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Optimization of HSA and NGF encapsulation yields in PLGA microparticles

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Abstract

The aim of this study was to prepare, in a reproducible manner, poly(D,L-lactide-co-glycolide) (PLGA) biodegradable microparticles with high encapsulation yield of nerve growth factor (NGF), a neurotrophic factor of interest in the treatment of neurodegenerative diseases. Human serum albumin (HSA) was co-encapsulated with NGF by the w/o/w emulsion solvent evaporation/extraction method to stabilize the primary emulsion and to protect NGF. The encapsulation yield optimization was first carried out with HSA alone since it was the major component in the final microspheres, as compared to NGF. The effects of ten process factors on HSA entrapment in PLGA microspheres were examined using a fractional factorial design. Four major factors were identified. The presence of carboxymethylcellulose sodium or mannitol in the internal aqueous phase, the increase of the internal aqueous volume and the concentration of HSA led to a decrease of the encapsulation yield. Two main factors, namely the internal aqueous phase volume and the proportion of acetone in the organic phase, previously identified as a predominant factor, were studied through a response surface methodology. The observed range of HSA encapsulation yield was 87.4–100%. NGF was encapsulated according to the optimal conditions found for the entrapment of HSA. The encapsulation yield was then $97.3 \pm 5.0\%$.

Keywords: NGF; Microparticle; Experimental designs; Emulsion/extraction process; PLGA; Protein delivery system

1. Introduction

The neurotrophic factors (NTF), among them the nerve growth factor (NGF), are proteins

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which cannot cross the blood brain barrier (Krewson et al., 1995) and of growing interest for the treatment of neurodegenerative diseases (Hefti, 1994). However, the neurotrophic receptors have a wide distribution even in non-neuronal tissues (Shibayama and Koizumi, 1996) and display roles other than those classically thought (Lewin, 1995; Scully and Otten, 1995) suggesting that therapeutics involving neurotrophic factors require to deliver them exclusively to the target cell population. To attain this objective a few groups previously developed an approach based on the intracerebral implantation of biodegradable poly(D,L-lactide-co-glycolide) (PLGA) sustained-release microspheres (Camarata et al., 1992; Menei et al., 1994; Mittal et al., 1994; Krewson et al., 1996). The feasibility of this approach was recently demonstrated in clinics by our group (Benoit et al., 1997).

The most appropriate method used to prepare protein-loaded microspheres was the w/o/w double emulsion technique. In this method, the drug was emulsified in an organic polymer solution which, in turn, was dispersed into an external aqueous phase. Based on this process, several studies reported the encapsulation of NGF or other NTFs in poly(D,L-lactide) (PLA) or poly(D,L-lactide-co-glycolide) (PLGA) matrices to investigate the effect of their sustained release on various biological phenomena (Camarata et al., 1992; Maysinger et al., 1993; Mendez et al., 1994; Mittal et al., 1994; Krewson et al., 1996). NTF encapsulation ratio was generally evaluated by assaying the protein in the release medium but very little information was given on the encapsulation process itself. Although conceptually simple, several factors influenced the characteristics of the final product; some of them such as the type of stabilizing agent, the presence of sodium chloride in the dispersing phase, etc., were studied (Uchida et al., 1995; Herrmann and Bodmeier, 1995; Blanco-Prieto et al., 1996; Conway and Alpar, 1996; Diaz et al., 1997; Rafati et al., 1997). But, the complexity of the method imposed to study several factors at once what was never achieved.

The aim of this study was to prepare, in a reproducible manner, 20 μm NGF-loaded PLGA microspheres with an optimized drug encapsulation yield using the experimental design approach. The encapsulation process retained was the w/o/w emulsion solvent evaporation/extraction method. Human serum albumin (HSA) was added to NGF since it was known as a NGF carrier used in aqueous solutions (Knepp et al., 1994) and a potential stabilizer that may be useful in the microencapsulation process. In a first step, a screening approach based on fractional factorial design was used to select the factors displaying the most significant effects on the albumin encapsulation yield. In a second step, the most significant factors were deeply investigated according to a response surface to optimize the albumin encapsulation yield. Finally, the NGF was introduced in the optimized process and the corresponding encapsulation yield was determined.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-co-glycolide) (PLGA 25/50) was supplied by Boehringer Ingelheim (Resomer® RG 502, BI Chimie, Paris, France). The gross compositions of the chains were 25% D-lactic units, 25% L-lactic units and 50% glycolic units. The mean molecular weight ($\bar{M}w$) as determined by steric exclusion chromatography, was 10000 ($I = 2.5$). HSA was purchased from Sigma (fraction V, 69 kDa, Sigma Chemical, St. Quentin Fallavier, France), ^{125}I-HSA from Amersham (HA injection BP, specific activity 2.5×10^{-3} μCi/μg, Les Ulis, France), NGF from Promega (2.5 S murine, 28 kDa, Charbonnières, France), ^{125}I-NGF from NEN (receptor grade 2.5 S NGF murine, specific activity 33–81 μCi/μg, Les Ulis, France) and poly(vinyl alcohol) (PVA) from Prolabo (Rhodoviol®, 4/125, 88% hydrolyzed, Paris, France). Methylene chloride and acetone (Prolabo, Paris, France) were used without further purification.

A sonicator (Microson XL2007, 100 W, 23 kHz, microprobe 3.2 mm, Misonix, Prolabo, Paris, France) and a mechanical stirrer (Heidolph RGH 500, Prolabo, Paris, France) were used to prepare the primary and secondary emulsions, respectively. Mannitol and CMC Na (apparent

viscosity of a 2% aqueous solution, 1500 mPa/s) were supplied by Cooper (Melun, France).

2.2. Preparation of microparticles

Microspheres were prepared by a w/o/w emulsion solvent evaporation/extraction method as previously reported (Marchais et al., 1996). In brief, an internal aqueous phase (citrate buffer 0.0016 M, pH 6.0, HSA 0.1%, w/v) containing additional HSA with NGF (30 μg NGF/2.5 mg HSA) or without NGF was prepared. This phase was emulsified in an organic solution composed of PLGA dissolved in methylene chloride (screening step) or methylene chloride/acetone mixture (optimization step) (2 ml). The first emulsion was obtained at 4°C by sonication at ouput 5 (5 W) in a polytetrafluoroethylene (PTFE) vial to limit the protein adsorption. The resultant emulsion was added with a syringe to 8–10°C polyvinyl alcohol aqueous solution (5%, w/v) under mechanical stirring (800 rpm). The double emulsion was poured into a large volume of extracting water (150 or 400 ml) under magnetic stirring to allow a rapid organic solvent removal. Finally, the resulting microparticles were collected by filtration (0.45 μm HVLP type filter, Millipore SA, St. Quentin-Yvelines, France), washed five times with 100 ml deionized water, freeze-dried (RP2V Serail, SGD, Argenteuil, France) and stored at 4°C.

2.3. Microparticle characterization

The size determination was carried out using a Coulter® Multisizer (Coultronics, Margency, France) after dispersion of microparticles in a conducting liquid (Isoton® II, Coultronics, Margency, France). Scanning electron microscopy (SEM) was used to observe the internal structure of particles. Freeze-dried microparticles were mounted on metal stubs using double-sided adhesive tape. The adhesive tape with stuck particles was firstly folded on itself and secondly roughly unfolded to fracture the microparticles. Then, samples were vacuum-coated with a carbon film (Ion Sputter JFC 1100, JEOL, Paris, France) and directly analysed by SEM (JSM 6301F, JEOL, Paris, France). In the screening step, the amount of entrapped HSA was determined as follows. Digestion of the microparticles was achieved by treating them (10 mg) in 2 ml 1 N NaOH at room temperature for 20 min. The solution was then neutralized with 0.1–1 N hydrochloric acid and diluted in PBS buffer (pH 7.4). The resulting solution was analysed in triplicate using the Bio-Rad protein assay. HSA standard solutions were prepared from 0 μg/ml to 20 μg/ml in PBS buffer (pH 7.4). The encapsulation yield (Y_1) was defined as follows:

$$Y_1 = \text{Experimental encapsulation ratio} / \text{Theoretical encapsulation ratio}$$

or,

$$Y_1 = \frac{\text{mg drug recovered in microparticles}}{\text{mg microparticles}} \times \frac{\text{mg(PLGA + drug + additives)}}{\text{mg drug introduced in the process}} \times 100$$

In the optimization step, a fraction of ^{125}I-labelled HSA (80 μg in 4 μl buffer) was added in the internal aqueous phase and mixed with unlabelled HSA. The amount of entrapped HSA in microspheres was evaluated by counting the fraction of ^{125}I-labelled HSA successfully entrapped in the particles. Finally, to determine the NGF encapsulation yield, a fraction of ^{125}I-labelled NGF (4 ng in 4 μl buffer) was added in the internal aqueous phase containing unlabelled NGF and HSA. The radioactivity was counted with a Minaxi autogamma 5000 series (Packard, Rungis, France). The encapsulation yield was defined as follows:

$$Y_1 = \frac{\text{recovered cpm in microparticles}}{\text{introduced cpm in the process}} \times \frac{\text{mg(PLGA + drug + additives)}}{\text{mg microparticles}} \times 100$$

2.4. Screening of process factors

The purpose of this study was to determine the relative influences of several process factors on the HSA-encapsulation yield (Y_1, %) in an experimental domain chosen beforehand. The particle size was also taken into account (Y_2, μm) to check

Table 1
Parameters studied in the screening step (fractional factorial design)

	Parameters studied	Levels	
		−1	+1
U_1	Volume of internal aqueous phase	200 μl	600 μl
U_2	Concentration of HSA in internal aqueous phase (w/v)	1.25%	2.5%
U_3	Quantity of PLGA in organic phase	50 mg	100 mg
U_4	Concentration of CMC Na in internal aqueous phase	0%	1%
U_5	Ultrasonic time	5 s	15 s
U_6	Mannitol in internal aqueous phase (w/v)	0%	1%
U_7	Volume of external aqueous phase	30 ml	70 ml
U_8	Emulsification time	1 min	5 min
U_9	Volume of extracting aqueous phase	150 ml	400 ml
U_{10}	Time of extraction	2 min	10 min

whether the obtained yields were not derived from a simple change in particle size.

Ten experimental factors were studied. Coded upper and lower limits ($X_i = +1$; $X_i = -1$) were attributed to each of the factors studied (Table 1). Selection of the extreme real values of the variables was carefully made taking into account the feasibility of the preparation while studying the largest range of variable conditions. The method of screening that consisted of assimilating factors to objects and classifying them with respect to their respective weight, was used. A fractional factorial design (2^{k-r}) was chosen (Box et al., 1978). The ten coded factors (X_1, X_2,...,X_{10}) were investigated in a 2^{10-6} type experimental design; only 16 experiments were needed to estimate the ten main effects b_i and five first-order interactions effects (b_{ij}, $i \neq j$) between the parameters studied (Table 2).

2.5. Optimization of the HSA and NGF encapsulation yields

Beyond the information provided by the fractional factorial design which defined the most significant factors, a response surface methodology, a Doehlert matrix (Doehlert, 1970), was used. This study was aimed at getting a knowledge of the studied responses in the whole domain defined by the two most significant factors, namely the internal aqueous phase volume and the percentage of acetone in the organic phase. A second-order polynomial model was used to make prevision in the chosen experimental domain. The equation of the model for the response Y_1 including the interaction and quadratic terms of each controlled variable was:

$$\eta_1 = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2$$

η_1 represented the encapsulation yield. The coefficients β_i and β_{ij} represented the estimations of the coefficients of the model.

To estimate the coefficients of the model, seven distinct experiments ($+4$ center replicated points to estimate the variability of the experimental results) were carried out (Table 3).

After verifying the validity of the model by the classical statistical tests (Box et al., 1978), the HSA encapsulation yield could be predicted in each point of the defined domain. The analysis of variance (ANOVA) was performed to determine the significance of the fitted equation. The multiple correlation coefficient statistical (R^2) gave the part of the variation of Y_1 explained by the model. The R^2 value should be as close as possible to 1.

3. Results

3.1. Screening of the process factors

By the least square method, the effect of the studied factors was determined from the experi-

Table 2
Factorial design (coded values) and experimental results of the parameter screening step (2^{10-6} fractional factorial design)

Trials	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	Y_1	Y_2
1	−1	−1	−1	−1	−1	+1	−1	+1	−1	−1	48.6	22
2	+1	−1	−1	+1	−1	−1	+1	+1	−1	+1	5	31
3	−1	+1	−1	+1	−1	+1	−1	−1	+1	+1	5	24
4	+1	+1	−1	−1	−1	−1	+1	−1	+1	−1	36.7/31.5	38/37
5	−1	−1	+1	+1	−1	+1	+1	+1	+1	−1	9.6	38
6	+1	−1	+1	−1	−1	−1	−1	+1	+1	+1	61.1	26
7	−1	+1	+1	−1	−1	+1	+1	−1	−1	+1	32.8	34
8	+1	+1	+1	+1	−1	−1	−1	−1	−1	−1	5	28
9	−1	−1	−1	−1	+1	−1	+1	−1	+1	+1	85.4	29
10	+1	−1	−1	+1	+1	+1	−1	−1	+1	−1	5	19
11	−1	+1	−1	+1	+1	−1	+1	+1	−1	−1	9.7	27
12	+1	+1	−1	−1	+1	+1	−1	+1	−1	+1	16.5	21
13	−1	−1	+1	+1	+1	−1	−1	−1	−1	+1	77.0	24
14	+1	−1	+1	−1	+1	+1	+1	−1	−1	−1	39.2	51
15	−1	+1	+1	−1	+1	−1	−1	+1	+1	−1	68.4	25
16	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	5	43

mental results with the help of the NEMROD® software (Mathieu and Phan-Tan-Luu, 1980). The relative importance of these effects was graphically represented on Fig. 1. The higher the effect of a factor in absolute value, the more significant it was on the HSA encapsulation yield or particle size. The sign of the effect indicated its direction. The incorporation of carboxymethylcellulose sodium (CMC Na) in the internal aqueous phase appeared to be the most predominant factor influencing Y_1. It decreased dramatically the encapsulation yield ($b_4 = -16.55$). Fig. 2A shows a batch of microparticles prepared without CMC Na in the internal aqueous phase, whereas Fig. 2B represents a batch prepared in the same experimental conditions but with 1% CMC Na. The encapsulation yields were respectively 94% and 21%. Interestingly, a similar internal morphology was observed in both cases. The presence of mannitol in the internal aqueous phase had also a negative effect on the encapsulation yield ($b_6 = -11.50$). Microparticles prepared with 1% mannitol (w/v) are shown on Fig. 2C, the encapsulation yield was 61%. In this case, the internal and external microsphere structures were modified, the internal network appeared more tortuous and the microsphere surface was lumpy in comparison to the standard batch (Fig. 2A). Nevertheless, the cavity size of the honeycomb-like structure was the same as that observed for the standard preparation. Another important factor was the internal aqueous phase volume ($b_1 = -10.35$). Increasing the internal aqueous phase volume led also to decrease the encapsulation yield. The last significant factor was the percentage of protein HSA in the aqueous phase ($b_2 = -9.65$). The other six parameters were less important. The increase of the quantity of PLGA in the organic phase ($b_3 = +5.5$) along with the ultrasonic time ($b_5 = +6.56$) led to a slight increase in the entrapment efficiency. The particle size (ranging from 19 to 51 μm) grew when the external aqueous phase volume ($b_7 = +6.34$) was increased, as well as the quantity of PLGA in the organic phase ($b_3 = +3.66$). The other factors were not significant on Y_2.

3.2. Optimization of the HSA and NGF encapsulation yields

Following the screening design, we focused on the acetone proportion in the organic phase (U_1) and the internal aqueous phase volume (U_2) while keeping constant the other factors (50 mg PLGA, 2.5 mg HSA, ultrasonic time 15 s, volume of external aqueous phase 30 ml, emulsification time

Table 3
Doehlert matrix in coded variables, experimental design and experimental results of the optimization step

Trials	X_1	X_2	Acetone (%)	Aqueous volume (ml)	Y_1 (%)
1	1.000	0.000	49	150	93.0
2	−1.000	0.000	1	150	88.6
3	0.500	0.866	37	250	90.1
4	−0.500	−0.866	13	50	96.2
5	0.500	−0.866	37	50	100.8
6	−0.500	0.866	13	250	87.4
7	0.000	0.000	25	150	98.9
8	0.000	0.000	25	150	96.9
9	0.000	0.000	25	150	96.7
10	0.000	0.000	25	150	99.3
11	0.000	0.000	25	150	101.7

1 min, volume of extracting aqueous phase 400 ml, time of extraction 10 min., without mannitol or CMC Na) (Table 3). The acetone proportion was not studied in the screening step because it was previously described as a critical parameter (Marchais et al., 1996). This factor was studied while keeping constant the organic phase volume (2 ml). The measured answer was the encapsulation yield (Y_1, %). The use of the radioactivity-based assay, for the evaluation of the HSA successfully entrapped in the microparticles, allowed the microparticle digestion to be avoided, that might result in an imperfect recovery of protein. The coefficients of the second-order polynomial model were estimated by the least square method with the results given by the 11 experiments (Table 3). It was as follows:

$$Y_1 = 98.7 + 2.68X_1 - 5.63X_2 - 7.9X_1^2 - 4.13X_2^2 - 1.09X_1X_2$$

The statistical analysis (Table 4: analysis of the variance for Y_1) showed that the model represented the phenomenon quite well and the variation of the response was correctly related to the variation of the factors. Graphical representation of the Y_1 isoresponse surface was shown on Fig. 3. The response surface showed an optimal point for 75 μl internal aqueous phase and 30% acetone. In these conditions, the encapsulation yield was $98.9 \pm 3.0\%$ ($n = 3$) for a 16-μm particle size. This value was close to the one obtained for the center point of the Doehlert matrix, $98.7 \pm 2.0\%$ ($n = 5$; trials 7–11). These two points defined a zone where the encapsulation yield was optimal. It must be noted that the considered zone is quite broad what reflects the robustness of the microparticle preparation.

Furthermore, the determination of the radioactivity recovery supplied additional information (Fig. 4). HSA adsorptions on filter, syringe, PTFE vial or sonicator probe were negligible. The radioactivity fraction found on the filter (2.3%) was likely due to adsorbed microparticles. Conversely, a level of radioactivity close to 11% was found in the dispersing phase after filtration and before washing. As the production yield was 87% and the encapsulation yield was closed to 100%, this result was attributed to a fraction of radiolabelled protein-loaded particles not retained by the 0.45-μm filter. The loss of radioactivity during the washing step (5×100 ml water) was found negligible indicating that the HSA was well encapsulated or associated to the polymer.

NGF was co-encapsulated with HSA in the aforementioned optimal conditions. Then, 30 μg of NGF were added to the internal aqueous phase. In these conditions, the encapsulation yield was $97.3 \pm 5.0\%$ ($n = 3$).

4. Discussion

The analysis of the response Y_1 (HSA encapsulation yield) showed that incorporation of

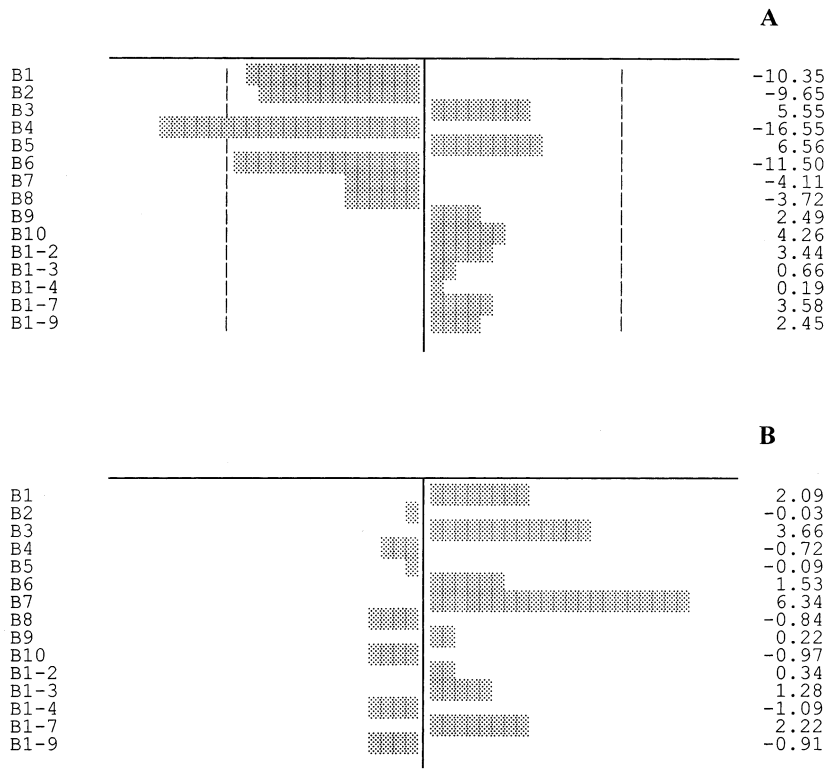

Fig. 1. Graphical analysis of the effects on the encapsulation yield (A) and microparticle size (B) in the parameter screening step.

CMC Na in the internal aqueous phase had the most significant effect on Y_1. This was partly in contradiction with previous works reporting that increasing the primary emulsion viscosity was a way to successfully retain the drug in the dispersed aqueous phase (Aftabrouchad and Doelker, 1994). The honeycomb-like structure, print of the primary emulsion, was unchanged irrespective of the presence of CMC Na. Thus, the poor HSA encapsulation efficiency was not due to a modification of the microparticle structure. The tensioactive and viscosifying properties of CMC Na contributed to the primary emulsion stability but the presence of CMC Na led to protein partitioning into the dispersing phase of the multiple emulsion. The second important factor observed was the presence, in the internal aqueous phase, of mannitol as previously proposed to protect the NGF in solution and during lyophilisation (Knepp et al., 1994). Since the size of the internal reservoirs as shown by SEM was not modified (1.5 μm), the primary emulsion was certainly not altered. The presence of sugar in the internal aqueous phase during the second emulsification step induced a difference in osmotic pressure between the two aqueous phases. As previously reported (Herrmann and Bodmeier, 1995), this promoted the influx of water from the dispersing phase towards the organic phase and resulted in a porous and lumpy microsphere surface. During the extraction and washing steps, this type of morphology would not prevent the leakage of free protein from the matrix. The results showed also that improving the encapsulation yield did not require high HSA concentrations. It must be pointed out that the amount of HSA was set at 2.5 mg in the optimization step. The resulting HSA/NGF ratio corresponded to the one often found when the combination of the two proteins was commercially supplied. The screening step showed that the increase of the polymer amount in the organic phase (U_3) and the

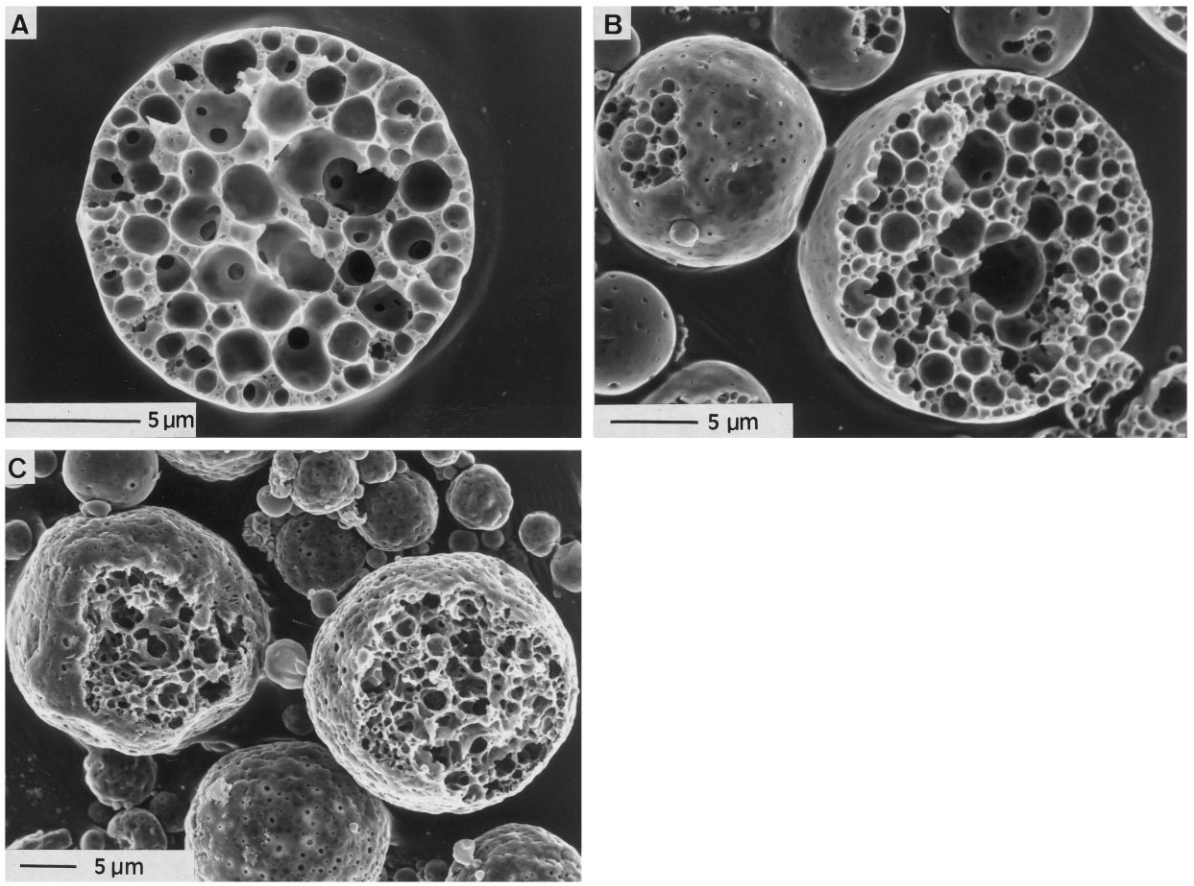

Fig. 2. Photomicrographs of microparticles prepared in different experimental conditions. (A) 150 µl internal aqueous phase, 2.5 mg HSA, 2 ml organic phase among which 0.5 ml acetone (encapsulation yield = 94%). (B) Formulation A + 1% (w/v) carboxymethylcellulose sodium in the internal aqueous phase (encapsulation yield = 21%). (C) Formulation A + 1% (w/v) mannitol in the internal aqueous phase (encapsulation yield = 61%).

time of the primary emulsion sonication (U_5) slightly enhanced the encapsulation yield. The increase of the organic phase viscosity and the sonication time resulting in a fine primary emulsion would be a possible explanation. Although, in contradiction with the screening step, the amount of coating polymer (U_3) was not increased in the optimization step in order to keep a NGF theoretical encapsulation ratio (0.06–0.1%) compatible with the ongoing animal experimentation. Even if the influence of the extraction aqueous phase volume (U_9) and the time of extraction (U_{10}) were not significant, these factors were fixed at 400 ml and 10 min. respectively, mainly to favour solvent elimination and to reduce their residual traces in the final particles. Finally, the effect of the factor interactions on the encapsulation yield was negligible. In other words, the effect of each factor was identical irrespective of the level of the others.

The strongest influence of the studied factors on the particle size (Y_2) was the external aqueous phase volume (U_7) and the quantity of PLGA in the organic phase (U_3) (Fig. 1B). No relationship was found between the size and the encapsulation yield in the studied conditions showing the effect of the different parameters on the encapsulation yield was not due to size modifications.

The optimization step showed that a reduction of the internal aqueous phase volume (corre-

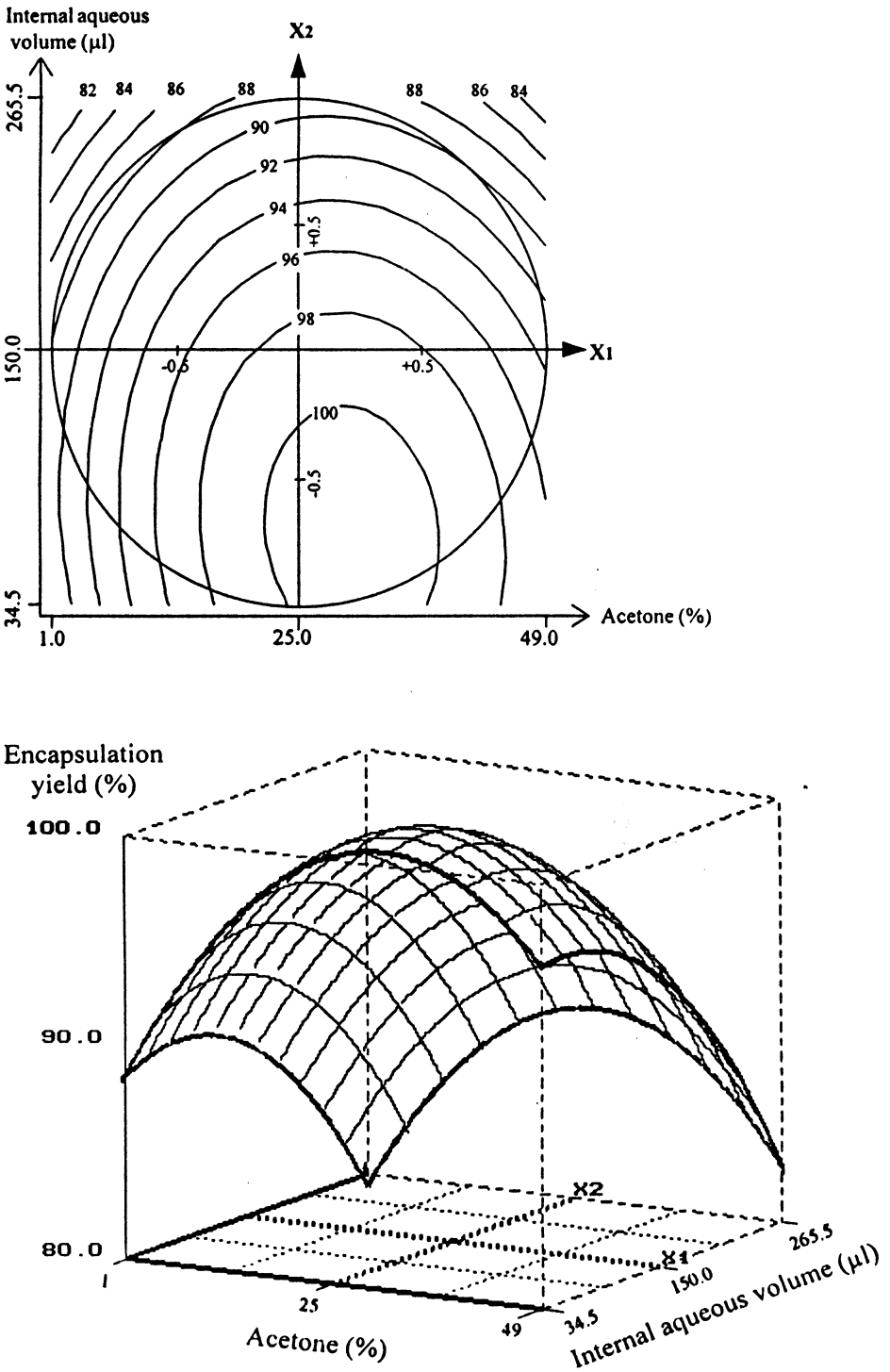

Fig. 3. Graphical representation (2D and 3D) of the experimental response Y_1 (encapsulation yield) in the optimization step (X_1 = acetone %; X_2 = internal aqueous volume μl).

Table 4
Results of the ANOVA for the response Y_1

Source of variation	S.S	D.F	Mean square	F	Significant
Regression	226.93	5	45.38	12.58	At $1‰ < \alpha < 1\%$
Residue	18.04	5	3.61		
Validity	1.40	1	1.40	0.34	59.5%
Error	16.64	4	4.16		
Total	2444.97	10			

sponding to a decrease of the aqueous phase/organic phase ratio) enhanced the encapsulation yield. This effect was not due to an increase of the HSA concentration since the negative effect of this factor was demonstrated in the screening step. In fact, the smaller the internal aqueous phase volume, the thicker the organic layer. As this layer acted as a diffusion barrier between the two aqueous phases, the protein retention was dependent on its thickness. Fig. 3 shows that the acetone proportion in the organic phase (U_1) and the internal aqueous phase volume (U_2) must be chosen between 25–40% and 50–100 μl, respectively, to improve the encapsulation yield (Y_1). The presence of acetone in the organic phase, used as cosolvent, was believed to cause a rapid precipitation of the polymer because it rapidly diffused in the aqueous phase (Bodmeier and McGinity, 1988). The kinetics of the polymer precipitation that governed the protein leakage, depended upon the respective amounts of acetone and methylene chloride in the organic phase.

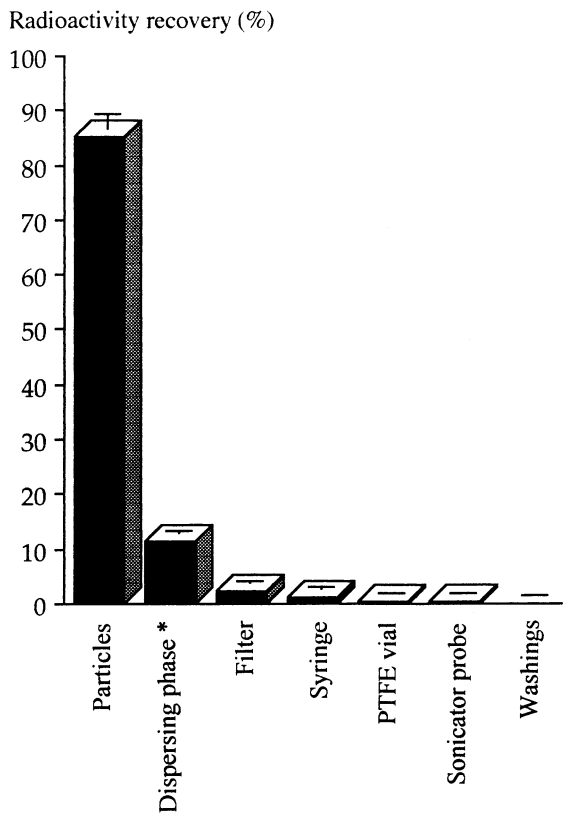

Fig. 4. Radiolabelled HSA recovery at the end of the emulsion/extraction process. * After filtration and before washing.

Because of the high potency of NGF, low theoretical loadings were used. Nevertheless, the encapsulation yield had to be close to 100% to avoid any costly loss. Since HSA was known to be a natural carrier for NGF, a good HSA encapsulation yield had to result in a high encapsulation yield for NGF. This was the case ($97.3 \pm 5\%$) and the presented data supported the interest of using the fractional factorial design along with a response surface methodology to optimize the entrapment efficiency of HSA and to obtain the ultimate objective.

These results were valid in the studied domain. In other experimental conditions, the effects of the process factors might be different. Moreover, the selected conditions for a good entrapment efficiency should be compatible with the keeping of the biological activity of NGF. This remains to be defined, even though the internal aqueous phase was buffered at pH 6 to improve the NGF stability (Knepp et al., 1994).

In conclusion, the strategy used in this study allowed us to optimize both the HSA and NGF encapsulation yields. More particularly, this study

outlined the importance of the volume and the composition of the internal aqueous phase, and the proportion of acetone in the organic phase to optimize the protein encapsulation yield. In the optimized conditions, the HSA and NGF encapsulation yields were closed to 100%. Their behaviour during the encapsulation process, was identical. HSA and NGF release kinetics and determination of NGF biological activity are now ongoing.

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