

Efficient size control of amphiphilic cyclodextrin nanoparticles through a statistical mixture design methodology

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ABSTRACT Purpose: the aim of the study was to investigate size control of amphiphilic β -cyclodextrin nanoparticles obtained by solvent displacement technique. **Methods:** An experimental design methodology for mixture design was undertaken using D-optimal approach with the following technique variables: water fraction X_1 (40-70% v/v), acetone fraction X_2 (0-60% v/v) and ethanol fraction X_3 (0-60% v/v). **Results:** The resulting quadratic model obtained after logarithmic transformation of data and partial least-square regression was statistically validated and experimentally checked. Also, the morphology of the colloidal nanoparticles from selected experiments was observed by cryo-transmission electron microscopy. **Conclusions:** This experimental design approach allowed to produce interesting amphiphilic β -cyclodextrin nanoparticles with a predicted mean size varying from 60 to 400 nm.

INTRODUCTION

In the pharmaceutical industry, natural or modified cyclodextrins are interesting excipients to reduce toxicity while improving stability, solubility and bioavailability of hydrophobic drugs. Amphiphilic β -cyclodextrins obtained by grafting hexanoic chains on the secondary hydroxyl groups have the ability to

self-organize yielding stable supramolecular assemblies using the nanoprecipitation technique. These amphiphilic β -cyclodextrin nanoparticles could be considered as promising carriers for drug molecules (1, 2). In a previous study, Géze *et al.* (3) have established the correlation between the chemical structure of amphiphilic β -cyclodextrin and their ability to form stable nanospheres. More recently, the long-term shelf stability of the nanoparticles was also monitored by photo correlation spectroscopy and cryo-transmission electron microscopy (4).

The nanoparticle capacity to associate drug is partially expected to be influenced by nanoparticle size. This latter parameter could be related to the nature and the amount of organic solvent involved during nanoparticle manufacturing. The mechanism of nanoparticle formation is complex and has only been partially understood (5) although numerous monographs and review articles have been written on the subject. Then, one of the major challenges for the design of the colloidal carriers is to define a solvent or a solvent mixture system suitable to obtain a desired size. However, one has to keep in mind that, the properties (Mw, polarity,...) of the drug when associated to amphiphilic β -cyclodextrins carriers, may also influence the size as well as the bulk structure of the nanoparticles.

The aim of the present work was to set up experimental procedures allowing the control of nanoparticle size during their manufacturing. This goal was achieved by using mixture design methodology in which an empirical correlation was found between the solvent proportion (water/acetone/ethanol) and the nanoparticle size. In addition, the morphology and internal structure of the nanoparticles were investigated using cryo-transmission electron microscopy (cryo-TEM) experiments.

MATERIALS AND METHODS

Natural β -cyclodextrin (β -CD), Kleptose®, was obtained from Roquette (Lestrem, France). Anhydrous pyridine (99%), dichloromethane (99%) and 2, 4-dimethylaminopyridine, hexanoyl chloride, were purchased from Aldrich. Tetrabutylammoniumfluoride and tetrahydrofuran were from Avogadro and Acros respectively. Ethanol and acetone at pharmaceutical grade, methanol, cyclohexane and chloroform were purchased from Sodipro (Grenoble, France). Water was distilled in our laboratory.

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Amphiphilic β -cyclodextrin synthesis

Amphiphilic β -cyclodextrin was prepared with a 3 step procedure from natural β -cyclodextrin according to a modified procedure described by Lesieur *et al.* (6) and Dubes *et al.* (7): i) protection of primary hydroxyl groups, ii) acylation of secondary face, iii) deprotection of primary face.

(I) Dried β -cyclodextrin (1g, 1eq.) was mixed with TBDMSCl (1g, 8eq.) in anhydrous pyridine (40ml) at room temperature under N_2 for 48 hours. The product was precipitated in ice-cooled water, filtered and washed twice with ice-cooled water. The dried crude product was then dissolved in a minimum quantity of chloroform and purified by column chromatography using chloroform/methanol/water (8/1.9/0.1, v/v/v,) as mobile phase. The pure Heptakis(6-O-tert-butylidimethylsilyl) cyclomaltoheptaose was obtained with yield of 76%.

(II) Hexanoyl chloride (3ml, 42 eq.) was added to a stirred solution of heptakis(6-O-tert-butylidimethylsilyl) cyclomaltoheptaose (1g, 1 eq.) and 2, 4-dimethylaminopyridine (3g, 49 eq.) in anhydrous pyridine (40ml). The mixture was heated at 70°C for 48 h, then cooled to room temperature and poured into a large volume of ice-cooled water. The aqueous phase was removed by settling and the syrupy residue taken up into dichloromethane. After washing with dilute sulphuric acid (2%), water and aqueous sodium hydrogen carbonate, the organic layer was dried (Na_2SO_4) and concentrated to a residue which was submitted to flash chromatography using cyclohexane/ethyl acetate (10/1) as mobile phase.

(III) Tetrabutylammonium fluoride 1M in tetrahydrofuran (3ml, 8 eq.) was added drop-wise to a stirred anhydrous tetrahydrofuran (28 ml, eq.) solution of the resulting product (1g, 1 eq.) obtained from step (II). The mixture was reacted at room temperature under N_2 for 44h. The crude product was then concentrated to dryness and submitted to flash chromatography using ethyl acetate/cyclohexane/ethanol (8/1.9/0.1, v/v/v,) as mobile phase.

Nanosphere preparation

The nanosphere suspensions were prepared using the nanoprecipitation technique firstly developed by LaMer and Dinegar (8) and applied to amphiphilic cyclodextrin by Skiba *et al.*(9) 20 mg of β -CDA were dissolved at 25°C in an organic solvent constituted by acetone or ethanol or in an acetone/ethanol mixture. The organic solution was poured slowly

through a silicon tube fitted with fine tip into distilled water subjected to a magnetic stirring. The organic solvents and water volume of each experiment unit were defined according to an experimental design worksheet (table 2). The nanospheres were formed immediately and the colloidal suspension obtained was subjected to evaporation under reduced pressure to remove organic solvent. The colloidal suspension obtained was filtered through a 0.8 μm membrane (Millex AA, Millipore, France) and stored in closed vials at room temperature.

Particle size measurements

The size of the nanospheres was measured using quasi-elastic light scattering (QELS) with a Zetasizer 3000 instrument (10 mW HeNe laser at 632.8 nm, K7132 correlator, Malvern Instrument, Malvern, UK). Experimental conditions were the following : temperature 25 \pm 0.1°C, reference angle 90°C, viscosity 0,899 \times 10⁻³ Pa.s, refractive index of the dispersant 1.330. The mean hydrodynamic diameter of the particles was calculated using a Contin algorithm with Zetasizer 3000. All the experimental units were analysed in triplicate.

Transmission electron microscopy

According to the procedure described elsewhere (10), specimens for cryo-TEM were prepared by quench-freezing thin liquid films of 0.1% (w/v) amphiphilic β -cyclodextrin nanostructure suspension into liquefied ethane (-171°C). Once transferred into a Gatan 626 cryoholder, the specimens were observed at low temperature (-180°C), using a Philips CM200 "Cryo" microscope operating at 80kV. Micrographs were recorded on Kodak SO163 films.

Experimental design

The influence of the water/acetone/ethanol mixture on the nanoparticle size was investigated by an experimental design methodology. In our objectives, the experimental design was chosen to support a quadratic regression model as adequate to estimate a potentially complex response function (equation 1).

$$\eta = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^{k-1} \sum_{j=i+1}^k \beta_{ij} X_i X_j + \sum_{i=1}^k \beta_{ii} X_i^2 + \varepsilon \quad (1)$$

Where η is the simulation model response, β_0 the constant, β_i the linear coefficients, β_{ij} the interaction coefficients, β_{ii} the quadratic coefficients, k the number of critical variables X_i and ε the random error term.

The solvent fraction levels, which limit the experimental field, were determined after some preliminary studies. First, due to the high solubility of β CDa in organic solvents, i.e., acetone and ethanol, nanoprecipitation that occurs via solvent shifting, was not satisfactory when the amount of water in the medium was under 40%. Secondary, when the amount of water in the medium was higher than 70%, the low final nanoparticle concentration in water, following removal of organic solvent, made the measurement difficult to perform (low limit of scattering intensity). Indeed, nanoparticles were finally so diluted in aqueous phase that an efficient measure was unfeasible with the Zetasizer 3000 instrument. No specific distinctions were postulated between the amount of acetone and ethanol amounts. The component limits are summarized in table 1.

Table 1: Settings levels for each mixture component.

labels	fraction	levels setting	
		low	high
X ₁	U ₁ , water	40% (v/v)	70% (v/v)
X ₂	U ₂ , acetone	0% (v/v)	60% (v/v)
X ₃	U ₃ , ethanol	0% (v/v)	60%(v/v)

Standard three-component design might require too many experiments because all the components are restricted by upper (X₂, X₃) or upper and lower (X₁) constraints. Then, mixture solvent formulation was investigated using a D-optimal design approach (11). D-optimal design was computed by MODDE v5.0 software (UMETRICS AB, Box 7960, SE-90719 Umeå Sweden) in twelve experiment units including two central replicates to estimate pure error. G-efficiency criterion was used to select the optimal worksheet design set. Finally, run sequence of experimental units was completely randomized to minimize the effect of noise and then safeguarding the experiment from systematic bias (table 2). All experiments were performed without replication.

Table 2: Design matrix (G-Efficiency = 69.63), experimental worksheet and corresponding nanoparticle size response.

Experimental unit	Coded variable			Natural variable			Size \pm SD (nm)	IP
	X ₁	X ₂	X ₃	U ₁ (ml)	U ₂ (ml)	U ₃ (ml)		
1	0	1	0	8	12	0	198 \pm 12	0.06
2	0	0	1	8	0	12	467 \pm 4	0.57
3	0.5	0.5	0	14	6	0	59 \pm 1	0.08
4	0.5	0	0.5	14	0	6	67 \pm 2	0.07
5	0.167	0	0.833	10	0	10	178 \pm 11	0.18
6	0.333	0	0.667	12	0	8	101 \pm 7	0.09
7	0.167	0.833	0	10	10	0	108 \pm 4	0.18
8	0.333	0.667	0	12	8	0	79 \pm 3	0.07
9	0	0.667	0.333	8	8	4	311 \pm 14	0.24
10	0	0.333	0.667	8	4	8	387 \pm 31	0.31
11	0.25	0.375	0.375	11	4.5	4.5	96 \pm 3	0.06
12	0.25	0.375	0.375	11	4.5	4.5	92 \pm 2	0.05

RESULTS AND DISCUSSIONS

The objective of the present study was to control the amphiphilic cyclodextrin self-assembly process to form nanoparticle with the desired size. First, the amphiphilic cyclodextrins were classically synthesized in three steps from the natural β -CD as summarized in figure 1: i) protection of primary hydroxyl groups, ii) acylation of the secondary face, iii) deprotection of primary face. The amphiphilic cyclodextrin products obtained was characterized as

a statistical mixture of β -CD variously acylated. The degree of acylation of the secondary alcohol is theoretically 14 for the β -cyclodextrin. However, it is perfectly known that in the presence of a large amount of DMAP self-condensation of acyl chains may occur and hence, leading to the increase of grafted hexanoyl chains on the β -CD (6, 7, 12). As described in figure 1, this synthesis leads to a statistical mixture of multisubstituted β -cyclodextrin containing under and over-acylated β -cyclodextrin by hexanoyl chains.

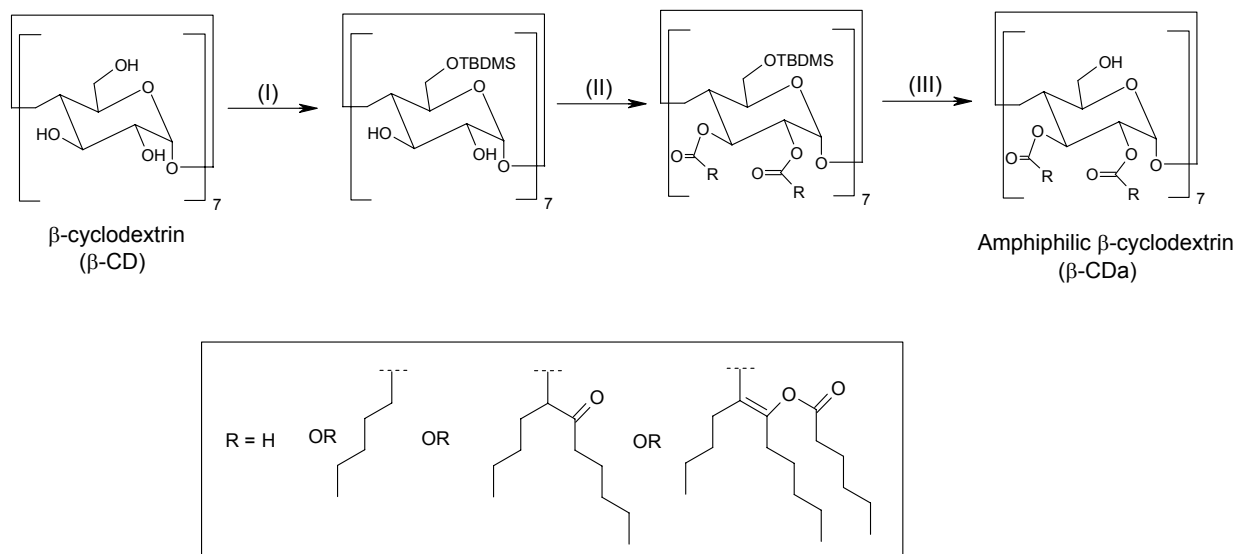


Figure 1: Schematic pathway for amphiphilic β -cyclodextrin synthesis

Then, the electrospray mass spectrometry characterization clearly demonstrated the presence of a mixture of numerous derivatives resulting from coupling of 8 to 23 alkanoyl chains (m/z $[M+Na] = 1941.8$ to 3412.9).

This product was used to obtain nanoparticles using nanoprecipitation procedure. The influence of water/acetone/ethanol mixture ratios involved in the nanoprecipitation technique on the nanoparticle size response was investigated by experimental design methodology. Five classical steps were carried out: choosing adequate model and performing the experimental worksheet as decrypted in experimental section, fitting the model coefficients, predicting the response and checking the adequacy of the model (13, 14).

Nanoparticle sizes, standard deviations (SD) and index of polydispersity (IP) of experimental worksheet are reported in table 2. The SD values are considered as low for all the experimental units. IP values below 0.25, generally signed the presence of monodisperse particle suspensions. This is the case for the nanosuspension of particles with mean size under 300 nm. For the samples n^o 2, 9 and 10, IP > 0.25 signed polydispersed suspensions.

The partial least-square process (PLS) which allows fitting the experimental response with the analytical model and parametric analysis methods, such as analysis of variance (ANOVA) or t-test, were based on assumptions that the population characteristics must be drawn from normally

distributed populations. Therefore, the experimental design data distributions were examined for departures from normality using a Shapiro-Wilk W test extended by Royston (15, 16) that thought to be one of the best tests of normality for small data samples. The p -value = 0.0120 obtained with this W test was less than 0.05 criterion, then the hypothesis that the data were normally distributed was rejected. The non-normal distributions of data may be "fixed" using appropriate transformation of the size response. Box-Cox transformation procedure (17) was investigated to find the best transformation that will approximately normalize the data. The best transformation indicated by the Cox-box procedure which minimize the residual sum of squares was obtained with a Log_{10} transformation of the nanoparticle size ($\text{Lambda max} = 0, 103942$).

The unknown coefficients β of the equation were estimated with PLS, which allows fitting the transformed size response with the analytical model. Corresponding coefficients were replaced in the following general equation 1:

$$\eta = 2,09988 - 0.133451X_1 + 0.256992X_2 + 0.355274X_3 + 0.013582X_{12} + 0.0479853X_{23} + 0.089019X_{13} - 0.05337X_1^2 - 0.0712576X_2^2 + 0.148889X_3^2 \quad (2)$$

The correlation coefficients R^2 and R^2_{Adjusted} describe respectively the fraction of variation of the response explained by the model and the fraction of variation of the response explained by the model adjusted for degrees of freedom. The coefficient of prediction Q^2 describes the fraction of variation of the response that can be predicted by the model.

Here, the coefficient of correlation $R^2=0.986$ and determination $R^2_{Adjusted}=0.975$ showed that quadratic model is well fitted by PLS. Besides, nanoparticle sizes are well predicted as indicated by the

coefficient of prediction $Q^2=0.879$. The summary of the analysis of variance (ANOVA) procedure based on F-test is shown in table 3.

Table 3: Regression analysis for the nanoparticle size with quadratic response surface model fitting. η_i is the predicted response of i^{th} experiment, y_i is the measured response of i^{th} experiment, \bar{Y} is the mean value of measured response, y_i^0 is the measured response of i^{th} experiment in central positions. \bar{Y}_0 is the mean value of response in central position. Variance column was the ratio of sum of squares by the corresponding degree of freedom.

Source	Degree of freedom	Sum of squares	Mean square (variance)	F value	Probability p
Regression	5	$\sum(\eta_i - \bar{Y})^2 = 1.01586$	0.203172	87.0796*	0.000
Residual	6	$\sum(y_i - \eta_i)^2 = 0.013999$	0.00233317		
Lack of Fit	5	$\sum(y_i - \eta_i)^2 - \sum(y_i^0 - \bar{Y}_0)^2 = 0.0138282$	0.00276564	16.1906**	0.186
Pure Error	1	$\sum(y_i^0 - \bar{Y}_0)^2 = 0.000170818$	0.00017082		

* Significant at the level 95%; $F_{0.05(5,6)} = 4.39$

** Significant at the level 95%; $F_{0.05(5,1)} = 230.16$

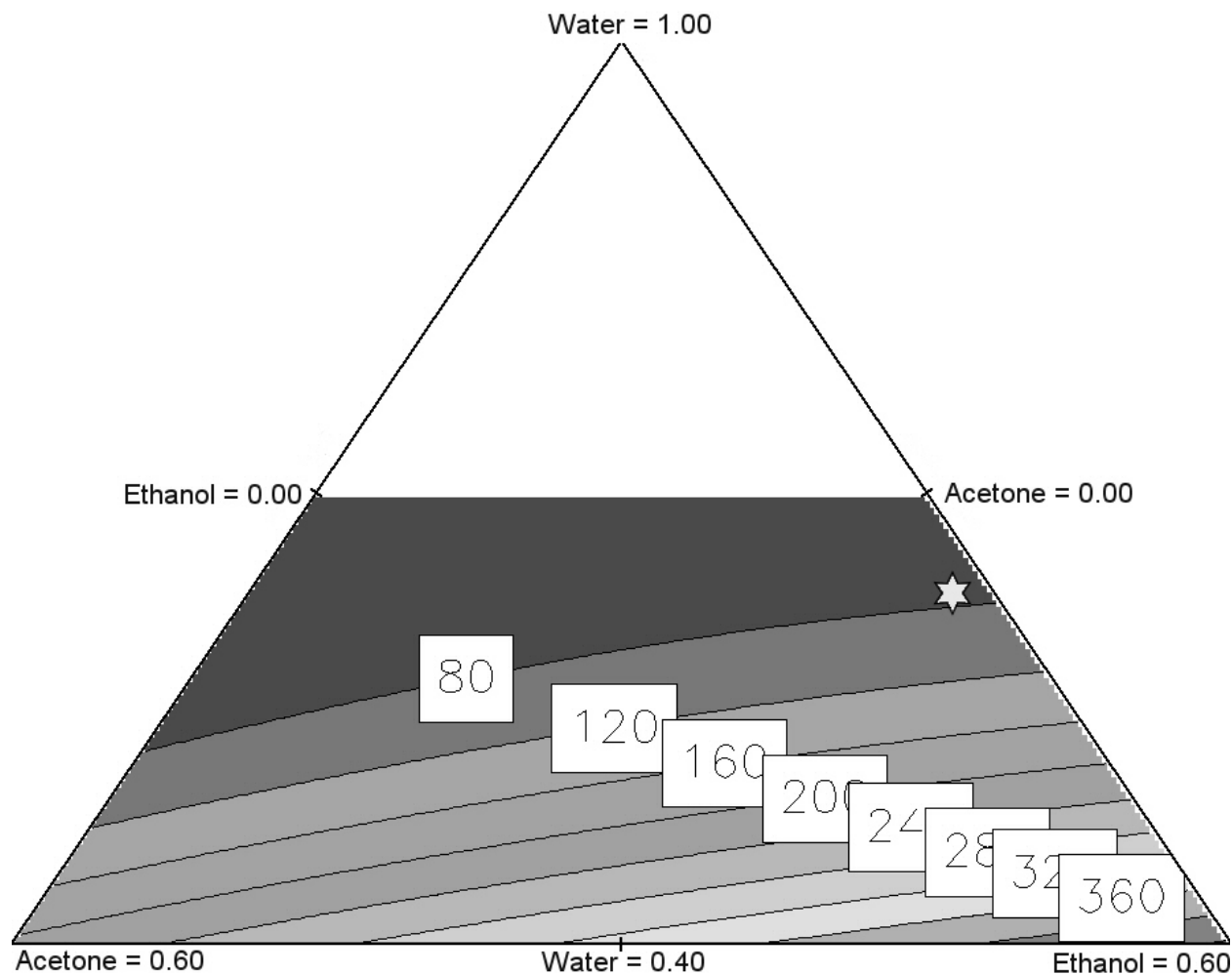


Figure 2: Ternary diagram for nanoparticle size. White star corresponding to the mixture water/acetone/ethanol=0.64/0.01/0.34 for a size of 82 nm.

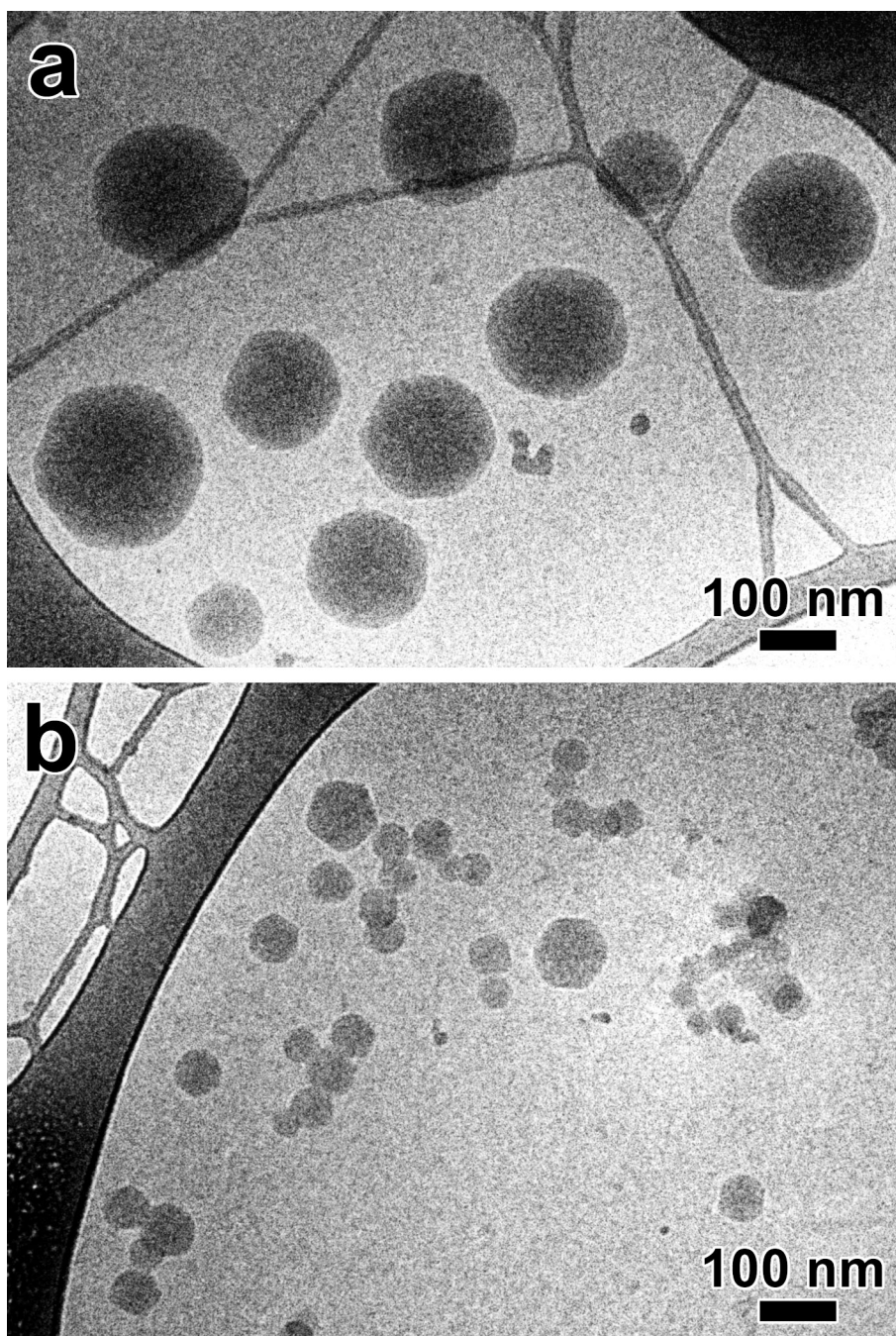


Figure 3: Cryo-TEM images of β -cyclodextrin nanoparticles in suspensions corresponding to experiment units 9 (a) and 12 (b), respectively.

The probability for the model regression is significant at 95% (P -value=0.000<0.05) and the probability for lack of fit is not significant at 95% (P -value=0.186>0.05). Hence, the quadratic model is statistically good and has no lack of fit within the range of variables used.

All the nanoparticles size and the corresponding solvent mixture were plotted in a ternary diagram (figure 2) using the equation 2. The solvent

proportions were explained in percentage unit scaled from 0 to 1 in accordance with the limits defined in table 1. Each point of the diagram corresponds to defined proportions of acetone, ethanol and water used in the experiment. The white area depicted on the ternary diagram is an experimentally unexplored zone of mixture. Each line plotting inside the diagram defines a specific size value (nm) labelled in the white square.

In order to decrease the nanosphere size, the nanoprecipitation should be investigated with high amount of water and low amount of organic solvent. This plot also indicates that the use of acetone as organic fraction is more efficient than ethanol to obtain small nanospheres.

An experimental test point focused on interesting mean size of 82 nm was carried out to confirm the model validation. The procedure used to predict the corresponding experimental conditions was the Nelder-Mead simplex method (18). The computation leads to the optimal following mixture water/acetone/ethanol=0.6428/0.01270/0.3445 (v/v/v). The mean size of nanoparticles obtained when applying these conditions was measured at 86 nm. This result was in accordance with the predicted value of 82 nm in 95% confidence interval. Consequently, the quadratic model was experimentally checked as good.

The morphology of the colloidal nanoparticles from selected experiments was studied by cryo-TEM.

Images of suspensions from experimental units 9 and 12 are shown in Figures 3a and 3b, respectively. In both cases, the particles are uniformly dense and spheroidal, although several of them exhibit a clear faceting. Such a faceting has already been reported by Gèze *et al.* (3) for β CDa particles. Those corresponding to experimental unit 9 are individual while some aggregation is observed in the suspension from experimental unit 12. The diameter measured from the cryo-TEM images ranges from 100 to 250 nm and 40 to 100 nm for particles from experimental units 9 and 12, respectively. The cryo-TEM images leads to a diameter slightly lower than that measured by QELS (table 2). This observation was because the values were obtained using different calculation procedures (intensity diameter for QELS and weight diameter for TEM); it is worth reminding that the QELS measurement integrates the ionic environment surrounding the particle surface. As this environment is not substantially denser than the embedding vitreous ice, it will not be detected in cryo-TEM images.(3)

CONCLUSION

In conclusion, this mixture design methodology led to a significant empirical correlation between the solvent proportion involved in amphiphilic cyclodextrin self assembling procedure and the corresponding nanoparticle size. With the output of this study, we are able to prepare nanoparticles with

predicted mean size varying from 60 to 400 nm. In the future, further studies will be carried out to establish the influence of the size of nanoparticles on their drug loading capacity and to check the long-term stability of these loaded nanoparticles.

REFERENCES

- [1] Duchêne, D.; Ponchel, G.; Wouessidjewe, D. Cyclodextrins in targeting application to nanoparticles. *Adv Drug Deliv Rev*, 36:29-40, 1999.
- [2] Duchêne, D.; Wouessidjewe, D.; Ponchel, G. Cyclodextrins and carrier systems. *J Control Release*, 62:263-268, 1999.
- [3] Gèze, A.; Putaux, J. L.; Choïnard, L.; Jéhan, P.; Wouessidjewe, D. Long-term shelf stability of amphiphilic β -cyclodextrin nanosphere suspensions monitored by dynamic light scattering and cryo-transmission electron microscopy. *J Microencapsul*, 21(6):607-613, 2004.
- [4] Gèze, A.; Aous, S.; Baussanne, I.; Putaux, J. L.; Defaye, J.; Wouessidjewe, D. Influence of chemical structure of amphiphilic β -cyclodextrins on their ability to form stable nanoparticles. *Int J Pharm*, 242:301-305, 2002.
- [5] Horn, D.; Rieger, J. Organic nanoparticles in the aqueous phase-theory, experiment, and use. *Angew Chemie Int*, 40: 4330-4361, 2001.
- [6] Lesieur, S.; Charon, D.; Lesieur, P.; Ringard-Lefebvre, C.; Muguet, V.; Duchêne, D.; Wouessidjewe, D. Phase behavior of fully hydrated DMPC-amphiphilic cyclodextrin systems. *Chem Phys Lip*, 106:127-144, 2000.
- [7] Dubes, A.; Bouchu, D.; Lamartine, R.; Parrot-Lopez, H. An efficient regio-specific synthetic route to multiply substituted acyl-sulphated β -cyclodextrins. *Tetrahedron Lett*, 42:9147-9151, 2001.
- [8] Lamer, V. K.; Dinegar, R. H. Theory, production and mechanism of formation of monodispersed hydrosols. *J Am Chem Soc*, 72:4847-4854, 1950.
- [9] Skiba, M.; Wouessidjewe, D.; Puisieux, F.; Duchêne, D.; Gulik, A. Characterization of amphiphilic β -cyclodextrin nanospheres. *Int J Pharm*, 142:121-124, 1996.
- [10] Durrieu, V.; Gandini, A.; Belcace, M. N.; Blayo, A.; Eiselé, G.; Putaux, J. L. Preparation of aqueous anionic poly-(urethane-urea) dispersions: influence of the nature and proportion of the urethane groups on the dispersion and polymer properties. *J Appl Polym Sci*, 94(2):700-710, 2004.
- [11] DuMouchel, W.; Bradeley, J. A simple Bayesian modification of D-optimal designs to reduce dependence on an assumed model. *Technometrics*, 36:37-47, 1994.
- [12] Höfle, G.; Steglich, W.; Vorbrüggen, H. 4-

- dialkylaminopyridines as highly active acylation catalysts. *Angew Chem Int Ed Engl*, 17:569-583, 1978.
- [13] Annadurai, G.; Sheeja, R. Y. Use of Box-Behnken design of experiments for the adsorption of verofix red using biopolymer. *Bioprocess and Biosystems Engineering*, 18(6):463-466, 1998.
- [14] Goupy, J., *Plans d'expériences : les mélanges*. DUNOD ed., 2000.
- [15] Shapiro, S. S.; Wilk, M. B. An analysis of variance test for normality (complete samples). *Biometrika*, 52:591-611, 1965.
- [16] Royston, P. An Extension of Shapiro and Wilk's W Test for Normality to Large Samples. *Applied Statistics*, 31:115-124, 1982.
- [17] Box, G. E. P.; Cox, D. R. An analysis of transformations. *J. Royal Stat. Soc.*, 26:211-243, 1964.
- [18] Nelder, J. A.; Mear, R. A simplex method for function minimization. *Comput J*, 7:308-315, 1965.