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Research paper

Evidence for restrictive parameters in formulation of insulin-loaded nanocapsules

Fabienne Cournarie[a], Monique Chéron[b], Madeleine Besnard[a], Christine Vauthier[a,*]

[a]*Laboratoire de Physico-chimie, Pharmacotechnie et Biopharmacie, Université Paris XI, Châtenay-Malabry, France*
[b]*Laboratoire de Physico-chimie Biomoléculaire et Cellulaire, Paris, France*

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Abstract

Poly(isobutylcyanoacrylate) nanocapsules with an oily core were originally proposed for lipophilic drug encapsulation [Int. J. Pharm. 28 (1986) 125] but insulin, a hydrosoluble protein, has also been successfully encapsulated by Damgé et al. [Diabetes 37 (1988) 246]. The aim of this work was to understand if several parameters were restrictive for the encapsulation of insulin into the oily core of the nanocapsules prepared by interfacial polymerization. The encapsulation efficiency of insulin was not affected by the type of insulin since the peptides adopted the same association state after their addition to the organic phase. Formulation parameters mainly affected the size of the nanocapsules obtained but did not influence the insulin encapsulation efficiency. In contrast, the order of introduction of insulin and of the monomer in the organic phase was shown to control the formation and the characteristics of the nanocapsules. The key parameters, which were found to clearly influence the encapsulation efficiency of insulin, were the pH of the aqueous insulin solution and the origin of the monomer. Both of these parameters can affect the rate of the interfacial polymerization. Consequently, the ability of insulin to be entrapped into the oil containing nanocapsules appeared to be governed more by the rate of the monomer polymerization.

Keywords: Insulin; Poly(isobutylcyanoacrylate) nanocapsules; Interfacial polymerization; pH

1. Introduction

The process for the manufacture of isobutylcyanoacrylate (IBCA) nanocapsules by interfacial polymerization has been first described by Al Khouri Fallouh et al. [1] and the influence of the main physicochemical and technological parameters on unloaded nanocapsule characteristics have been largely studied since. Parameters that influence the size and the formation of the nanocapsules have been determined: temperature and pH of the aqueous dispersing phase, concentration of absolute ethanol, stirring rate of the aqueous dispersing phase, flow rate of injection of the organic phase into the aqueous dispersing phase, surfactant concentration, nature of the oil [1–4]. Poly(isobutylcyanoacrylate) (PIBCA) nanocapsules with oily core were originally proposed for lipophilic drug encapsulation, for which encapsulation efficiency reached 100% [1,5], whereas other encapsulation techniques have been proposed for entrapping water-soluble compounds, among them the double emulsion/solvent evaporation method [6–8] and interfacial polymerization of alkylcyanoacrylate in spontaneously forming water-in-oil microemulsions [9–11]. However, the process for the manufacture of oil containing IBCA nanocapsules has been adapted to the encapsulation of insulin by Damgé et al. [12]. By optimizing the ratio of the different components, these authors reached an insulin encapsulation efficiency of almost 100%. Aboubakar et al. [13] demonstrated that such a high encapsulation efficiency cannot be explained by a specific interaction between insulin and the polymer forming the nanocapsule wall. In addition, the insulin molecule was not chemically modified during the nanoencapsulation process, and insulin was found to be located in the oily core of the nanocapsule. To the present time no further studies have considered which parameters could control the high encapsulation efficiency of insulin reported previously. Indeed, most of the previous

* Corresponding author. Laboratoire de Physico-chimie, Pharmacotechnie et Biopharmacie UMR CNRS 8612, Université Paris XI, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, 92 296 Châtenay-Malabry Cedex, France. Tel.: +33-1-46-83-53-86; fax: +33-1-46-61-93-34.
E-mail address: christine.vauthier@cep.u-psud.fr (C. Vauthier).

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works on insulin-loaded PIBCA nanocapsules have been concerned with biological activity and in vivo investigation [12,14–20].

During the preparation, a large number of parameters may influence the nanocapsule characteristics whereas reproducibility is essential for transposition of the process to an industrial scale. The purpose of our work was to investigate if some parameters can be restrictive for the encapsulation of insulin into the oily core of the nanocapsules. In this view, the effect of the method of preparation of both the insulin solution and the organic phase on the nanocapsule characteristics were investigated for the first time. Three insulins, differing either from their structure or from their origin, were used as well as a monomer from two suppliers. The effect of these parameters was evaluated on the insulin encapsulation efficiency, nanocapsules size and zeta potential.

2. Materials and methods

2.1. Materials

Insulin from bovine pancreas was purchased from Sigma (St Quentin Fallavier, France) and recombinant human insulins (Humalog® and Umulin®) were generously supplied by the Eli Lilly Company (Indianapolis, USA). Hydrochloric acid (HCl), sodium hydroxide (NaOH), potassium phosphate (KH_2PO_4) (Sigma, St Quentin Fallavier, France), were used for the preparation of insulin solutions. IBCA used as a monomer was either kindly supplied by Loctite (Dublin, Ireland) or purchased from Ethnor (Issy les Moulineaux, France), Miglyol® 812 N (medium-chain triglycerides) was a gift from Hüls (Puteaux, France), Lutrol® F68 (Poloxamer 188) used as a surfactant was obtained from BASF (Ludwigshafen, Germany) and absolute ethanol was purchased from Carlo Erba (Rodano, Italy). For HPLC determination of insulin, trifluoroacetic acid (TFA) (Sigma, St Quentin Fallavier, France) and acetonitrile (Carlo Erba, Rodano, Italy) were used. Potassium chloride (KCl) (Sigma, St Quentin Fallavier, France) was used for Zeta Potential measurements. Hydroquinone was purchased from Fluka (St Quentin Fallavier, France). MilliQ® water (Waters Millipore, Saint-Quentin en Yvelines, France) was used throughout.

3. Methods

3.1. Preparation of insulin-loaded nanocapsules

Insulin-loaded nanocapsules were prepared by interfacial polymerization of IBCA according to the method proposed by Damgé et al. [12]. An insulin solution was prepared as follow: 25 mg of insulin (625 IU) was dissolved in acidic water (400 μl water + 200 μl HCl 0.2 N). A buffer was then added (500 μl of KH_2PO_4 1/15 M) and the solution was completed to a final volume of 2.5 ml with water. The pH of this solution was 2.5 and the insulin concentration 10 mg/ml (250 IU/ml). Insulin solutions of different pH values (1.8, 3, 3.5, 4) were then prepared adding either HCl or NaOH. An organic phase was obtained by adding the following components, absolute ethanol (25 ml), Miglyol 812® (1 ml), IBCA (125 μl) and the previously prepared insulin solution (500 μl), at different orders, as shown in Table 1, and at different stirring rates (from 250 to 500 rpm). This organic phase was finally added to an aqueous phase (50 ml) containing Lutrol® F68 (0.25%), as soon as the last ingredient was added in the organic phase, and maintained at a temperature below 20 °C. Based on preliminary experiments and on the literature [1], the stirring rate of the aqueous phase was fixed to 750 rpm. The organic phase was either dropped into the aqueous phase or injected via a silicone catheter. The flow of injection of the organic phase into the aqueous phase varied from 6.1 to 141 ml/min. Nanocapsules, which formed immediately, were stirred for 30 min. The suspension of nanocapsules was adjusted to 0.4 mg (10 IU) insulin/10 mg polymer/ml by rotoevaporation under vacuum. Insulin free solutions were prepared following the procedure described for the insulin solution but without insulin. Insulin free solutions at pH 2.5 and at pH 3–4 were used to prepare unloaded nanocapsules and to investigate the influence of the addition of the aqueous solution into the organic phase, respectively. All the results concerning the characterization of the nanocapsules were obtained from at least 10 different preparations.

3.2. Determination of the nanocapsule size

The mean diameter and the nanocapsule size distribution were measured by Quasi Elastic Light Scattering (scattering angle 90°) with a Nanosizer N4 plus (Coultronics, France). Measures were done three times on each sample.

3.3. Dissolution of nanocapsules and determination of the total insulin recovered

To determine the total amount of insulin recovered after nanocapsule preparation, 1 ml of the nanocapsule suspension was allow to dissolve in 1 ml of acetonitrile for 24 h. The amount of insulin was evaluated by HPLC only on samples in which nanocapsules were fully dissolved.

3.4. Determination of insulin encapsulation efficiency

The determination of insulin encapsulation efficiency was evaluated by the indirect method. Non-encapsulated insulin was measured in the polymerization medium after removing the nanocapsules by ultracentrifugation at 112,000 × *g* for 90 min (Beckman Model L7-55 centrifuge, 70 I-Ti rotor, Beckman Instruments, Palo Alto, CA). The encapsulation efficiency of insulin was calculated from

Table 1
Effect of the order of introduction of the different components during the preparation of the organic phases on the characteristics of the nanocapsules

Method	Organic phase	Diameter (nm)	% Insulin recovered after nanocapsule dissolution	Nanocapsule solubility in acetonitrile after 24 h	Encapsulation efficiency (%)
A	1. Ethanol				
	2. IBCA[a]?	360 ± 30	100	Totally	
	3. Miglyol			soluble	60 ± 10
	4. Insulin[b]?			polymer	
B	1. Ethanol				
	2. Miglyol			Totally	60 ± 10
	3. IBCA[a]	363 ± 32	100	soluble	
	4. Insulin[b]			polymer	
C	1. Ethanol				
	2. Insulin[b]			Insoluble	
	3. Miglyol	284 ± 28	70	polymer	ND
	4. IBCA[a]			remains	
D	1. Ethanol				
	2. Insulin[b]			Insoluble	
	3. IBCA[a]	286 ± 27	70	polymer	ND
	4. Miglyol			remains	

ND, not determined.
[a] IBCA from Loctite.
[b] Bovine insulin solution at pH 1.8.

the difference between the total amount of insulin and the non-encapsulated insulin retrieved from the polymerization medium.

3.5. Dosages of insulin

Dosages of insulin were performed by HPLC using a reverse phase column C18 (μBondapack, Waters Millipore, France, ref P/N 27324). The solvent consisted of 0.1% TFA in water (solvent A) and 0.1% TFA/49.9% water/50% acetonitrile (solvent B). Elution was performed at a flow rate of 1 ml/min with a linear gradient (10–100% solvent B over 80 min). The absorbance was measured using a UV detector at 220 nm (Waters 486). Each sample was analyzed three times.

3.6. Zeta potential

Nanocapsule zeta potential was determined by laser Doppler anemometry (Malvern Zetasizer IIc, France). The principle is based on the Doppler shift caused by the movement of particles across interference fringes that are produced by intersection of two laser beams. Nanocapsules (20 μl) were suspended in 1 mM NaCl (2 ml, pH 6.5) and the measurements were made at 25 °C.

For some experiments, insulin (in a quantity corresponding to 25% of non-encapsulated insulin or to 0% of encapsulation) was added to the suspension of unloaded nanocapsules, stirred for 2 h before proceeding to measurements. Each sample was measured 10 times.

3.7. NMR studies

^{1}H-NMR and ^{13}C-NMR (200 MHz) spectra were recorded on a Bucker AC 200P spectrometer, after dissolution of the monomers from Loctite and Ethnor in $CDCl_3$ and DMSO-d6 (Sigma, St Quentin Fallavier, France).

3.8. Spectroscopic studies

UV-visible spectra of IBCA were recorded on a UV/Vis spectrometer Lambda 11 (Perkin Elmer, Norwalk, USA) at a scanning rate of 10 nm/min. Visible spectra were obtained on pure monomers whereas UV spectra were recorded on the monomers diluted in acetonitrile at concentrations of 10 mg/ml and at 67 μg/ml. Visible and UV spectra given by acetonitrile solutions of hydroquinone, a very well known polymerization inhibitor used to stabilize monomers, were recorded respectively at concentrations of 4.5 mg/ml and 22 μg/ml.

3.9. Circular dichroism studies

Circular dichroism spectra were recorded with a Jasco J-810 (Jasco, Tokyo, Japan) dichrograph equipped with a peltier type temperature control system. Insulin solutions at different pH values were scanned from 300 to 230 nm at a scanning rate of 10 nm/min and at a temperature of 25 °C using a thermostated cuvette holder. The insulin solutions were subsequently introduced in the ethanol containing

the oil and those resulting organic phases were scanned from 300 to 230 nm and from 240 to 200 nm. A 0.1- and a 0.5-cm path-length quartz suprasil cuvette were used, respectively, for the higher and the lower wavelength region to obtain optimum resolution of the spectra. The generated ellipticity values were subsequently converted to molar circular–dichroic absorption ($\Delta\epsilon$) using the equation $\Delta\epsilon = \theta/(32980Cl)$ (where θ is the observed ellipticity, C the molar insulin concentration and l the path-length of the cell). Spectra are expressed in $\Delta\epsilon$ function of the wavelength. $\Delta\epsilon$ (M^{-1} cm^{-1}) is the differential molar dichroic absorption coefficient.

4. Results

4.1. Influence of the process of nanocapsule preparation

4.1.1. Preparation of the organic phase

The influence of the preparation of the organic phase was explored according to two parameters, considering successively the order of introduction of the different components and the rate of stirring. Organic phases of the same composition were prepared in different ways as described in Table 1 and injected into the aqueous phase to form the insulin-loaded nanocapsules. The size, the drug loading efficiency and the solubility of the resulting nanocapsules in acetonitrile were investigated. The order of introduction of monomer and Miglyol influenced neither the encapsulation efficiency nor the size of the nanocapsules formed (methods A and B in Table 1). The nanocapsules were found to be totally soluble in acetonitrile. However, when insulin was introduced before the monomer (methods C and D in Table 1), nanocapsules formed were smaller and were not totally soluble in acetonitrile after 24 h since undissolved polymer remained in the dispersion. Moreover, the total amount of the insulin introduced in the preparation of nanocapsules was not recovered by HPLC and encapsulation efficiency could not be determined. From these results it appears that it is essential to introduce insulin as the last component when preparing the organic phase. Therefore, all the following experiments were performed according to method A.

Since the organic phase needs to be added to the aqueous phase immediately after addition of the last ingredient, its stirring rate was varied from 250 to 500 rpm to investigate the influence on the nanocapsule characteristics. Indeed, the homogeneity of the organic phase may influence those characteristics. As shown in Table 2, the size of the nanocapsules formed was influenced by the rate of stirring whereas the encapsulation efficiency was not affected by this parameter. Therefore, to produce reproducible batches of nanocapsules, the rate of stirring was chosen to produce the smallest nanocapsules and kept at 375 rpm for all the following experiments.

Table 2
Influence of the rate of stirring of the organic phase on nanocapsule formation and characteristics (bovine insulin solution at pH 1.8 and IBCA from Loctite)

Stirring rate (rpm)	Mean diameter (nm)	Encapsulation efficiency (%)
250	420 ± 50	59 ± 12
375	335 ± 25	60 ± 10
500	417 ± 33	61 ± 11

4.2. Method of addition of the organic phase in the aqueous phase

First, the influence of the flow rate of the injection of the organic phase into the aqueous phase was investigated. The results reported in Table 3 show that the faster the flow rate, the smaller and the narrower in size distribution the nanocapsules were. In contrast, the flow rate did not influence the encapsulation efficiency.

Second, the influence of the way of addition of the organic phase into the aqueous phase was investigated. More precisely, it was studied if the presence or the absence of an air/water interface could influence the formation and the characteristics of the nanocapsules. The results showed that when the organic phase was dropped into the aqueous phase, the presence of an air/water interface promoted the formation of aggregates. In contrast, no aggregate was formed by injecting the organic phase directly into the water phase using a silicone catheter and avoiding the presence of the air/water interface. For the best success of the method, a high flow rate for the addition of the organic phase should be recommended and the organic phase should be injected directly into the aqueous phase.

4.3. Effect of the type of insulin and of the monomer source

For this part of the work, the organic phase used in the preparation of the nanocapsules was obtained following method A (Table 1) and with a stirring rate of 375 rpm. The organic phase was injected in the aqueous phase at a flow rate of 141 ml/min using the silicone catheter.

With the aim of investigating the reproducibility of the insulin encapsulation method, we studied the encapsulation of different insulins using monomers from two suppliers. Moreover, we investigated the effect of the pH of the insulin

Table 3
Influence of the flow of injection of the organic phase into the aqueous phase on nanocapsules size (bovine insulin solution at pH 1.8 and IBCA from Loctite)

Flow rate (ml/min)	Mean diameter (nm)	Encapsulation efficiency (%)
6.1	465 ± 60	57 ± 20
14.1	374 ± 25	52 ± 15
141	360 ± 20	60 ± 10

solution on the encapsulation efficiency. Experiments were performed with insulin solutions of pH up to 4 to insure complete dissolution of insulin in the aqueous solution. Indeed, above pH 4, insulin starts to precipitate since the pH of the solution approaches its pH_i, which is about 5.4. It should be noted that when insulin solutions at pH 3–4 were added into the organic phase, an increase in turbidity due to a Tyndall effect appeared, while after addition of solutions at the same pH but without insulin, the organic phase remained totally clear. Thus the size of nanocapsules, insulin encapsulation efficiency and zeta potential were only evaluated for insulin solutions prepared between pH 1.8 and 2.5 to be sure to work with fully dissolved insulin in both the aqueous solution and after addition into the organic phase. Working with the monomer from Loctite, the encapsulation efficiency of insulin varied from 58 to 76% while the pH of the insulin solution was respectively comprised between 1.8 and 2.5 independently of the type of insulin (Table 4). The diameters of the nanocapsules reported in Table 4 show that they were not affected by the pH of the insulin solution. The zeta potential of nanocapsules prepared with an aqueous solution at pH 2.5 (insulin-loaded and unloaded) was -10.0 ± 0.3 mV for Umulin® nanocapsules, -8.4 ± 0.4 mV for Humalog® nanocapsules and -9.3 mV $\pm$ 0.7 mV for unloaded nanocapsules. As shown by these results, all the nanocapsules presented a very similar zeta potential of around -10 mV. To investigate whether non-encapsulated insulin could induce measurable variation of zeta potential of the nanocapsules, free insulin (Umulin®) was added in the dispersing medium of unloaded nanocapsules to mimic the encapsulation efficiency of 75% (25% of insulin in the external medium) and of 0% (100% of insulin in the external medium). The zeta potentials measured for those preparations were respectively, -9.02 ± 0.3 mV and -13.6 ± 0.3 mV indicating that the zeta potential of the unloaded nanocapsules was not dramatically affected after addition of free insulin in the dispersing medium. The same results were obtained with Humalog® and bovine insulin.

By using a monomer from a different supplier (Ethnor), and keeping all the other parameters constant, the encapsulation efficiency obtained when working with an insulin solution at pH 2.5, increased from 75% up to 95% as indicated in Table 4. It is important to note that the results were, in this last case, much more reproducible, as suggested by the lower values of standard deviations.

Finally, it should be noted that nanocapsules were mainly formed in all the preparations but the presence of a small pellet after ultracentrifugation suggested that a small amount of nanospheres was also produced [4].

4.4. Characterization of the different insulins

The association states of the insulins used in this study were investigated by circular dichroism, first in the aqueous solution at pH 2.5 and at a concentration of 10 mg/ml, and second after addition of this solution into the monomer free organic phase where the final concentration was 0.2 mg/ml. In the aqueous solution, a negative maximum was observed for all insulins at 275 nm (Fig. 1A). The CD spectra of Umulin® and bovine insulin were identical whereas the band of Humalog® was less pronounced. After addition of the insulin aqueous solution into the organic phase, the previously observed dichroic band completely disappeared (Fig. 1B) whereas an absorption band at 210 nm was noted and revealed almost the same intensity for all insulins (Fig. 2).

4.5. Characterization of the monomer

Macroscopically, the monomer from Loctite was perfectly colorless while the monomer from Ethicon presented a slight yellowish color. ^{1}H- and ^{13}C-NMR analysis did not reveal any difference and showed all characteristic peaks due to the chemical structure of IBCA (data not shown). Ultra-violet visible spectra of the monomers are presented in Fig. 3. In the visible domain, the monomer supplied by

Table 4
Characteristics of insulin-loaded nanocapsules prepared with IBCA from Loctite and Ethnor

Nature of insulin		Monomer source			
		Loctite			Ethicon
	pH of insulin solution	1.8	2.5	3, 3.5, 4	2.5
Sigma	Encapsulation efficiency (%)	60 ± 10	75 ± 10	Organic phase slightly turbid	ND
	Size (nm)	360 ± 20	359 ± 20		ND
Umulin®	Encapsulation efficiency (%)	59 ± 11	76 ± 9	Organic phase slightly turbid	95 ± 3
	Size (nm)	355 ± 23	353 ± 21		295 ± 9
Humalog®	Encapsulation efficiency (%)	58 ± 12	74 ± 11	Organic phase slightly turbid	92 ± 4
	Size (nm)	352 ± 20	350 ± 18		300 ± 10

ND, not determined.

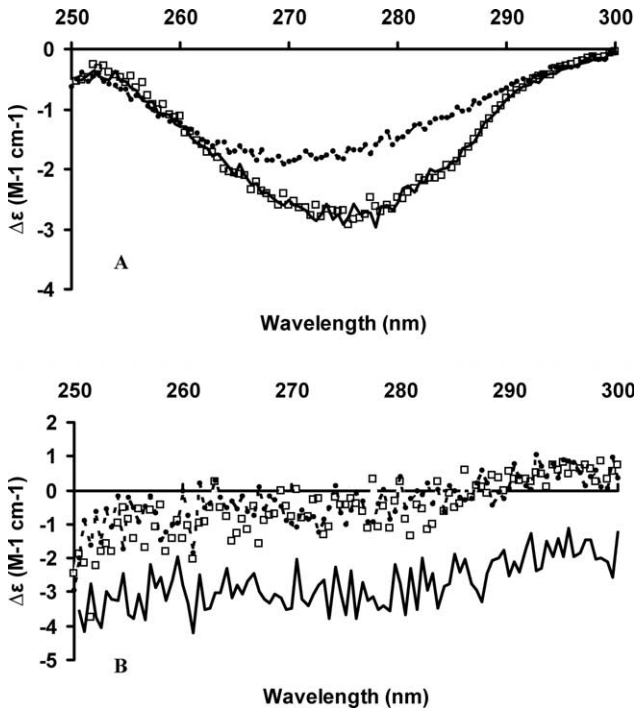

Fig. 1. Circular dichroic spectra of Umulin® (squares), Humalog® (dots) and bovine insulin (solid line), A in aqueous solution (10 mg/ml), B after addition in the organic phase (0.2 mg/ml) between 250 and 300 nm.

Ethicon showed an absorption peak at 466 nm in agreement with the yellowish color (Fig. 3A). In the UV domain, both monomers showed very similar absorption spectra with a first peak around 210 nm (data not shown) and a second peak around 300 nm (Fig. 3B). As an example of polymerization inhibitor used as stabilizer of IBCA [21], the UV spectrum of hydroquinone was also monitored. It showed an absorption peak at 300 nm (Fig. 3C) and no absorption in the visible domain (data not shown).

5. Discussion

To develop the preparation of insulin-loaded nanocapsules up to an industrial level, it is necessary to control all the parameters that affect the characteristics of the resulting

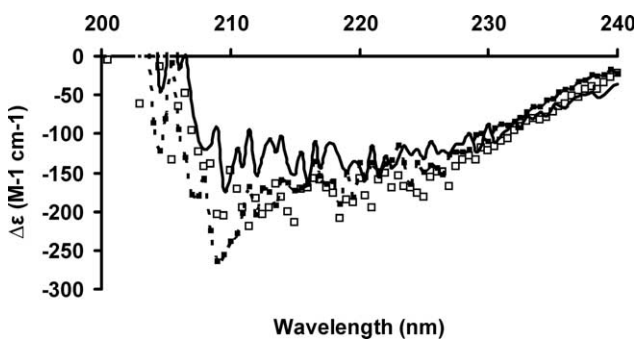

Fig. 2. Circular dichroic spectra of Umulin® (squares), Humalog® (dots) and bovine insulin (solid line) after addition in the organic phase (0.2 mg/ml) between 200 and 240 nm.

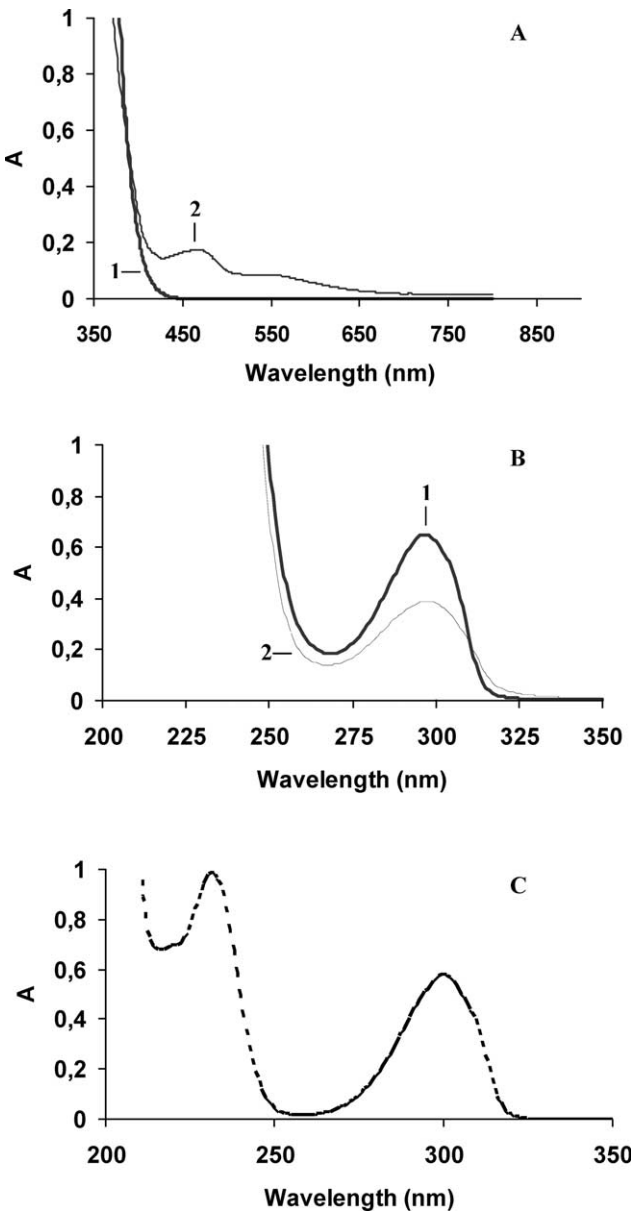

Fig. 3. UV/visible spectra of pure monomer (A), monomer at 10 mg/ml (B) and hydroquinone at 22 μg/ml (C). (1) Loctite monomer, (2) Ethnor monomer.

nanocapsules in terms of both their size and insulin encapsulation efficiency. The first part of our study devoted to investigate some of the process parameters showed that parameters, such as the stirring rate of the organic phase and the flow rate of injection of the organic phase into the aqueous phase, influenced the size of the nanocapsules in agreement with previously reported data obtained for unloaded nanocapsules [1]. However, the preparation of the organic phase and especially the order of addition of the insulin and of the monomer appeared to play a critical role in the final characteristics of the nanocapsules. Indeed, a loss of insulin regarding the total initial amount used in the preparation of the nanocapsules and a lack of solubility of the resulting insulin-loaded nanocapsules in acetonitrile

were observed only when insulin was added to the organic phase before the monomer (methods C and D in Table 1). In contrast, when insulin was added at last and just before injecting the organic phase into the aqueous phase (method A and B in Table 1), the total amount of insulin was recovered and the nanocapsules can be fully dissolved in acetonitrile. As reported by Leonard et al. [22], Damgé et al. [23], and Aboubakar et al. [13], insulin can react with the monomer used in the preparation of the nanocapsules leading to an insoluble polymer in acetonitrile. Thus it can be postulated that the introduction of insulin in the organic phase before (method C and D) or after (method A and B) the monomer could respectively promote or avoid a reaction of insulin with the monomer leading to insoluble or soluble nanocapsules.

Beside nanocapsules, a small amount of nanospheres were always produced whatever the condition of fabrication. Indeed, the use of ethanol, a protic solvent, was shown to promote the formation of nanospheres [24]. The commonly accepted mechanism for the formation of PIBCA nanocapsules is interfacial polymerization of the organic phase upon mixing with the aqueous phase [1,2]. The formation of nanospheres can be due to either the fragmentation process of the polymeric interfacial film, which does not contain any oil droplets [2], or to polymerization nuclei, already formed in the organic solvent [24]. Chouinard et al. [4] proposed that nanospheres may be formed by nanoprecipitation of a preformed polymer in a large excess of non-solvent at the oil/water interface. This supposes that the polymerization of IBCA started in the organic phase. If ethanol was shown to induce the formation of small oligomers in the organic phase [13], it is believed that this mechanism may only be marginal in the conditions used here for the preparation of insulin-loaded nanocapsules. In our experiments, no evidence of polymerization process starting in the organic phase was highlighted, the solution being perfectly clear after addition of every compound and with insulin solutions at pH 1.8 and 2.5. In these conditions, a colloidal milky suspension was obtained only after addition of the organic phase into the aqueous phase.

Zeta potential of the colloidal particles is very sensitive to small changes in the electrical properties of the particle surface. Thus, it is assumed that adsorption of insulin on the nanocapsule surface will cause a modification of the nanocapsule zeta potential. The insulin-loaded nanocapsules, the unloaded nanocapsules and the unloaded nanocapsules in the presence of free insulin added in the external medium showed almost the same zeta potential value. These results mean that if insulin is adsorbed onto the nanoparticle surface, the amount was so low that no modification of zeta potential was induced. They are in agreement with those of Aboubakar et al. [13]who observed only a slight variation of zeta potential when insulin was added in the external medium at a concentration representing 100% of the amount of insulin used for the nanocapsule formation. Significant variations of zeta potential appeared with much higher concentrations of free insulin added in the dispersing medium. Thus, the results obtained in our experimental conditions show that zeta potential of nanocapsules is not a good indicator to make the difference between insulin truly encapsulated in the oily core of the nanocapsule and insulin which may be adsorbed at the nanocapsule surface.

The literature reports the encapsulation of different kinds of insulin in poly(alkylcyanoacrylate) nanocapsules prepared by interfacial polymerization. For instance, highly purified semicrystalline zinc insulin from Novo Nordisk [16,17,20], insulin Velosulin® for Nordisk [14], and bovine insulin from Sigma [13] were found to be successfully encapsulated with an encapsulation efficiency up to almost 100%. In contrast, commercially available Umulin® solutions from Lilly and regular porcine insulin solutions from Organon, which both contained insulin preservative agents, were encapsulated with lower efficiency (about 55%) [12]. The effect of the insulin type has never been taken into account in any of the previous studies. Thus, here we tested the influence of the insulin type using crystalline forms of insulin from three different origins. In aqueous solution, insulin exists as an equilibrium mixture of monomers, dimers, hexamers and possibly some higher associated states depending particularly on species of insulin, zinc content, pH, concentration and solvent [25]. It is interesting to note that, the primary structure of Humalog® is identical to that of Umulin® except for the transposition of two amino acids (proline and lysine) at positions 28 and 29 in the C-terminus region of the B chain. As a result, Humalog® has a lower ability to self-associate into dimers and hexamers and dissociate much faster than Umulin® [26].

Circular dichroism experiments evidenced a higher associated state for Umulin® and bovine insulin compared to Humalog® in aqueous solution at pH 2.5 and at a concentration of 10 mg/ml. Indeed, the band at 275 nm provides an indication of insulin self-association [27–29]. The negative band at 275 nm was assigned to tyrosine and phenylalanine aromatic residues in the B23–28 region of the antiparallel structure formed between the monomers of insulin in the hexamer structure. Attenuation of this band is attributed to insulin hexamer dissociation [30]. The negative maximum at 275 nm, observed with a lower intensity for Humalog® than for Umulin® and bovine insulin, attests of the ability of Humalog® to dissociate in solution. The difference in the association state of the insulins, clearly shown in aqueous solution, totally disappeared after addition of the insulin solutions into the organic phase. In the organic phase, the monomeric form became the predominant species of insulin, since the band appearing at 210 nm was associated to the α-helical structure of monomers. The dissociation can be attributed either to the ethanol which, as organic solvent, can counteract associations [25] or to the dilution (insulin 0.2 mg/ml). These results suggested that, whatever the association state of insulin in the aqueous solution was, the addition in the organic phase leads to monomerization of

the peptide. Therefore, the encapsulation depends on the monomer species. In addition, insulin monomers exhibit hydrophobic regions at the surface of the molecule, which are normally hidden in associated states [31,32]. This hydrophobicity could be favorable for hydrophobic interactions with the oily phase leading to high encapsulation efficiency. Because insulin is a peptide, its solubility is affected by the pH. Above pH 4, insulin was not soluble in water and above pH 3, insulin was not soluble in the organic phase, as suggested by the apparition of a Tyndall effect. When insulin was added at pH 1.8, it induced a decrease of the insulin encapsulation efficiency compared to pH 2.5. The role of the pH on the polymerization of alkylcyanoacrylate has been discussed previously in the case of the emulsion polymerization of isohexylcyanoacrylate and IBCA in which various concentrations of sulfur dioxide were added. It was found that the pH can modulate the efficacy of sulfur dioxide to control the anionic polymerization of alkylcyanoacrylates [33,34]. As suggested by Aboubakar et al. [13], a slower polymerization reaction slows down the formation of the polymer envelop around the oil droplets. Thus, insulin has more time to partition in water and to escape the oily core of the nanocapsules that form. Consequently, the encapsulation decreases.

The present study also highlighted a clear effect of the source of the monomer on the insulin encapsulation efficiency. The encapsulation efficiency, as well as the reproducibility of the encapsulation, was higher with the monomer obtained from Ethnor than with the monomer provided by Loctite. According to the results of the NMR analysis, both monomers displayed the expected chemical structure for IBCA, meaning that they were of the same species. In contrast, UV-visible spectra revealed some differences. The absorption peak appearing at 300 nm presented the same shape and maximum wavelength than hydroquinone, a free-radical polymerization inhibitor founded in the composition of the IBCA provided by Ethnor [21]. The presence of this absorption peak suggested that this component was actually added as stabilizing agent to both monomers. The comparison of the height of the peak for monomer solutions at the same concentration indicated that the concentration of hydroquinone was higher in the monomer provided by Loctite than in the monomer provided by Ethnor. Another difference between both monomers could be highlighted in the wavelength range of the visible domain. An absorption peak with a maximum at 466 nm appeared with the monomer provided by Ethnor, in agreement with its yellowish color. These analyses revealed that, whereas the major component of the monomers provided by the two suppliers corresponding to the expected IBCA, other compounds, which nature varies with the monomer suppliers, were added as traces at different concentrations. Such components can be polymerization inhibitors that affect the interfacial polymerization of the IBCA during the preparation of the nanocapsules. Indeed, Behan et al. [35] pointed out that little attention is usually given to the reactivity of the monomer despite the fact that monomers are stabilized by polymer inhibitors whose nature and quantities vary from batch to batch and from suppliers [36]. Here, both the pH and the source of the monomer were found to affect the encapsulation efficiency of insulin. Therefore, to achieve successful and reproducible encapsulation of insulin, it is important to work with reproducible batches of monomers and with insulin aqueous solutions prepared at a defined pH.

6. Conclusion

The aim of this work was to investigate if some parameters can be restrictive for the encapsulation of insulin into the oily core of the nanocapsules obtained by interfacial polymerization of IBCA. This work highlighted for the first time that two major parameters control the encapsulation of insulin: the pH of the insulin solution and the quality of the monomer in terms of the nature and the quantities of the added polymerization inhibitors. Those parameters may affect the reactivity and the polymerization of the monomer. As a consequence, it seems that the faster the interfacial polymerization of IBCA occurs, the higher the encapsulation efficiency of insulin is. Thus, those parameters should be kept under control to obtain high loaded insulin nanocapules with reproducible characteristics.

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