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Preparation factors affecting the properties of polylactide nanoparticles: a factorial design study

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PLGA nanoparticles were produced using a w/o/w emulsification solvent evaporation method incorporating pilocarpine HCl as a model drug. The influence of four preparation parameters on the particle properties was studied. The factors studied were the concentration of the stabilizer in the outer water phase, the presence of buffer in the outer water phase, the amount of drug relative to the amount of polymer and the type of PLGA used. Particle size was influenced by the concentration of PVA in and the addition of buffer to the outer water phase. The ratio drug/polymer had an effect on the drug entrapment.

1. Introduction

One of the main problems in ophthalmic drug delivery is the rapid elimination of conventional eye drops resulting in a low bioavailibility of the drug applied. Rapid reflex blinking, induction of lachrymation caused by irritation and the relatively large volume of the administered eye drop lead to a high rate of lachrymal drainage. Moreover, due to its structure the cornea is a very difficult barrier to pass [1]. As a result of the rapid elimination of eyedrops the medication has to be administered frequently resulting in a diminished patient compliance. Absorption of the drained drug at the nasal mucosa can also cause marked side-effects [2].

Therefore alternative administration systems are investigated which allow the dosage form to stay longer at the eye surface and to improve the resorption of the drug. Examples of such systems are microemulsions, gels, inserts and particulate systems such as liposomes, micro-and nanoparticles.

The aim of the present study was to formulate nanoparticles of the polymer poly(lactic-co-glycolic acid) (PLGA). PLGA is a copolymer of lactic acid and glycolic acid. Because of its biocompatibility and biodegradability it is used and investigated for a wide range of applications. In the field of ophthalmology, PLGA has been used mainly for the formulation of sustained release preparations for intravitreal use and also for topical applications [3–5]. The model drug to be incorporated was pilocarpine HCl, a parasympathomimetic drug employed in the treatment of glaucoma.

A w/o/w solvent evaporation technique is used to produce the PLGA nanospheres. The influence of four preparation parameters on particle properties is investigated. These four factors are the concentration of the stabilizer polyvinyl alcohol (PVA) in the outer water phase, the presence of buffer in the outer water phase, the amount of drug relative to the amount of polymer and the type of PLGA used. The particle properties investigated are the size and drug entrapment. Particle size will determine the distribution of the particles in the tear film and the lower cul-de-sac during blinking. The rate at which the particles are cleared through the lachrymal puncti after instillation may also be size-dependend. Finally, a possible penetration of the nanoparticles themselves into the cornea by endocytosis will depend on the particle size [6].

To investigate the influence of the four preparation parameters on these two particle properties, a factorial design is used. With this technique the influence of the four factors investigated as well as their interactions can be studied with a minimal number of experiments.

2. Investigations, results and discussion

2.1. Particle size measurements

The factors investigated in the present study and their values at the upper (+) and lower (−) level examined are presented in Table 1. A symbol is assigned to each factor and will be used for further reference in all Tables and graphics.

The results of the particle size measurements are summarized in Table 2. Particle sizes ranging from 400 to 1400 nm were measured. To estimate the effects of the investigated parameters on the particle size the measure-

Table 1: Factorial design: investigated factors and levels

Symbol	Factor	Value at + level	Value at − level
A	Ratio pilocarpine HCl/PLGA	1/20	1/5
B	Type of PLGA polymer	Resomer® 503 H	Resomer® 503
C	Presence of buffer in outer water phase	Buffer present	No buffer present
D	Concentration PVA in outer water phase	1%	2%

Table 2: Particle size measurement results A: Ratio pilocarpine HCl/PLGA B: Type of PLGA polymer C: Presence of buffer in outer water phase D: Concentration of PVA in outer water phase

Factors				Particle size Zave (nm)	
A	B	C	D	Mean	Standard deviation
+	+	+	+	608	52
+	+	−	−	428	7
+	−	+	−	906	15
−	+	+	−	710	63
+	−	−	+	427	21
−	+	−	+	465	67
−	−	+	+	599	27
−	−	−	−	422	9
+	+	+	−	966	8
+	+	−	+	421	16
+	−	+	+	680	27
−	+	+	+	598	39
+	−	−	−	469	80
−	+	−	−	481	33
−	−	+	−	800	192
−	−	−	+	406	23

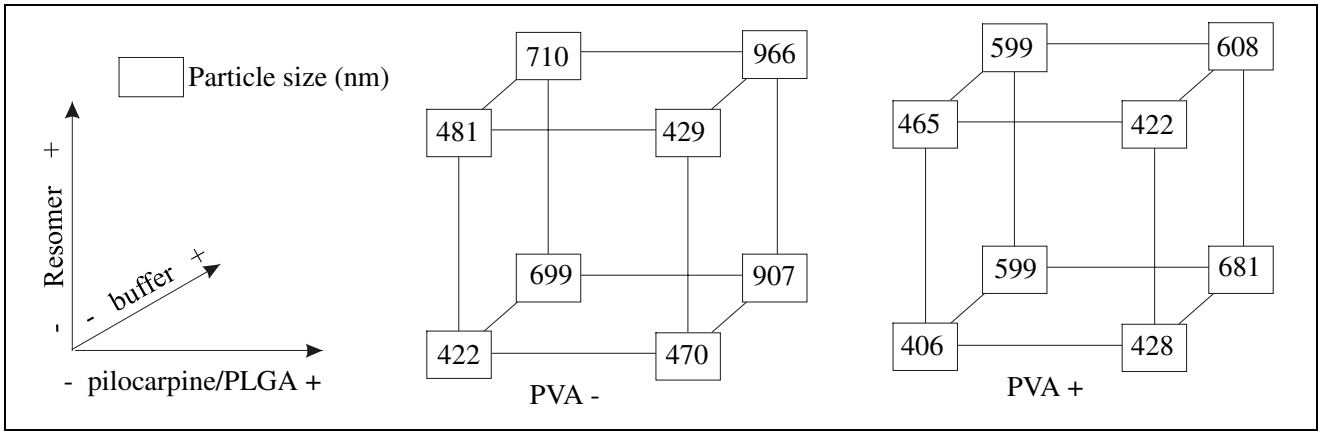


Fig. 1: Graphical representation of the particle size measurements

ments can be presented graphically as in Fig. 1. The mean values of the particle size measurements are represented at the corners of two cubi. This makes a visual evaluation of the most important effects possible. To estimate the effect of the concentration of PVA, for example, one has to compare the results in the right cube to those in the left cube. The effect of addition of buffer to the outer water phase can be estimated by comparing the results in the front of the cubi to those in the back. Looking at the measurement data in this way reveals that two factors have an important influence on particle size: the concentration of PVA and the presence of buffer salts in the outer water phase. This is confirmed by the normal probability plot which is shown in Fig. 2. The size of the effects can be calculated and is given in Table 3. The significance of the effects is tested by means of a t-test. The presence of buffer has an effect of 50% on the particle size, indicating that when buffer is added to the formulation, the particle size increases by 50%. The factor PVA has an effect of −29.9%. This means that when the PVA concentration is changed from its lower (−) value (conc. 2% w/v) to its upper (+) value (conc. 1% w/v) particle size decreases by almost 30%. The opposite effect was observed by Rafati et al. and Erden et al. [7–8]. They argue that the increase of PVA concentration causes the emulsion to be more stable. In the present work the lowest PVA concentration was probably already sufficient to stabilize the emulsion. Further addition of PVA causes the particle size to increase.

The addition of buffer salts also seems to affect the stabilization of the emulsion as particle size increases with 50% after addition of buffer. Possibly, the addition of salts has a negative effect on the stability of the emulsion. The interaction of factor C (buffer) and D (concentration PVA) has a smaller, but still significant effect. A graphical representation of this interaction is shown in Fig. 3. At the low level of factor C (no addition of buffer) the influence of factor D is only 21 nm (= 451 nm – 430 nm). However, when buffer is added during preparation of the nanoparticles the influence of the concentration of PVA changes to 224 nm (= 846 nm – 622 nm).

To investigate wether the influence of the concentration of PVA and the addition of buffer salts on the particle size could be explained as an effect on the viscosity of the outer water phase, the kinematic viscosity of the solutions used in the preparations was measured (see Fig. 4). These results show a similar interaction between concentration of PVA and addition of buffer as is observed for the particle size. At low PVA concentrations (D+) the addition of buffer salts to the solution has no effect on the viscosity. However, at a concentration of 2% PVA (w/v), adding buffer causes the viscosity to rise. Thus, the particle size changes in a similar way as does the viscosity. When the energy output of the ultrasonic probe is kept constant an increased viscosity of the outer water phase may lead to greater emulsion droplets and subsequently to larger particles.

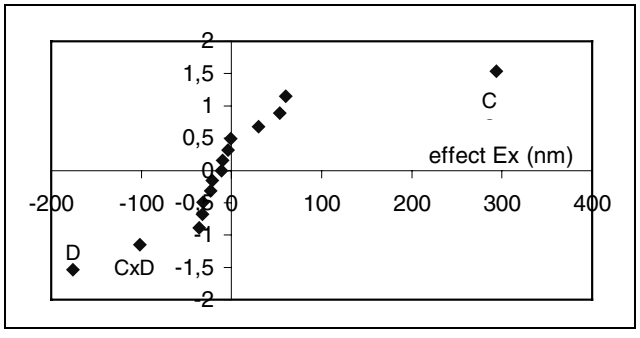
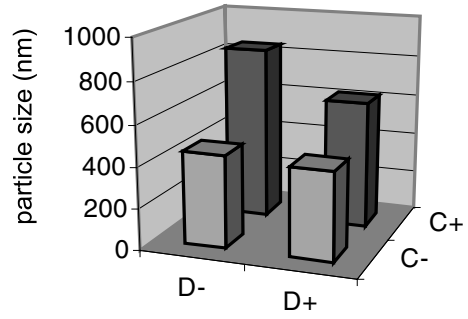


Fig. 2: Half normal probability plot for particle size; C: Presence of buffer in outer water phase D: Concentration of PVA in outer water phase; C × D: interaction between factor C and D

Table 3: Size and significance of effects of preparation parameters on particle size; S: significant; NS: not significant; ×: interaction between factors

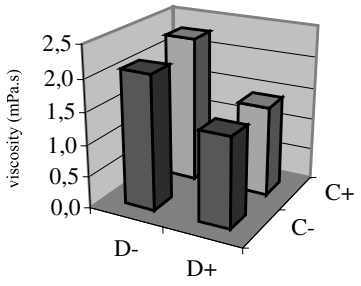
Factor	E_x (nm)	E_x (%)	$T = E_x/(SE)_e$	Significance
A	53.3	9.1	1.729	NS
B	−3.9	−0.7	−0.127	NS
C	293.5	50.0	9.524	S
D	−175.7	−29.9	−5.700	S
A × B	−11.0	−1.9	−0.357	NS
A × C	59.9	10.2	1.942	NS
A × D	−35.9	−6.1	−1.165	NS
B × C	−21.6	−3.7	−0.701	NS
B × D	−0.9	−0.2	−0.029	NS
C × D	−101.9	−17.4	−3.305	S
A × B × C	30.2	5.2	0.981	NS
A × C × D	−31.8	−5.4	−1.033	NS
A × B × D	−23.2	−3.9	−0.751	NS
B × C × D	−9.8	−1.7	−0.316	NS
A × B × C × D	−31.9	−5.4	−1.035	NS

$\sum s_i^2$	60777.55
s_p^2	3798.60
$(SE)_e$	30.82
t (n = 32. p = 0.05)	2.037



	D-	D+
C-	451	430
C+	846	622

Fig. 3: Graphical representation of the interaction between factor C and D



	D-	D+
C+	2,29	1,41
C-	2,10	1,41

Fig. 4: Graphical representation of the effects of factor C and D on the viscosity of the PVA solutions

2.1. Drug entrapment

Drug loading measurement results and effect calculations are summarized in Figs. 5 and 6 and in Tables 4 and 5. Drug entrapment is defined as:

$$\text{Drug entrapment (\%)} = \frac{\text{mass of drug in nanoparticles} \times 100}{\text{mass of drug used in formulation}}$$

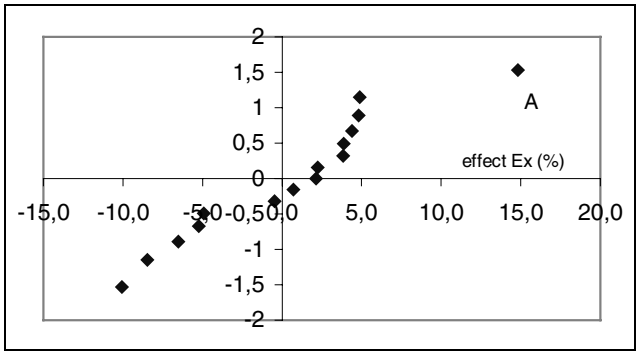


Fig. 6: Half normal probability plot for drug entrapment; A: Ratio pilocarpine HCl/PLGA B: Type of PLGA polymer C: Presence of buffer in outer water phase D: Concentration of PVA in outer water phase

Table 4: Drug entrapment measurements A: Ratio pilocarpine HCl/PLGA B: Type of PLGA polymer C: Presence of buffer in outer water phase D: Concentration of PVA in outer water phase; SD: standard deviation

Factors				Drug entrapment (%)				
A	B	C	D	replica 1	replica 2	replica 3	mean	standard deviation
+	+	+	+	49.1	32.8	59.2	47.0	13.3
+	+	−	−	*29.8*	51.6	55.4	45.6	2.6
+	−	+	−	54.5	32.1	49.8	45.4	11.8
−	+	+	−	33.8	20.1	41.7	31.8	10.9
+	−	−	+	*31.1*	96.3	75.0	67.4	15.1
−	+	−	+	33.9	32.4	40.3	35.5	4.1
−	−	+	+	40.0	32.9	28.6	33.8	5.7
−	−	−	−	38.1	32.1	*7.3*	25.8	4.2
+	+	+	−	28.9	11.8	89.8	43.5	41.0
+	+	−	+	88.0	48.7	81.9	72.8	21.1
+	−	+	+	*3.5*	36.9	57.6	32.6	14.6
−	+	+	+	34.4	44.8	48.0	42.4	7.1
+	−	−	−	48.5	63.2	59.0	56.8	7.5
−	+	−	−	36.2	43.1	46.4	41.8	5.2
−	−	+	−	35.7	46.0	43.1	41.6	5.2
−	−	−	+	41.5	35.1	43.5	40.0	4.4

Drug entrapments were measured varying from 3.5% tot 89.8%. For some preparations, very large differences were found between replica's.

The poor reproducibility of the encapsulation of pilocarpine HCl in PLGA nanoparticles results in high values for the variance of the three replicates and consequently in high values for s_p^2 and $(SE)_e$. This causes the t-test values to be low and no effects could be shown to be significant. Therefore the large differences between replicates should

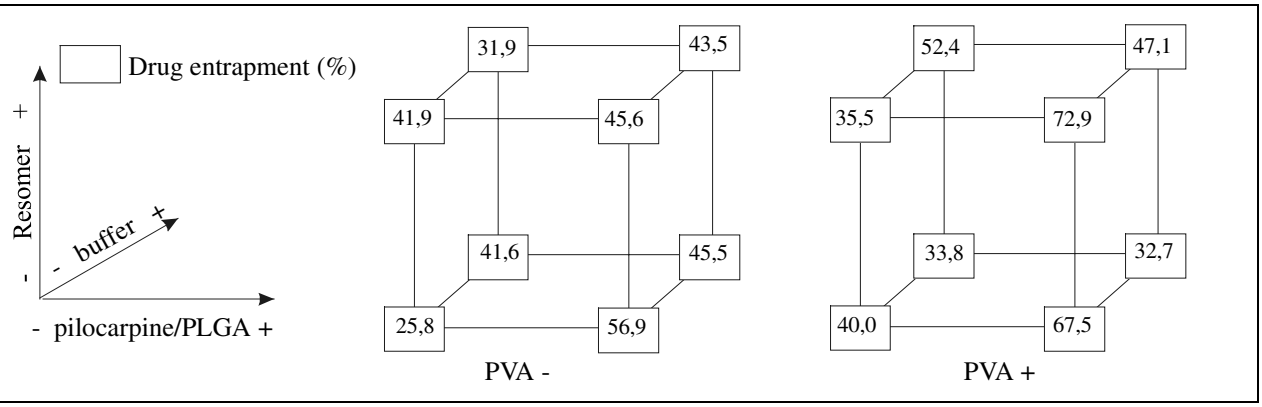


Fig. 5: Graphical representation of drug entrapment measurements

Table 5: Size and significance of effects of preparation parameters on drug entrapment; S: significant; NS: not significant; ×: interaction between factors

Factor	E_x	E_x (%)	E_x / $(SE)_e$	significance
A	18.75	39.77	2.536	S
B	−2.13	−4.53	−0.289	NS
C	−11.05	−23.43	−1.494	NS
D	6.84	14.50	0.925	NS
A × B	−2.43	−5.16	−0.329	NS
A × C	−10.35	−21.95	−1.400	NS
A × D	6.50	13.79	0.879	NS
B × C	1.31	2.78	0.178	NS
B × D	−0.08	−0.18	−0.012	NS
C × D	−4.82	−10.22	−0.652	NS
A × B × C	2.18	4.64	0.296	NS
A × C × D	−5.86	−12.43	−0.793	NS
A × B × D	−1.84	−3.90	−0.249	NS
B × C × D	5.10	10.82	0.690	NS
A × B × C × D	−2.31	−4.89	−0.312	NS

$\sum s_i^2$	3499.5
s_p^2	218.72
$(SE)_e$	7.39
t (n = 32. p = 0.05)	2.037

be further investigated. However, when the results printed in italics in Table 4 are considered as outliers and left out of the calculations, one effect seems to be significant, factor A being the ratio pilocarpine HCl/ PLGA. The size of the effect of this factor is 33.7%. This would mean that the drug entrapment increases with 33.7% as the concentration of pilocarpine HCl in the inner water phase is increased from 2.5% to 10% (m/v). The addition of buffer did not increase drug entrapment, although this technique has been noted to be useful for other drug molecules. The pH is set to such a level that the drug to be encapsulated appears in the non-ionised, less aqueous soluble, form. This should decrease the migration of the drug molecule from the inner to the outer water phase. This technique has already been successfully applied to increase the encapsulation of ketoprofen [9], loperamide [10], phenobarbital sodium [11] and lactoglobulin [12]. For pilocarpine no increase in drug entrapment was found. A possible explanation is that the free base of pilocarpine HCl is still fairly soluble in water. Also, the pH level of the buffered solution was 7.5 at which not all pilocarpine is in its basic form. Further increase of the pH value, however, would catalyse the degradation of the drug [13].

3. Experimental

3.1. Materials

The PLGA polymers used were Resomer® 503 and Resomer® 503 H from Boehringer Ingelheim (Ingelheim, Germany). The inherent viscosity of the Resomer polymers was 0.32–0.44 dl/g (0.1% in chloroform at 25 °C). Pilocarpine HCl was obtained from Federa (Brussels, Belgium). PVA (M.W. 30000-70000) was from Sigma Chemicals (St-Louis, MO, USA). For the preparation of the borate buffer boric acid (Certa, Braine l'Alleud, Belgium) and NaOH (Merck, Darmstadt, Germany) were used. Methylene chloride was obtained from Aldrich (Gillingham, Dorset, UK). Deionized, freshly distilled water was used throughout the study.

3.2. Experimental design

The experimental design used in this study is a four factor two level full factorial design. The influence of four preparation parameters (factors) is investigated by choosing two levels for each factor and preparing particles with all the possible combinations of levels for all the factors. This results in $2^4 = 16$ different experiments. All preparations were made in triplicate, yielding a total of 48 preparations. The investigated factors and their values at the upper (+) and lower (−) level are presented in Table 1. The experiments are randomised to diminish the influence of time-dependend factors.

After the particles are produced the following responses are measured: particle size and drug entrapment.

To estimate which factor has an effect on a certain response graphical methods are used, such as representing the measurement data in cubi as in Figs. 1 and 5 and by drawing normal probability plots. To calculate the effect E_x of a factor all measurements where the factor is at its lower (−) level are substracted from all the measurement where the factor is at its upper (+) level and subsequently divided through half of the number of measurements used in the calculation. For this study this results in the following formula:

$$E_x = \frac{\sum x(+) - \sum x(-)}{16/2} .$$

To estimate the significance of the effects a t-test is performed.

The test statistic t equals:

$$t = \frac{E_x}{(SE)_e} .$$

E_x is the effect of a factor and $(SE)_e$ is the standard error on the effect. As each preparation is made in triplicate the variance on the results from these replicates is used to estimate $(SE)_e$.

After calculating the variance for each triplicate, the pooled variance s_p^2 for all replicates is calculated.

$$s_p^2 = \frac{\sum_{i=1}^{N} (n_i - 1)\, s_i^2}{\sum_{i=1}^{N} (n_i - 1)} = \frac{2 \cdot \sum_{i=1}^{N} s_i^2}{16 \cdot 2} = \frac{\sum_{i=1}^{N} s_i^2}{16}$$

with n_i being the number of replicates

$(SE)_e$ is then calculated using the following equation:

$$(SE)_e = \sqrt{\frac{2s_p^2}{n}} = \sqrt{\frac{2s_p^2}{8}} = \sqrt{\frac{s_p^2}{4}}$$

with n being N/2 = 16/2 = 8

The calculated test statistic t is compared to a tabulated t-value for 32 $(= (3-1) \cdot 16)$ degrees of freedom at a significance level of 95% $(\alpha = 0.05)$. If the calculated t-value is greater than the tabulated t-value the effect is considered to be significant.

3.3. Particle production

PLGA particles are prepared using an emulsification solvent evaporation method. The required amount of pilocarpine HCl (2.5% or 10% w/v) is dissolved in 2.0 ml of distilled water. This solution is 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, USA) for 1 min at 80 W. This primary W/O emulsion is poured into 50 ml of an aqueous PVA solution (1 or 2% w/v) and sonication is continued for 30 s. Finally, the W/O/W emulsion is poured into 400 ml of a PVA solution (0.3% w/v) and stirred with a propeller (IKA Eurostar digi-visc, IKA Labortechnik, Staufen, Germany) for 2 h at 700 rpm to allow the dichoromethane to evaporate and the PLGA to precipitate as particles. The particle suspension is then stored in a refrigerator.

For the preparations with buffer, boric acid (0.31% w/v) is dissolved in the PVA solutions and NaOH solution (0.2 M) is added to reach a pH level of 7.5.

3.4. Physical measurements

3.4.1. pH

The pH of solutions was determined at room temperature with a Orion 420 A pH meter (Orion, Beverley, MA, USA).

3.4.2. Viscosity

The viscosity of solutions was measured by means of an appropriate viscosimeter (KPG Viskosimeter, Schott-Geräte, Mainz, Germany). Samples were measured at room temperature (25 °C) and each sample was measured at least four times.

3.5. Particle evaluation

3.5.1. Size measurements

Particle size is determined using photon correlation spectroscopy (PCS) with a Zetasizer 3000 (Malvern Instruments, Malvern, UK). Before each measurement the samples are diluted 25 times with distilled water. Two ml

of the diluted sample are put in a quartz cuvette and placed in the Zetasizer. For each sample, the particle size (Zave) is measured at least 3 times. The duration of the measurements is determined by the software of the zetasizer. The average values of the measurements are used for further calculations.

3.5.2. Drug entrapment

Drug entrapment is determined indirectly by measuring the amount of pilocarpine HCl which was not encapsulated. A small volume of the particle suspension is diluted five times in distilled water. Five ml of this dilution is brought in an ultrafiltration device (Vivascience, Lincoln, UK) and centrifuged for 10 min at 3000 g. The M.W.C.O. of the PES membrane of the ultrafiltration device is such that nanoparticles and PVA polymer chains are retained at the filter surface while free pilocarpine HCl molecules can pass through. After centrifugation the device is filled with five ml of distilled water and centrifuged for another 10 min at 3000 g. This rinsing procedure is repeated once more. The rinsing solutions are added to the first filtrate and destilled water is added to a total volume of 20.0 ml.
The pilocarpin HCl concentration is than determined by means of UV spectroscopy at 215 nm using a spectrofotometer U 2001 (Hitachi, Japan). Once the amount of free pilocarpin HCl is determined the amount of encapsulated pilocarpine can be calculated, as well as the drug entrapment.

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