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Biocompatible stabilizers in the preparation of PLGA nanoparticles: a factorial design study

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Abstract

Poly(lactic-co-glycolic-acid) nanoparticles are often produced using the w/o/w emulsification solvent evaporation method. In most cases poly(vinyl alcohol) (PVA) is used as stabilizer of the emulsion. The goal of this study was to compare a series of polymers to PVA in a 2^2 full factorial design study. The influence of the concentration of PVA and the polymers tested on particle size and zeta potential value was evaluated before and after freeze-drying of the prepared particles. Nanoparticles were obtained with most polymers when they were used in combination with PVA. Leaving PVA out of the formulation, however, increased the size of the particles over 1 μm. Two exceptions are poloxamer and carbopol, which can be considered as valuable alternatives to PVA. Zeta potential values were usually slightly negative, the most extreme zeta potential values were measured when poloxamer and carbopol were employed. The use of gelatin type A made it possible to achieve positive values. The original 2^2 full factorial design study was further expanded to a central composite design for poloxamer and carbopol, in order to fit the measured data to a quadratic model and to calculate response surfaces.

Keywords: PLGA; Nanoparticles; Emulsification solvent evaporation; Stabilizer; Experimental design; Response surface

1. Introduction

Because of their biodegradability and biocompatibility, polylactic acid and its copolymers with glycolic acid (PLGA) are widely employed for the preparation of sustained release preparations (Anderson and Shive, 1997). They are used for the production of implants, inserts and particulate systems. Especially micro- and nano-particles made of PLGA copolymers are widely investigated for the controlled release of classical drug molecules as well as peptides and proteins. The administration routes vary from parenteral (Das et al., 2000), oral (Coombes et al., 1997), dermatological (De Galon et al., 2001) pulmonary (O'Hara and Hickney, 2000) and nasal (Tobio et al., 1998) to ocular (Veloso et al., 1997; Moritera et al., 1992, 1991).

Several methods were proposed for the preparation of PLGA microspheres, such as extrusion (Zhang et al., 1994), spray drying (O'Hara and

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Hickney, 2000) and supercritical fluid extraction (Kompella and Koushik, 2001). The technique mostly used, however, is the emulsification solvent evaporation method (O'Donnell and McGinity, 1997). It involves the solution of the PLGA polymer in an organic solvent, emulsifying the PLGA solution in a non solvent (mostly water) and precipitating the PLGA polymer as particles by evaporating the organic solvent. Lipophilic drugs are incorporated by dissolving them in the organic solvent along with PLGA. For hydrophilic drugs the w/o/w emulsification solvent evaporation is used, dissolving the drug into the inner water phase of the double emulsion.

In most cases, a stabilizer is added to the formulation in order to stabilize the emulsion formed during particle preparation. These stabilizers, however, can also influence the properties of the particles formed. The type and concentration of the stabilizer selected may affect the particle size. Being present at the boundary layer between the water phase and the organic phase during particle formation, the stabilizer can also be incorporated on the particle surface, modifying particle properties such as particle zeta potential and mucoadhesion (Scholes et al., 1999; Feng and Huang, 2001). Both size and zeta potential value are important physicochemical particle properties, as they determine the physical stability as well as the biopharmaceutical properties of the preparation. Drug release rate, biodistribution, mucoadhesion and cellular uptake can all be influenced by the type and concentration of the stabilizer used.

In the literature, poly(vinyl alcohol) (PVA) is the most popular stabilizer for the production of PLGA nanoparticles. In the present study other polymers were tested as stabilizers. These polymers were incorporated as such and in combination with PVA. The aim was to study how the use of other polymers in the preparation of nanoparticles would affect particle size and zeta potential value. The effect of the presence or absence of PVA during preparation as well as the effect of the concentration of the alternative stabilizers was evaluated. Considering the stability of PLGA particles, the effect of the freeze-drying process on the particle size and zeta potential was also studied.

The polymers evaluated as stabilizers in this study are cellulosic derivatives methylcellulose (MC), hydroxyethylcellulose (HEC), hydroxypropylcellulose (HPC) and hydroxypropylmethylcellulose (HPMC), as well as gelatin type A and B, carbomer and poloxamer. Some of these polymers have been reported as adjuvants in the preparation of PLGA particles, such as poloxamer (Scholes et al., 1999; De Rosa et al., 2000; O'Hara and Hickney, 2000; Couvreur et al., 1997), gelatin (Tobio et al., 1998; Arshady, 1991), HPMC (Gabor et al., 1999; Sansdrap and Moës, 1993), MC (Arshady, 1991) and carbopol (Wang et al., 1991). Moreover, HPMC, poloxamer and carbomer are interesting compounds because of their mucoadhesive properties (Takeuchi et al., 2001). Gelatin type A and B were both selected because of their difference in isoelectric point, resulting in different electrical charges. A difference in zeta potential of the particles produced with gelatin type A and B was thus expected.

Each polymer was compared to PVA using a 2^2 full factorial design. This rational methodology allows for the determination of the influence of the factors investigated and their interactions requiring a minimum of experiments. Moreover, the design was expanded to a central composite design, enabling the modelling of the responses as a function of the parameters investigated. This allows an estimation of the particle properties for a certain combination of polymer and PVA within the experimental region.

2. Materials and methods

2.1. Materials

The PLGA polymer used was Resomer® 503 H with a molecular weight of 34000 (Boehringer Ingelheim, Germany). Poly(vinyl alcohol) (PVA) MW 30000–70000 was supplied by Sigma, USA. As alternatives for PVA, the following polymers were employed: methylcellulose (MC): Methocel® MC, 4000cP (Fluka, USA); hydroxyethylcellulose (HEC): Natrosol® 250G (Aqualon, USA); hydroxypropylcellulose (HPC): Klucel® 99H (Aqualon); hydroxypropylmethylcellulose

(HPMC): Benecel® MP 943 R (Aqualon); gelatin type A: from porcine skin, bloom 175 (Sigma); gelatin type B: from bovine skin, bloom 225 (Sigma); carbomer: Carbopol® 980 NF (BF Goodrich, USA) and poloxamer: Lutrol® F68 (BASF, Germany). Dichloromethane was purchased from Sigma-Aldrich (Germany).

2.2. Experimental design

2.2.1. 2^2 full factorial design with centerpoint

2.2.1.1. Design of the experiments. Various polymers were evaluated as stabilizers in the production of PLGA nanoparticles using a two level full factorial design with centerpoint. The two factors investigated were the concentration of PVA and the concentration of the polymer tested in the outer water phase. For the concentration of PVA, the upper (+), centerpoint (0), and lower (−) level values are 1% w/v, 0.5% w/v and 0% w/v, respectively, as was described in an earlier study (Vandervoort and Ludwig, 2000). The concentrations of the polymers tested were chosen as follows. At the centerpoint, an aqueous solution having the same viscosity as a 1% w/v PVA aqueous solution was set. These concentrations were derived from capillary viscosimetric measurements. For the upper level this concentration was multiplied by two, for the lower level it was divided by two.

Points 1 and 2 are preparations in which the polymer tested is used simultaneously with PVA. In experiments 3 and 4 no PVA is present during PLGA particle preparation.

The same design was applied for all polymers investigated. At the centerpoint preparations were made in triplicate in order to estimate the experimental error. After obtaining preparations 1–5, the particles' physical properties were measured. Effects of and interactions between parameters were calculated.

2.2.1.2. Calculation of effects and interactions. To calculate the effect E_x of a factor x all measurements where the factor is at its lower (−) level were subtracted from all those where the factor was at its upper (+) level and subsequently divided through half of the number of measurements used in the calculation. This results in the following formula (Box et al., 1978):

$$E_x = \frac{\sum x(+) - \sum x(-)}{4/2} \quad (1)$$

The effect of PVA on the particle size, for example, was estimated by subtracting the sum of the particle sizes measured at points 3 and 4 from the sum of those measured at points 1 and 2 and dividing the result by 2.

Interactions between components were also calculated. An interaction between PVA and a polymer tested is present when the effect of the polymer is not the same at the two levels of PVA. To estimate an interaction between two factors one has to calculate the effect of the first factor at the lowest level of the second factor and subtract it from the effect of the first factor at the highest level of the second factor. An interaction between two factors is symbolized as factor 1 × factor 2.

To estimate the significance of the effects a t-test is performed. The test statistic t equals:

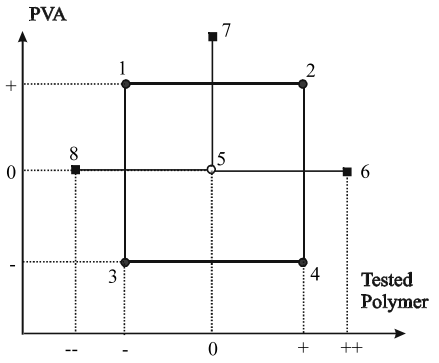
$$t = \frac{E_x}{(\mathrm{SE})_e} \quad (2)$$

E_x is the effect of a factor and $(\mathrm{SE})_e$ is the standard error on the effect. The replicates of the centerpoint were used to estimate $(\mathrm{SE})_e$. In this case, $(\mathrm{SE})_e$ equals s, the standard deviation on the results measured at the centerpoint.

The calculated test statistic t is compared to a tabulated t-value at a significance level of 95% ($\alpha = 0.05$). If the calculated t-value is higher than the tabulated t-value the effect is considered to be significant.

2.2.2. Central composite design

For the two most promising polymers, the 2^2 full factorial design was expanded to a central composite. The advantage of this method is that by adding just three extra 'star' points to the initial factorial design, extra levels for both factors are created resulting in four levels for PVA and five levels for the polymers tested. This allows for the fitting of the experimental data to a quadratic model. Once the model is calculated it



	Level		Conc. (%w/v)	
point	PVA	Polymer	PVA	Polymer
1	+	-	1	C/2
2	+	+	1	Cx2
3	-	-	0	C/2
4	-	+	0	Cx2
5	0	0	0,5	C
6	0	++	0,5	$C+C\sqrt{2}$
7	++	0	1,21	C
8	0	--	0,5	$C-(C/2)\sqrt{2}$

C: concentration giving the same viscosity as a 1% PVA aqueous solution measured by capillary viscometry

Fig. 1. Central composite design (●) 2^2 full factorial design; ○ centerpoint; (■) star points.

can be used to predict a certain response, in this case zeta potential value and particle size, for a known composition of stabilizers in the preparation. The 2^2 full factorial design and its expansion to a central composite design is presented in Fig. 1.

The following quadratic model was used to fit the data:

$$\text{Response} = a + b \times \text{PVA} + c \times \text{PVA}^2 + d \times \text{Polymer} + e \times \text{Polymer}^2 + f \times \text{PVA} \times \text{Polymer} \quad (3)$$

To perform the statistical analysis of the data, the Statistica® software was employed (Statsoft, Tulsa, OK).

2.3. Preparation of polymer solutions

The concentrations of the polymer solutions used in the present study are presented in Table 1. PVA, MC and HEC solution were prepared by dispersing the polymer in distilled water at 70 °C under magnetic stirring. In the case of HPC the water was brought to a temperature of 50 °C, while HPMC, poloxamer and carbopol were dispersed in distilled water at room temperature. The carbopol dispersion was afterwards neutralized by adding a NaOH solution to a pH value of 7. The gelatin solutions were prepared by dispersing the gelatin in cold distilled water, allowing the gelatin particles to swell, and afterwards heating the dispersion to 50 °C under magnetic stirring (Thermolyne HP46820-26, Dubuque, IO).

2.4. Particle preparation

The PLGA particles were prepared using a w/o/w emulsification solvent evaporation method (Vandervoort and Ludwig, 2000). Two microliters of distilled water (w1) were emulsified with a solution of PLGA (10% w/v) in 10 ml of dichloromethane using an ultrasonic probe (Branson Sonic Power S.A., Danbury, CT) for 1 min. This primary w1/o emulsion was poured into 50 ml of a stabilizer solution (w2) and sonication was continued for 30 s. Finally, the w1/o/w2 emulsion was poured into a larger volume (400 ml) of a stabilizer solution (w3) in order to increase the distance between emulsion droplets and to minimize coalescence and aggregation of the particles being formed. The preparation was stirred with a

Table 1
Concentrations (% w/v) of polymers used in the experimental design

Polymer	Level of polymer in design				
	−	0	+	++	−−
PVA	0.000	0.500	1.000	1.210	
MC	0.038	0.076	0.152		
HEC	0.050	0.100	0.200		
Carbopol	0.006	0.012	0.024	0.029	0.003
HPMC	0.038	0.076	0.152		
HPC	0.018	0.036	0.072		
Gelatin A	0.234	0.467	0.934		
Gelatin B	0.305	0.609	1.218		
Poloxamer	1.100	2.199	4.398	5.325	0.640

propeller (IKA Eurostar digi-visc, IKA labortechnik, Staufen, Germany) for 2 h at 700 rpm to allow the dichloromethane to evaporate and the PLGA to precipitate as particles. The particle suspension was then stored in a refrigerator. Part of the suspension was kept as such for direct size and zeta potential measurements. Another part of the preparation was freeze-dried immediately after preparation (GT-2a, Leybold-Heraeus, Germany).

The concentrations of the polymers in the first outer water phase w2 are mentioned in Table 1. The concentrations in the second outer water phase w3 are those used in w2 but divided by three.

2.5. Physical measurements

2.5.1. Particle size

Particle size was determined by photon correlation spectroscopy (PCS) with a Zetasizer 3000 (Malvern Instruments, Malvern, UK). Non freeze-dried samples were diluted ten times with distilled water. The lyophilized product was resuspended in distilled water using mild magnetic stirring before measuring. The Z ave of each sample was measured at least five times and the mean value was calculated and used for the factorial design and response surface calculations.

2.5.2. Particle zeta potential

To determine particle zeta potential values laser doppler anemometry (LDA) was used. Before measuring, each sample was diluted ten times with distilled water. Freeze-dried samples were resuspended in distilled water under magnetic stirring. About 10 ml of dispersion was injected into the capillary of the Zetasizer 3000 (Malvern Instruments). Each sample was measured at least ten times. The average values were employed for the calculations of the factorial design and the response surfaces.

3. Results and discussion

3.1. 2^2 Full factorial design with centerpoint

3.1.1. Particle size

A graphical representation of the particle size of PLGA nanoparticles obtained is given in Fig. 2. Particle sizes ranging from 300 to 3000 nm were measured. For most polymers, the sizes measured in points 3 and 4 of the design are larger than those measured in points 1 and 2. This means that when PVA is left out of the formulation, particle size increases. This can be explained by the fact that most polymers tested are not such good stabilizers, resulting in the formation of larger droplets during emulsion preparation and subsequently larger precipitated particles. The size of the effects is presented in Table 2.

For most preparations, the addition of PVA to the formulation has a negative effect on the particle size, meaning that particle size decreases as the PVA concentration increases. Two exceptions are carbopol and poloxamer. In the case of carbopol the effect of the concentration of PVA is slightly positive, both before and after freeze-drying. This can be explained by the fact that carbopol itself is a good stabilizer. Adding PVA to the formulation causes an increase in the viscosity of the outer water phase, resulting in larger emulsion droplets and larger particles. In the case of poloxamer the effect of PVA is almost zero before freeze-drying and + 107 nm after freeze-drying.

The effect of the concentration of the polymers tested is negative or positive. A positive effect would imply that increasing the concentration causes the emulsion to have larger droplets, hence leading to larger particles. A negative effect means that increasing the concentration causes the emulsion to be more stable, hence leading to smaller particles. The stabilizing properties of the polymers can be explained by either their viscosity, their ability to lower surface tension or their three dimensional structure at the interface (Couvreur et al., 1997).

Freeze-drying has either a significant positive effect, or a non-significant smaller effect. It could be expected that particle size would increase after lyophilization, because nanoparticles tend to aggregate during this process. If the aggregated particles do not separate during redispersion, then larger particle sizes will be measured. Polymers with cryoprotecting properties such as poloxamer and carbopol should, when present at the nanoparticle surface, protect the particles from

Table 2
Effects of the concentration of PVA, the polymers tested and freeze-drying on the particle size of the PLGA nanoparticles

Factor	Stabilizers							
	MC	HEC	Carbopol	HPMC	HPC	Gelatin A	Gelatin B	Poloxamer
Effect of the stabilizer concentrations on the particle size before freeze drying (nm)								
Conc. PVA	**−729**	**−596**	**+79**	**−1249**	**−356**	**−375**	**−1234**	0
Conc. polymer	**+608**	**+72**	**−21**	**+1076**	**+88**	−177	**−861**	−38
PVA × polymer	**−602**	−41	**+31**	**−957**	**+1**	+31	**+812**	**+184**
Effect of the stabilizer concentrations on the particle size after freeze drying (nm)								
Conc. PVA	**−553**	**−953**	**+44**	**−1055**	**−951**	**+689**	**−2062**	+107
Conc. polymer	**+923**	**+407**	**+107**	**+1004**	**+444**	**+468**	**−577**	**+194**
PVA × polymer	**−471**	**−351**	**+64**	**−1137**	**−335**	**+580**	**+696**	**+138**
Effect of the freeze-drying process on the particle size (nm)								
Freeze-drying	+206	**+152**	+22	+73	**+266**	**+716**	−10	+81

The effects and interactions that were statistically significant are printed bold ($P = 0.05$); '×' symbolizes an interaction between factors.

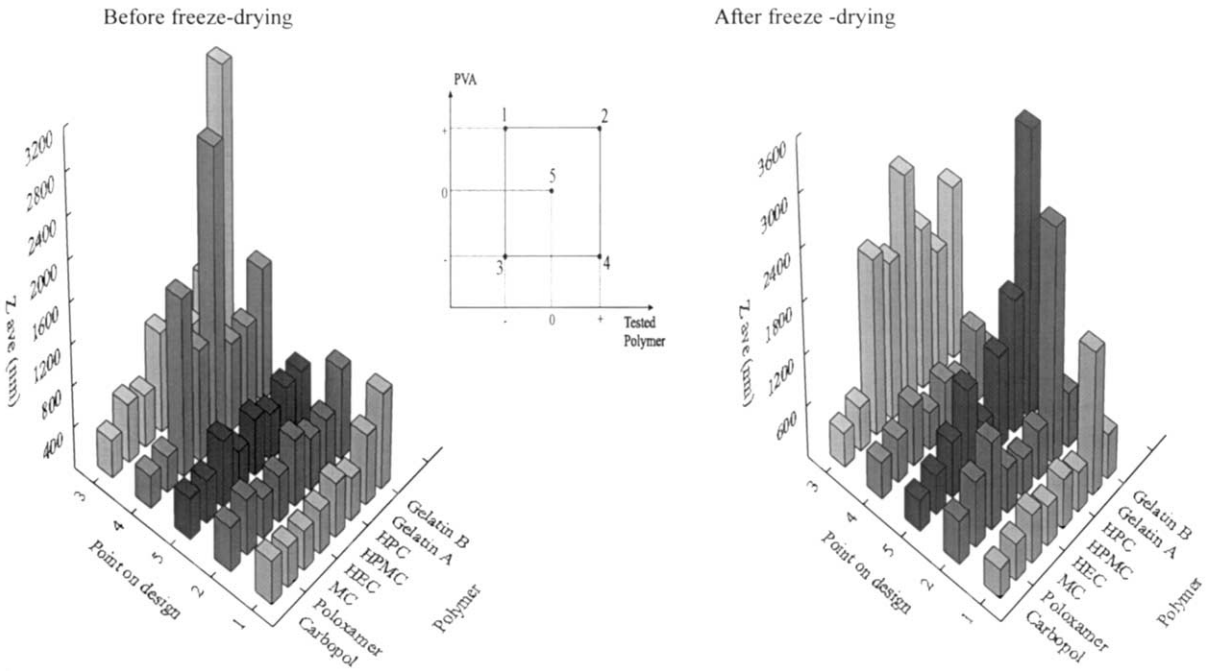


Fig. 2. Graphical representation of the particle size of PLGA nanoparticles before and after freeze-drying.

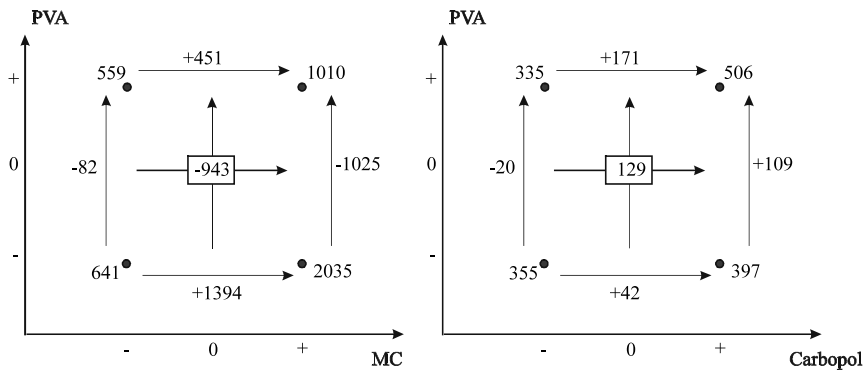


Fig. 3. Interaction effect between MC and PVA and Carbopol and PVA on PLGA nanoparticle size (nm).

aggregation and make sure the redispersion requires a minimum of energy, being attributed to the formation of a steric barrier between the particles during lyophilization or to a stabilization of the particle dispersion due to electrostatic repulsions, which will be discussed in Section 3.1.2 (Quintanar-Guerrero et al., 1998).

Some of the interactions are also significant and their effect is sometimes quite large, indicating that the effect of the polymer tested without the presence of PVA in the preparation is different from the effect when PVA is present. The interaction effect is illustrated with the plots presented in Fig. 3. In the first plot the size data obtained from

the experiments with MC and PVA are printed at the points representing the experiments (the centerpoint was left out of the drawing). At the lowest (−) level of PVA the particle size obtained at a low concentration of MC is 641 nm, while at the higher concentration 2035 nm was measured. Consequently, the effect of the concentration of MC is + 1394 nm. Making the same calculations at the high (+) level of PVA results in an effect of + 451 nm. Thus going from the low to the high level of PVA concentration, the effect of MC changes from + 1394 to + 451 nm. The interaction is calculated as 451 − 1394 nm = − 943 nm. This kind of effect is called a negative interaction.

In the second plot an example of a positive interaction is presented. At the low PVA level the effect of the concentration of carbopol is only + 42 nm, compared to + 171 nm at the high PVA level. In this case the effect of the first factor (carbopol) increases as the level of the second factor (PVA) changes from − to + . This positive interaction can be calculated as 171 − 42 nm = + 129 nm. The data for the interactions found in Table 2 are equal to the ones calculated here, but divided by two, on the analogy of Eq. (1).

In conclusion, for most polymers tested the presence of PVA is needed in order to produce nanoparticles. Two polymers seem to be able to yield nanoparticles without the addition of PVA as stabilizer: carbopol and poloxamer. The particle size is an important particle property, as it can influence the biopharmaceutical properties of the particle preparations. Smaller particles have a larger free surface, which can lead to a faster release of a drug incorporated (Gabor et al., 1999). The biodistribution of the particles may also depend on the particle size. A possible endocytosis of the particles, for example, is size dependent (Zimmer et al., 1991; Calvo et al., 1994). Interaction with the mucous membranes is also partly determined by particle size (Takeuchi et al., 2001). From the results presented above, it can be concluded that particle size can be controlled by either altering the concentration of PVA or the concentration of the other stabilizer in the formulation. However, in order to establish the relationship between the combination of concentrations of stabilizers and the particle size, more experimental points are needed, as will be discussed in Section 3.2.

3.1.2. Zeta potential

The results of the zeta potential measurements are presented in Table 3. Zeta potential values of the prepared PLGA particles vary between 0 and − 50 mV. Most particles have slightly negative zeta potential values. Particles produced with poloxamer and carbopol, however, have more pronounced negative zeta potential values. The only stabilizer yielding nanoparticles with a positive zeta potential value is gelatin type A.

The size of the effects and interactions are shown in Table 4. The effect of the concentration

Table 3
Zeta potential values (mV) of the various PLGA nanoparticle preparations

	Before freeze-drying					After freeze-drying				
Point on design	1	2	3	4	5	1	2	3	4	5
Level of PVA	+	+	−	−	0	+	+	−	−	0
Level of tested polymer	−	+	−	+	0	−	+	−	+	0
MC	−16.9	−12.0	−4.5	−3.7	−17.8	−14.0	−12.7	−4.5	−4.5	−13.7
HEC	−21.5	−19.3	−17.6	−21.0	−20.5	−13.9	−10.8	−6.6	−0.2	−16.4
Carbopol	−19.6	−19.5	−50.6	−52.5	−27.0	−19.8	−27.2	−46.5	−39.7	−34.8
HPMC	−15.1	−13.5	−2.5	−1.4	−14.6	−18.8	−9.4	−2.6	−0.9	−18.4
HPC	−20.2	−16.9	+0.1	−2.2	−16.7	−13.0	−10.5	−2.4	−0.8	−14.1
Gelatin A	+14.2	+12.8	+13.3	+14.3	+12.9	+15.2	+13.0	+10.6	+12.5	+8.7
Gelatin B	−8.2	−5.7	−5.3	−4.3	−10.0	−9.2	−4.8	−10.8	−5.1	−8.9
Poloxamer	−20.3	−23.3	−49.4	−41.9	−28.0	−15.8	−10.6	−53.6	−40.0	−20.7

Table 4
Effects of the concentration of PVA and the tested polymers on the zeta potential value (mV) of the PLGA nanoparticles prepared

	MC	HEC	Carbopol	HPMC	HPC	Gelatin A	Gelatin B	Poloxamer
Before freeze-drying								
Conc. PVA	**−10.4**	−1.1	**+32.0**	**−12.4**	**−17.5**	−0.3	−2.2	**+23.9**
Conc. polymer	+2.9	−0.6	−0.9	+1.4	+0.5	−0.2	+1.8	**+2.3**
PVA × polymer	+2.1	+2.8	+1.0	+0.3	+2.8	**−1.2**	+0.8	**−5.3**
After freeze-drying								
Conc. PVA	−8.9	**−9.0**	**+19.6**	**−12.4**	**−10.2**	**+2.6**	+1.0	**+33.6**
Conc. polymer	+0.7	**+4.8**	−0.3	**+5.6**	+2.1	−0.1	**+5.1**	+9.4
PVA × polymer	+3.5	**+9.5**	−3.7	**+13.9**	**+9.0**	**+0.9**	**+3.8**	−7.7

The effects and interactions that were statistically significant are printed bold ($P = 0.05$).

of PVA seems to be not significant or negative for most polymers, indicating that the zeta potential value will be more negative when PVA is added to the formulation. However, the effect of PVA is + 23.9 mV before and + 33.6 mV after freeze-drying in the case of poloxamer, and + 32.0 mV before and + 19.6 mV after lyophilization in the case of carbopol. This means that for these polymers the most negative zeta potential values are measured when PVA is left out of the formulation. The effect of the concentration of the polymers tested is less pronounced than that of PVA. In fact, before freeze-drying, the only polymer where the concentration was found to have a significant influence on the zeta potential value of the PLGA nanoparticles was poloxamer. This can probably be explained by the fact that the differences between the upper and lower levels of the polymers tested are less extreme than those of PVA. Indeed, when one compares the upper and lower level of PVA, one compares preparations with (1% w/v) and without (0% w/v) PVA. The polymers tested, however, are present in all preparations of the design, the only difference between their levels being their concentration.

The zeta potential value is an important particle characteristic as it can influence both particle stability as well as particle mucoadhesion. In theory, more pronounced zeta potential values, being positive or negative, tend to stabilize particle suspensions. The electrostatic repulsion between particles with the same electrical charge prevents the aggregation of the spheres (Feng and Huang, 2001). The stabilization of the particle suspension during freeze-drying of the preparations with poloxamer and carbopol, which was demonstrated in Section 2.5.1, can thus be explained by the fact that these preparations also show the largest zeta potential values. Mucoadhesion, on the other hand, can be promoted by a positive zeta potential value. The mucus layer itself is at a neutral pH value an anionic polyelectrolyte (Bayens and Gurny, 1997). Consequently, the presence of positively charged groups on the particles could lead to electrical charge interactions between the mucus and the particles. In this study, the only positively charged particles obtained were those prepared with gelatin type A as stabilizer. Unfortunately, the size of these particles was quite large. But the particles prepared with carbopol or poloxamer also offer interesting possibilities, as their anionic surfaces could be coated with cationic polymers, thus improving their mucoadhesive properties (Takeuchi et al., 2001).

As was noted for the particle size, zeta potential values can be controlled by either altering the concentration of PVA or the concentration of the alternative stabilizer in the formulation. To establish the relationship between the combination of concentrations of stabilizers and the particles zeta potential value, the experimental design was expanded to a central composite design for the two most promising stabilizers.

3.2. Central composite design

3.2.1. Introduction

The 2^2 full factorial design was expanded to a central composite design in the case of poloxamer and carbopol because both polymers induced a pronounced zeta potential value, do not require the presence of PVA to form PLGA nanoparticles and possess good cryoprotecting properties.

The following quadratic model was used to fit the data:

$$\text{Response} = a + b \times \text{PVA} + c \times \text{PVA}^2 + d \times \text{Polymer} + e \times \text{Polymer}^2 + f \times \text{PVA} \times \text{Polymer} \quad (4)$$

The response (zeta potential value or particle size) is described as the sum of a basic value (a) and four other terms of which the value depends on the concentration of PVA and the polymer investigated. The influence of the concentration of PVA is split in a linear term (b) and a quadratic term (c). Parameters d and e are the linear and quadratic terms for the tested polymer, while f is a measure for the interaction between PVA and the polymer tested.

After preparing the particles and measuring their zeta potential values and particle sizes, the data is fit into the model and parameters a to f are calculated. This creates the possibility to predict the zeta potential value and the particle size of any combination of PVA and polymer concentration within the experimental region. The models can also be used to optimize a certain response or even particle size and zeta potential value at the same time. One could, for example, calculate the combination of PVA and polymer tested that results in the smallest particles, or the most negative zeta potential value and in doing so change the particle properties depending on the particle size and zeta potential, required in relation to bio- or mucoadhesion, distribution and release rate required for a certain drug or route of administration.

3.2.2. Poloxamer

The concentrations of poloxamer used for the preparation of the nanoparticles were presented in Table 1. After performing zeta potential and size measurements the data was fit to a quadratic model of which the parameters are summarized in Table 5. A graphical representation of the measured points and the response surfaces is given in Fig. 4 and Fig. 5.

The parameter values for the zeta potential values indicate that the zeta potential rises when PVA is added to the formulation. The effect of the concentration of poloxamer (parameter d) is also positive, but to a much smaller extent. Consequently, the most negative zeta potential values can be obtained by leaving PVA out of the formulation and using low concentrations of poloxamer. The interaction term f is also quite small. These observations are consistent with the observations of the 2^2 full factorial design. The terms c and e which represent the curvature of the response surfaces reveal that most of the curvature is found along the axis of the concentration of PVA, as can be seen in Fig. 4.

Table 5
Parameters of fitted model in the case of poloxamer used as stabilizer in the preparation of PLGA nanoparticles

	Parameter					
	a	*b*	*c*	*d*	*e*	*f*
Zeta potential (mV)						
Before freeze-drying	−54.5	+52.4	−23.9	+6.1	−0.8	−2.6
After freeze-drying	−57.7	+86.4	−48.5	+3.9	0.0	−2.2
Particle size (nm)						
Before freeze-drying	+667.0	−345.4	+56.9	−137.2	+16.5	+109.2
After freeze-drying	+420.1	−176.4	−22.4	+62.3	−12.0	+93.6

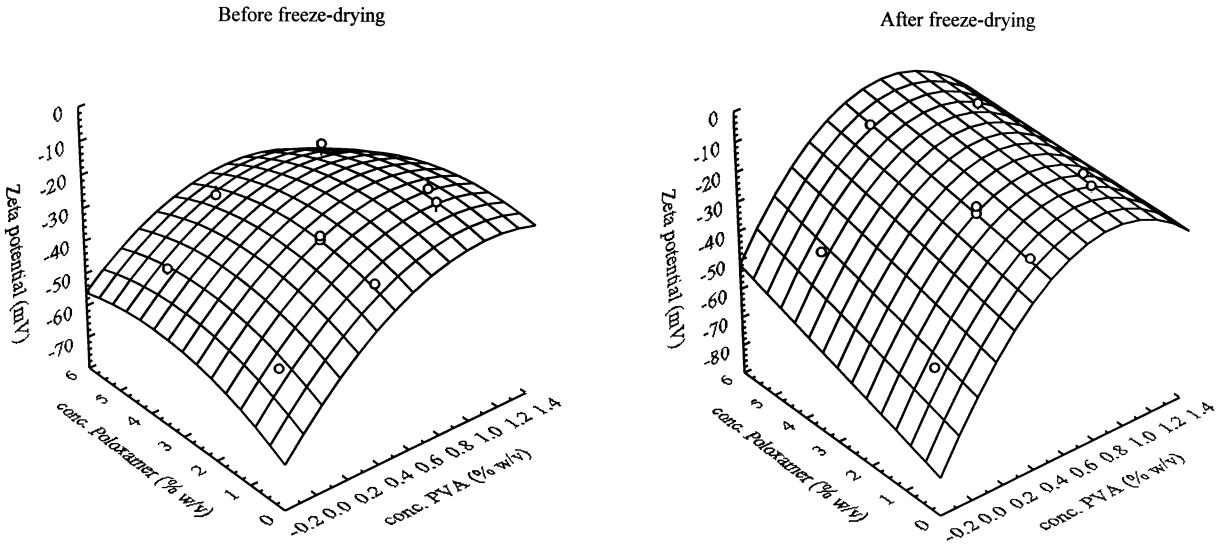


Fig. 4. Response surfaces for zeta potential before and after freeze-drying of PLGA nanoparticles prepared with poloxamer and/or PVA as stabilizers.

Table 6
Parameters of fitted model in the case of carbopol used as stabilizer in the preparation of PLGA nanoparticles

	Parameter					
	a	*b*	*c*	*d*	*e*	*f*
Zeta potential (mV)						
Before freeze-drying	−63.5	55.5	−26.4	2004.0	−61 194.4	5.2
After freeze-drying	−40.7	16.9	−7.7	−13.7	−12 074.0	226.4
Particle size (nm)						
Before freeze-drying	293.2	188.5	−97.7	5269.8	−116 573.6	−1763.3
After freeze-drying	396.0	−56.0	−86.0	2993.0	−216 375.0	12 068.0

The response surfaces for the particle size show the strong interaction effect between PVA and poloxamer. In the plot of data measured before freeze-drying of the preparation one can see that the effect of PVA on the particle size is negative at low levels of poloxamer (the curve is going down), while at higher poloxamer concentrations the particle size of the nanoparticles increases when the concentration of PVA increases. This gives the response surface a twisted view. Parameter *f* is a measure for this interaction effect. Factors *c* and *e* have quite small values, resulting in a small curvature of the surfaces.

3.2.3. Carbopol

The concentrations of carbopol used for the preparation of the PLGA nanoparticles were presented in Table 1. The size of the parameters *a* to *f* is given in Table 6. A graphical representation of the measured points and the response surfaces is illustrated in Fig. 6 and Fig. 7.

As with poloxamer, the influence of the addition of PVA on the zeta potential value of the PLGA nanoparticles is clear. Parameters *c* and *e* become smaller after freeze-drying of the preparation, resulting is a less curved surface, as can be seen in Fig. 6. Some parameters have very high values, such as parameters *d* and *e* before freeze-

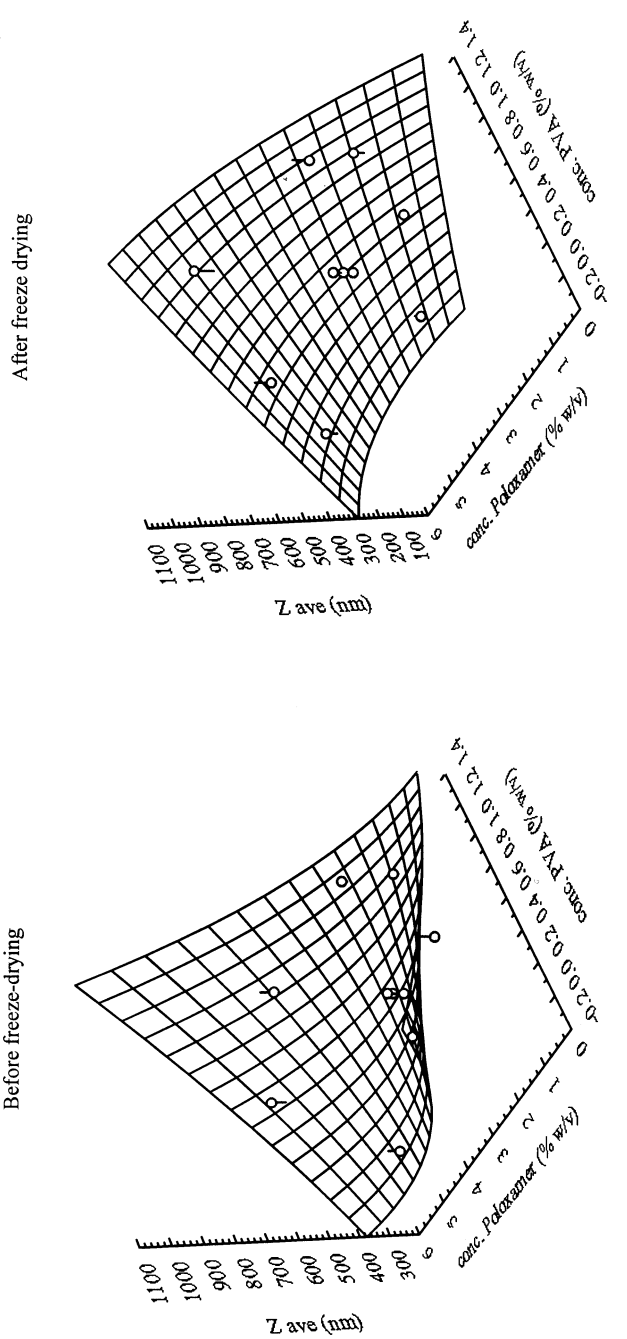


Fig. 5. Response surfaces for particle size before and after freeze-drying of PLGA nanoparticles prepared with poloxamer and/or PVA as stabilizers.

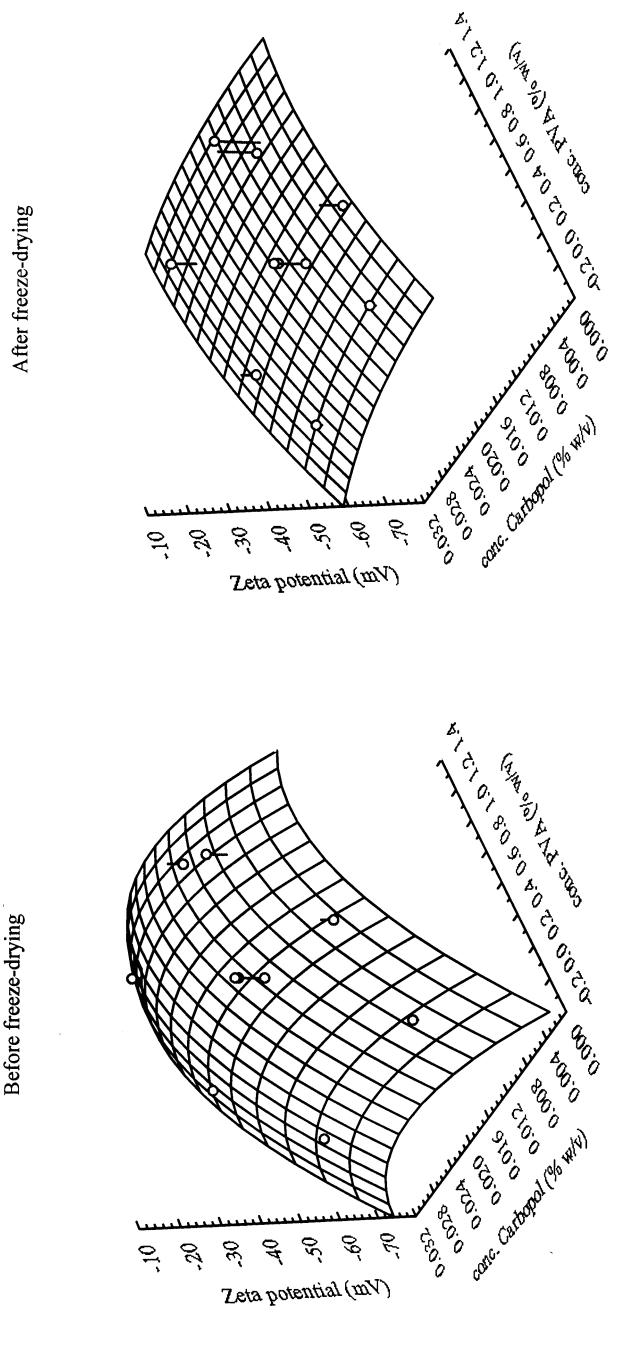


Fig. 6. Response surfaces for zeta potential before and after freeze-drying of PLGA nanoparticles prepared with carbopol and/or PVA as stabilizers.

drying and *e* and *f* after freeze-drying. These parameters, however, do have opposite signs, thereby decreasing their influence on the zeta potential of the nanoparticles. Parameters *d* and *e*, for example determine the influence of the concentration of carbopol on the zeta potential. When the concentration of carbopol in the preparation increases, the zeta potential value should increase dramatically according to factor *d*. The negative value of factor *e*, however, compensates for this effect. Consequently, although some parameters have very high values, the response surfaces do not look so different from the ones drawn in Fig. 4.

The most striking effect observed in Fig. 7 is the much more twisted look of the response surface after freeze-drying PLGA particles prepared using PVA and carbopol. This is confirmed by the much higher value of the parameter *f* after freeze-drying the preparation. This positive interaction effect on the particle size was also discussed in Section 3.1.1. The smallest particles are obtained at either high levels of carbopol and low levels of PVA or at low levels of carbopol and high levels of PVA. This can be explained by the fact that both carbopol and PVA are good stabilizers for the emulsion formed during the preparation of the particles. A high concentration of carbopol or PVA alone is enough to stabilize the emulsion resulting in small nanoparticles. At the lowest level of both carbopol and PVA, the concentration of stabilizer is below optimal, resulting in a poorer stabilization of the emulsion, and consequently in larger emulsion droplets and larger PLGA nanoparticles. At the highest concentration of PVA and carbopol, however, there is more than enough stabilizer present, but the viscosity of the stabilizer solution is higher. Consequently, at a constant energy input of the ultrasonic probe, when the w/o emulsion is added to the w2 water phase, larger emulsion droplets are formed in the w/o/w emulsion. Although these droplets are then stabilized by carbopol and PVA, larger particles are obtained.

4. Conclusions

After testing various polymers as alternatives for PVA as stabilizers in the production of PLGA

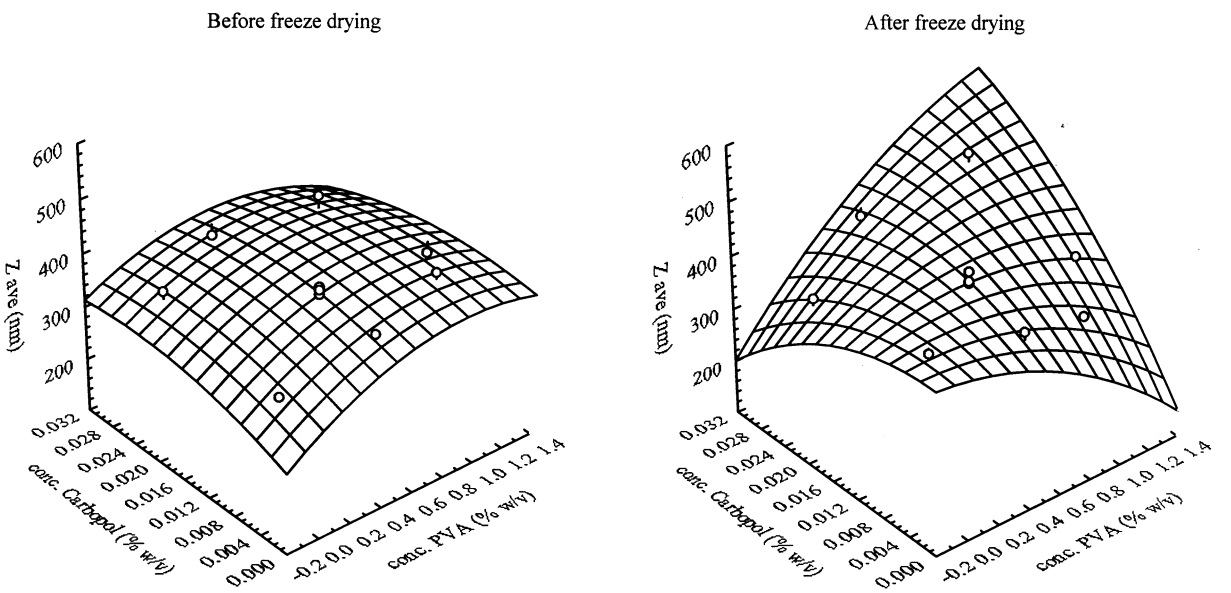


Fig. 7. Response surfaces for particle size before and after freeze-drying of PLGA nanoparticles prepared with carbopol and/or PVA as stabilizers.

nanoparticles using the w/o/w emulsification solvent evaporation technique the following conclusions can be drawn.

Nanoparticles were obtained with most polymers when they were used in combination with PVA. Leaving PVA out of the formulation in most cases increases the size of the particles over 1 μm. This indicates that most polymers tested are not able to stabilize the emulsion as well as PVA does. Two exceptions are poloxamer and carbopol, which can be considered as valuable alternatives for PVA. A further study of these two polymers using a central composite lead to the fitting of the responses to a quadratic model. This creates the possibility of calculating a formulation for PLGA nanoparticles with a desired particle size and zeta potential value, depending on the biopharmaceutical properties required.

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