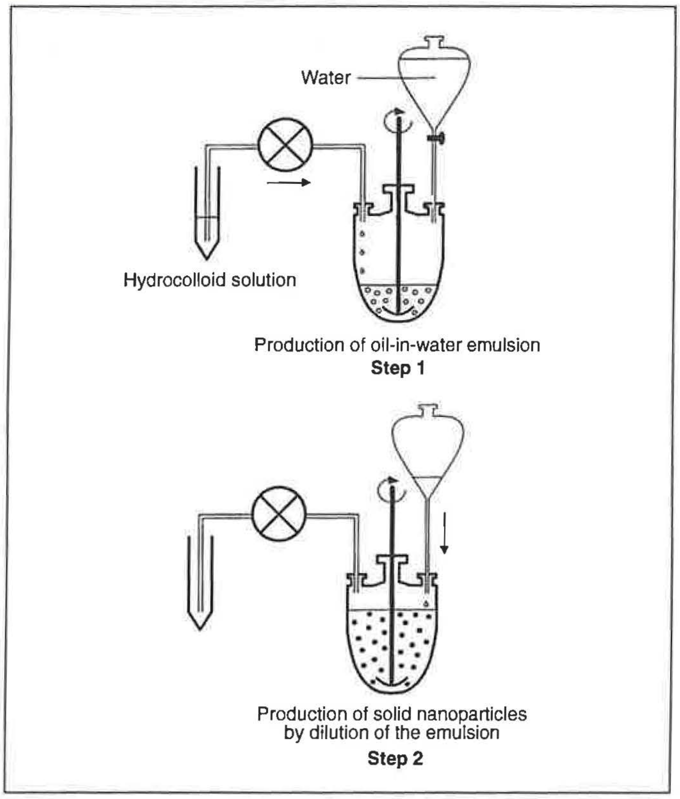

Fig. 1 Schematic procedure for preparation of the nanoparticles

stirring at 1200 rpm. A water-in-oil emulsion was first obtained and the emulsion underwent a phase inversion upon complete addition of the aqueous gel. Since benzyl alcohol is miscible at a ratio of 1:25 (m/v) with water, enough water (or buffer) (660 g) was subsequently added to the emulsion in order to allow the diffusion of the organic solvent into the water, leading to the precipitation of the polymer. The overall process which is depicted in Fig. 1, took less than 10 min.

Drug-loaded nanoparticles were produced as described above, except that a determined amount of chlorambucil was first dissolved in the benzyl alcohol. Only the PLA nanoparticle suspensions were concentrated and purified by cross-flow filtration as previously described (8) using a Sartocon® Mini device (Sartorius, Göttingen, Germany) mounted with a polyolefin cartridge filter with 100 nm pore size. The elimination of benzyl alcohol in the filtrate during the purification procedure was monitored spectrophotometrically at 258 nm (reference wavelength: 400 nm; confirmation wavelengths: 252 and 264 nm) using a diode array spectrometer HP 8452A (Hewlett Packard, Waldbronn, Germany). After collecting 10 l of filtrate, the nanoparticle suspension was completely rid of water-solubilized benzyl alcohol.

The PLA nanoparticles were finally frozen at –55 °C for 10 min and freeze-dried for 24 h at 0.05 mBar in a Lyolab BII (Secfroid, Aclens, Switzerland).

2.2.2 Nanoparticle characterization

The nanoparticle mean size and polydispersity (index expressed from 0 to 9) were assessed with a Coulter Nano-Sizer® (Coulters Electronics, Harpenden, Hertfordshire, UK). Each value is the average of 3 measurements.

The nanoparticles were also examined by scanning electron microscopy (SEM). An aqueous dispersion of the nanoparticles was finely spread over a slab and was dried under vacuum. A 20 nm thick gold layer was applied on the dried nanoparticles and the sample was observed by SEM using a JSM-6400 scanning microscope (JEOL, Japan).

The residual PVAL content of purified nanoparticles was determined by colorimetry as previously reported (6). Each assay was performed in triplicate.

2.2.3 Viscosity determination

A Bohlin® Controlled Stress Rheometer with a cone-plate CP 1/40 (Bohlin Rheology GmbH, Mühlacker, Germany) was used for the measurement of the gelatin solution viscosity. A stress viscometry test was applied to the sample which was placed on the stationary lower plate. The temperature was controlled during the test with a Bohling® extended temperature unit and the controlled torque was applied on the rotatable upper geometry using a drag-cup motor principle. A shear stress of 8.3 Pa was selected and the shear rate measured at increasing temperatures (2.5 °C min^{-1}; 29–51 °C). The strain delay was of 20 s, the integration time of 20 s and the measurement interval of 10 s.

2.2.4 Drug loading and entrapment efficiency

After freeze-drying, 15 mg of nanospheres were dissolved in 100 ml of chloroform and the solution was sonicated for 15 min. The chlorambucil content was assayed spectrophotometrically at 258 nm (reference wavelength: 500 nm; confirmation wavelengths: 254 and 262 nm). The drug loading and entrapment efficiency were calculated according to Eqs. 1 and 2, respectively:

$$\text{Drug loading (\%)} = \frac{\text{amount of drug in nanoparticles}}{\text{amount of nanoparticles}} \times 100 \qquad \text{(Eq. 1)}$$

$$\text{Entrapment efficiency (\%)} = \frac{\text{percent drug loading}}{\text{percent of the initial content} \times (1 - \text{fraction of residual PVAL})} \times 100 \qquad \text{(Eq. 2)}$$

Because of the adsorption of a certain amount of PVAL onto the nanoparticles during the manufacturing process, the correction factor 1/(1-fraction of residual PVAL) was introduced in Eq. 2 to avoid an underestimation of the entrapment efficiency. Each value is the average of triplicate determinations.

3 Results and Discussion

In earlier work (5) the concentration of hydrocolloid in the external phase has shown to be the predominant parameter governing the nanoparticle mean size. Accordingly, the influence of the percentage of two hydrocolloids, namely PVAL and gelatin (Fig. 2) and the amount of external phase added to the organic solution (Fig. 3) on the nanoparticle size were investigated. As shown in Fig. 2, it is possible to produce nanoparticles as small as approximately 70 nm (73 ± 4 nm) by increasing the PVAL concentration of the aqueous phase. The smallest nanoparticles reported by the salting-out procedure had an average size of 170 nm (5). Fessi et al. (2) have succeeded in producing 90 nm PLA nanoparticles with the precipitation method by adding 10% (v/v) of water to an acetone solution of polymer. The polymer solution was then poured under stirring in a poloxamer aqueous solution, inducing the precipitation of the polymer. The possibility of producing very small nanoparticles is attrac-

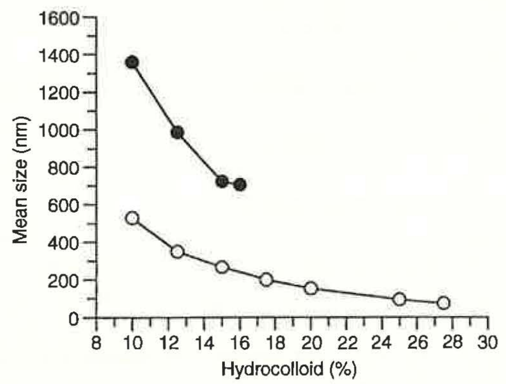

Fig. 2 Influence of the percentage of hydrocolloid in the external phase on the mean size of the nanoparticles
External phase: PVAL (○) (100 g) or gelatin (●) (40 g) at different concentrations; internal phase: Eudragit® S100 14.3%, 21 g
Mean ± SD (SD smaller than symbols), n = 3

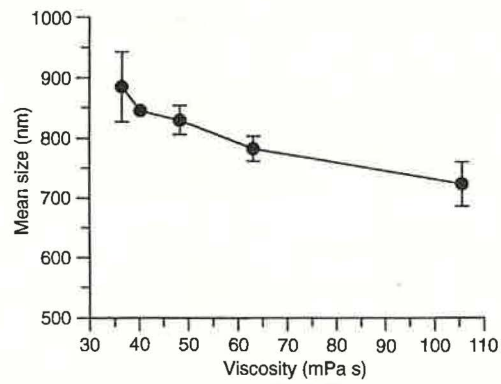

Fig. 4 Influence of the external phase viscosity on the mean size of the nanoparticles
External phase: gelatin 15%, 40 g; internal phase: Eudragit® S100 14.3%, 21 g
Mean ± SD, n = 3

tive since it has been previously found that the targeting of carriers to non-phagocytic cells is limited to carrier sizes below 150 nm. (9). Furthermore, the success in targeting efficiently organs other than the liver and spleen with poloxamer coated nanoparticles, strongly depends on the nanoparticle size. For instance, the targeting of a carrier to the bone marrow is best achieved with carrier sizes below 150 nm as far as the rabbit model is concerned (10).

Gelatin does not have stabilizing properties as marked as PVAL since the smallest nanoparticles produced in this case have an average size of 700 nm. Furthermore, the gelatin solution has to be kept at least at 30 °C to remain fluid. Gelatin exhibits an advantage over PVAL in that it is widely used in parenteral applications. Using this new technique, it seems relatively easy to modifiy the nanoparticle surface by simply changing the hydrocolloid. With the salting-out process, the selection of a stabilizing colloid other than PVAL is limited due to compatibility problems (11). The external/internal phase ratio (Fig. 3) has only a slight influence on the nanoparticle mean size. Hence, once the phase inversion occurs, any excess of PVAL has practically no effect on reducing the nanoparticle size and only prolongs the purification process. Therefore, an external/internal phase ratio of 1.9 which allows a complete phase inversion, was selected for the following experiments.

The influence of the viscosity of the external phase on the nanoparticle mean size was also investigated (Fig. 4). The gelatin solution was heated to different temperatures (30–50 °C) in order to modify the external phase viscosity, and added to the organic solution.

Increasing the external phase viscosity decreases the nanoparticle mean size, but only to a limited extent. During the emulsification stage of the nanoparticle preparation, the system should obey to the laws governing the emulsions properties. The viscosity of the external phase of an emulsion (η_c) affects the Reynolds number and thus the prevailing mechanism governing the droplet breakup i.e. laminar or turbulent flow. Generally, the droplet mean size decreases with increasing η_c with a less pronounced effect when a turbulent flow is involved (12).

The nanoparticle size can also be adjusted by varying the percentage of polymer in the internal phase (Fig. 5).

Contrary to the procedure described by Allémann et al. (5), the increase in polymer concentration in the internal phase raises the nanoparticle mean size. Increasing the PLA concentration from 0.5% to 14.3% changes the organic phase density from

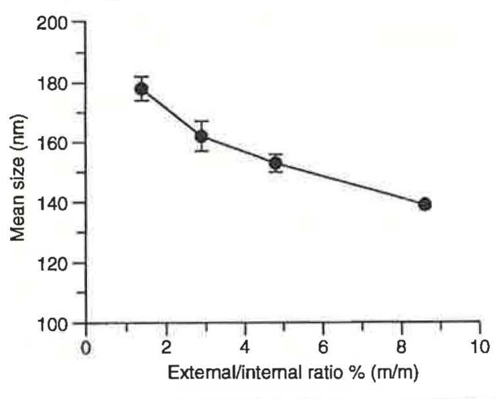

Fig. 3 Influence of the external/internal phase ratio on the mean size of the nanoparticles
External phase: PVAL 20%, different amounts; internal phase: Eudragit® S100 14.3%, 21 g
Mean ± SD, n = 3

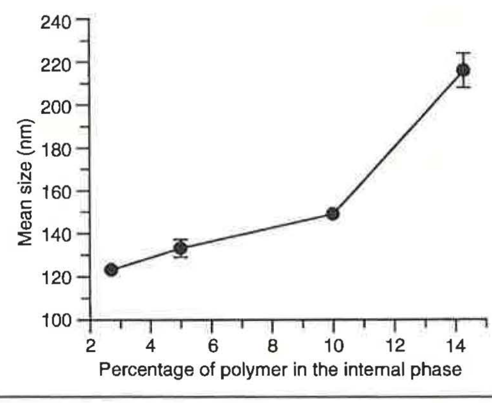

Fig. 5 Influence of the percentage of PLA in the internal phase on the mean size of the nanoparticles
External phase: PVAL 17%, 40 g; internal phase: PLA 14.3%, 21 g
Mean ± SD, n = 3

Table 1 Examples of nanoparticles produced by the emulsification-diffusion process
External phase: PVAL 15%, 40 g; internal phase: polymer 14.3%, 21 g

Example No.	Polymer	Treatment	Mean size (nm)	Polydispersity[a]
1	Eudragit® S100	stirring at 1200 rpm	244	1
2	Eudragit® S100	stirring at 1200 rpm and concomitant sonication	244	0
3	Eudragit® S100	stirring at 5000 rpm	141	2
4	PCL	stirring at 1200 rpm	264	1
5	PLGA 85:15	stirring at 1200 rpm	253	1

[a]See section "Materials and Methods"

1.042 to 1.061. Since the external phase has a density of 1.040, the difference between the density of the 2 phases tends to increase with increasing PLA concentrations. According to Stoke's law (13), the internal and external phase should have approximately the same density to ensure an optimal stability of the dispersion. Therefore, the nanoparticle mean size rise could be partly attributed to density variations of the internal phase. On the opposite, when nanoparticles are produced using acetone as organic solvent (5), the nanoparticle size decreases with increasing Eudragit® S concentrations. In this case, increasing the polymer concentration from 10 to 20% decreases the difference of density between the internal and external phases from 0.021 to 0.002. It should be stressed that the reference to the Stoke's law is simply used to help understanding the above-mentioned phenomenon, even though this approach cannot be strictly applied to these extremely small emulsion droplets due to the Brownian motion.

Besides PLA (Fig. 5), the nanoparticles can be produced using other biodegradable polymers (Table 1, examples No. 4 and 5) in the internal phase without any marked modification of the nanoparticle mean size. One should also note that the sonication of the system (example No. 2) during the preparation process does not allow any further reduction of the particle size. However, by increasing the stirring rate to 5000 rpm (example No. 3), it becomes possible to decrease substantially the particle mean size and consequently, to lower the amount of PVAL needed to produce very small nanoparticles.

The scanning electron micrograph of PLA nanoparticles (Fig. 6) following purification and freeze-drying shows relatively monodispersed smooth nanospheres. These nanoparticles (213 nm, polydispersity index of 2) were prepared using 17% PVAL in the external phase. The residual PVAL remaining at the surface of the particles amounted to 9.79 ± 0.22% (m/m) of the total mass of freeze-dried product. In a previous study (6), it has been shown that the residual PVAL amount was roughly linearly related to the specific surface area of the particles. This hydrophilic layer allows a very fast redispersion of freeze-dried nanoparticles in water. During the purification process, 97.4% of the benzyl alcohol was eliminated after the purification of the particles using 4 l of water. Since the nanoparticles

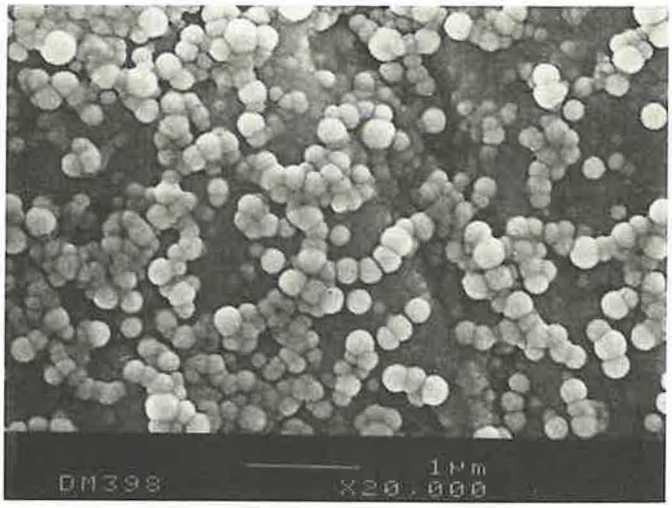

Fig. 6 Scanning electron micrograph of PLA nanoparticles

Table 2 Characterization of chlorambucil loaded PLA nanoparticles
External phase: PVAL 14% in water or buffer, 40 g; internal phase: PLA 12.8% and chlorambucil 10 or 15%, 21 g

Batch No.	Initial drug content (% m/m)	External phase[a]	Size before freeze-drying (nm)[b]	Size after freeze-drying (nm)[b]	Batch weight yield (%)c	Entrapment efficiency (%)	Drug loading (m/m %)	Residual PVAL (m/m %)
1	10	unbuffered	282	283	83.7	63.0	5.94	5.76
2	10	buffered, pH 5[d]	271	294	82.3	62.0	5.87	5.28
3	15	unbuffered	284	295	90.7	60.1	8.52	5.50

[a]Includes the dilution phase
[b]Polydispersity 2
[c]Batch yield after preparation, purification and freeze-drying
[d]Phosphate buffer 1/15 M

are washed with 10 l of water and are subsequently freeze-dried, the residual amount of benzyl alcohol should be very low.

An attempt was made to load these nanoparticles with a cytostatic drug (Table 2). Chlorambucil is a very slightly water-soluble drug according to USP XXVII. It is an alkylating agent active at very low doses and is used for its antineoplasic properties, mainly in the treatment of leukemias and lymphomas. Among the side-effects reported, central neurotoxicity renders this drug potentially interesting for an incorporation into nanoparticles (7).

As shown in Table 2, the entrapment efficacy is acceptable and a relatively important drug content does not alter the nanoparticle mean size. Chlorambucil is an acid and thus has even a lower solubility in acidic media. Therefore, as previously observed, the acidification (3) and alkalinization (6) of the external phase can modifiy the entrapment efficacy, depending on the drug. However, preparation of the nanoparticles using a PVAL solution at pH 5 (batch No. 2) and a dilution solution at the same pH did not increase the entrapment efficacy as compared to an external phase without pH adjustment (batch No. 1). These results may be explained by a rapid drug saturation of the external phase of both acidic and neutral media. Actually, most of the drug loss is likely to occur during the extensive washing procedure. The entrapment efficacy remains approximately the same whether a 10 or 15% initial drug content is used suggesting, for these concentrations, that its incorporation mechanism depends on its partition coefficient (14). As most of the methods based on the emulsification of an organic solvent in an aqueous medium, this technique should be preferentially used to entrap lipophilic compounds (6, 9). Other methods such as those based on the dispersion of natural macromolecules (15) or those involving polymerization reactions (16, 17) are more appropriate for the incorporation of hydrophilic drugs.

4 Conclusion

The present study has shown that it is possible to produce drug-loaded nanoparticles with the emulsification-diffusion process using benzyl alcohol as organic solvent. Due to the partial miscibility of this solvent with water, the use of salting-out agents is no longer required. Furthermore, benzyl alcohol is a generally accepted solvent for injectables and can be present in pharmaceutical preparations in concentrations up to 2% (7). Accordingly, the purified particles can contain residual benzyl alcohol without being a potential health hazard. Finally, it has to be mentioned that this new manufacturing procedure requires large amounts of dilution water. However, this is no major problem, because the nanoparticle suspension can be easily concentrated during the purification process (8).

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