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Research Article

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Experimental problems in the application of UV/visible based methods as the quantification tool for the entrapped/released insulin from PLGA carriers

V.L. Lassalle* and M.L. Ferreira

Abstract

BACKGROUND: Controlled release of medicaments from biodegradable polymers remains the most convenient way for their sustained release. Although a number of articles have been published, experimental work involving the preparation of polymer-based carriers and release procedures are not described with sufficient level of detail to allow other researchers to reproduce the experiments and to compare published results with their own. In this contribution the experimental background of the entrapment and release of insulin from PLGA carriers is described and the problems found at each step related to UV the visible method used to quantify them are addressed in detail.

RESULTS: The quantification of entrapped insulin by UV/visible methods was affected by aggregation. The design of the release experiment influenced the results regarding the entrapment efficiency (EE) and the maximum percentage of released insulin. It was also found that the presence of colloidal polymeric particles, insufficient centrifugation times and the kind of solvent used in the release test might lead to mistakes in the percentage of liberated insulin when UV/visible based methods are employed.

CONCLUSIONS: This contribution demonstrates that serious discrepancies in the EE and percentage of released protein may arise if some key experimental factors are not taken into account. Therefore, the analysis presented here tries to point out important aspects of this topic currently not reported, unnoticed or not properly analyzed in the open literature. The results are useful for the entrapment of any protein on any polymeric device using UV/visible based methods to quantify them.

Keywords: sustained release; insulin; poly (lactic-co-glycolic acid); *in vitro* release; protein entrapment

INTRODUCTION

With the advent of biotechnology, poly (lactic acid) (PLA), poly (glycolic acid) (P GA) and the copolymers poly (lactic-co-glycolic) acids (PLGA), have received increasing attention as systems for the sustained release of drugs; and among the most important therapeutic proteins and peptides being explored is insulin.[1–4] Several polymeric devices such as nano/microparticles, nano/microcapsules, hydrogels and complexes have been investigated to entrap/link insulin and other proteins; therefore, the number of publications related to this issue has grown considerably. However, the broad dispersion noted in published articles with regards to the entrapment efficiency and maximum released protein (reported in terms of percentage of cumulative release relative to the entrapped amount of protein) during the incubation period, led to concerns about the accuracy of the procedures implemented. In fact, the precise quantification of the proteins entrapped in the studied device as well as the measurement of the released drug still appear to be difficult tasks and major obstacles are encountered in relation to the analysis, specially when UV/visible based methods are employed to these purposes.[5–7] Published literature referring to this specific matter is limited and, in general, not enough details are supplied. Consequently, the discrepancies found cannot be explained since they are difficult to evaluate if the problems presented are operational or there are alternative explanations that were not included in the description of the experiments. A few recent articles deal with particular features of the entrapment and release of proteins from PLGA-based devices. For example, Bilati *et al*. reviewed the effect of the processing parameters on the stability of the entrapped proteins during the formation of biodegradable nano/microparticles and during the release process. They explored the influence of the solvents, the sampling method, the polymeric matrix and the presence of additives on the quality and stability of the selected protein. However, they have not investigated the effect of such factors on the reported entrapment efficiency and on the percentage of liberated protein.[8]

Giteau *et al*. reported the influence of experimental conditions on the release profile of proteins from PLGA nano/microparticles with the aim of achieving complete release of the therapeutic agent. They evaluated the release medium, the sampling method

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and other factors affecting the stability of the proteins and proposed some strategies to avoid protein destabilization and to promote the complete release of the drug from the carrier. Aspects concerning protein stability during the encapsulation step were not addressed in that article.[9]

In the present work a careful study of the variables involved in the entrapment/release of insulin on/from PLGA-based carriers has been undertaken, showing the effect of the parameters studied on the reported results when UV visible based methods are employed to quantify the protein content. Original data are given in order to demonstrate the experimental difficulties found with protein quantification and to explain the origin of the discrepancies detected in the published results.

The importance of the analysis of the protein in solution by UV/visible methods, before quantification, was emphasized. The tendency of the insulin to aggregate strongly affects the data, so factors like the solvent and the protein concentration were evaluated in order to avoid mistakes.

The other important point on which this contribution is focused is the design of the release experiment. In general, incubation conditions able to reproduce the *in vivo* environment are employed, but the analysis of a number of factors, such as the kind of media, sampling method, centrifugation step, presence of colloidal particles, effect of the PLGA, is essential to achieve reproducible and accurate results. It is worth noting that a study of this nature is difficult to find in the available literature, so that the comparison between results of different researchers becomes difficult.

The goal of this article is to demonstrate that confusing results may be achieved if an accurate analysis of the experimental conditions is not performed. The way and level to which such parameters alter the results are clearly stated, and consequently a number of strategies designed to avoid/detect them are proposed, supplying the details needed to compare and reproduce the strategies. This work emphasizes the need to clearly establish the procedures used in these kinds of studies.

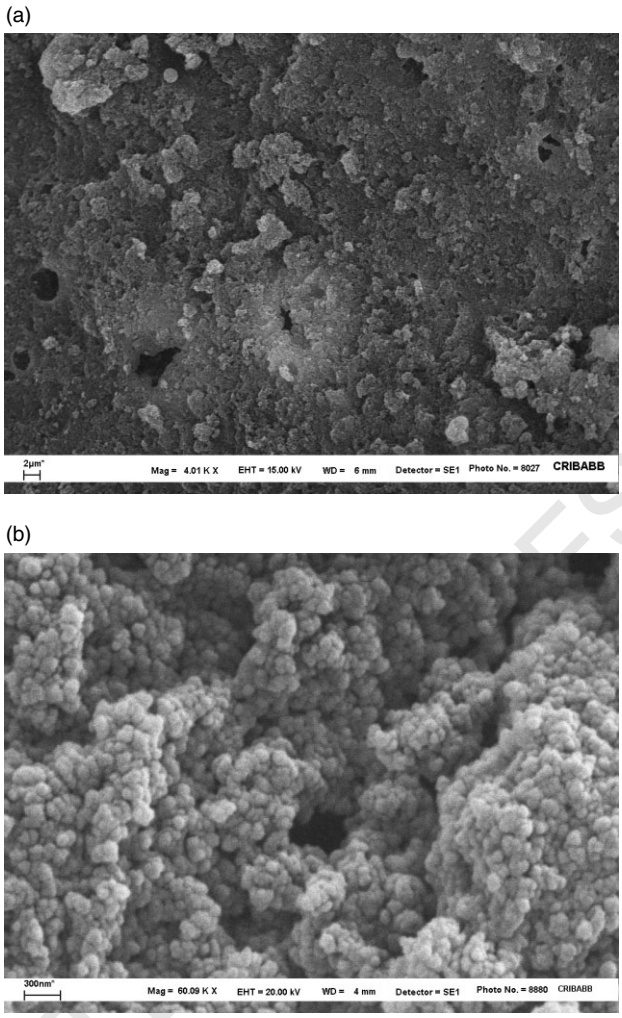

Figure 1. SEM micrographies of (a) raw PLGA; (b) PLGA/insulin complex.

EXPERIMENTAL

Materials

The PLGAs were prepared by enzymatic polymerization using D/L lactic acid 85% (Sintorgan S.A (Argentina)) and glycolic acid (Fluka Chemika (Switzerland)) as monomers and immobilized *Candida antarctica* Lipase B, Novozyme 435 (Novozymes, Denmark) as biocatalyst. The polymerization protocol has been reported previously,[10] and the number average molecular weight of the PLGAs employed ranged between 1500 and 10 000 Da with a molar LA/GA ratio of 50/50.

Analytical grade dichloromethane (CH_2Cl_2) and ethanol were provided by Dorwill (Argentina, SA); the buffer solution of pH 7 (disodium hydrogenophosphate) was from Merck. The 0.1 mol L^{-1} solution of phosphate saline buffer (PBS, pH = 7.4) was prepared from 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 4.3 mmol L^{-1} Na_2HPO_4 and 1.4 mmol L^{-1} KH_2PO_4. The commercial solution of porcine neutral insulin was supplied by Betasint U-40 (Beta Laboratories, Argentina).

Preparation of insulin/PLGA complexes

Insulin/PLGA complexes were prepared using 25–30 mg of PLGA (dissolved in 3 mL of CH_2Cl_2) and 1.44 mg of insulin (1mL of aqueous insulin commercial solution) pre-treated with 1 mL of ethanol under stirring for 20 min to avoid protein aggregation. The PLGA : insulin weight ratio was kept almost constant in all experiments at 17 : 1. Both solutions (organic and aqueous/ethanolic) were contacted by adding the insulin to the polymeric solution using a syringe. 0.5 mL of buffer pH = 7 was added to maintain a neutral media during the procedure. The formation of the complexes was allowed over 24 h at 37 °C under stirring, evidenced by the formation of a cloudy emulsion. Then, precipitation was performed in 10 mL of bidistilled water and the CH_2Cl_2 was eliminated by evaporation. The solid was recovered by filtration and washed several times with bidistilled water. The supernatant was separated to measure the entrapment efficiency (EE) of insulin on the polymeric matrix.

Scanning electron microscopy (SEM) analysis demonstrated that the complexes obtained were near spherical nanoscale particles, highly agglomerated, as observed in Fig. 1, where the image of the complex (Fig. 1(b)) is compared with that of the PLGA matrix (Fig. 1(a)).

Quantification of entrapped insulin

The amount of insulin entrapped on PLGA was calculated indirectly, as the relative EE taking into account the initial amount of insulin from the supernatant of the entrapment procedure.

The protein concentration was obtained using UV/visible spectroscopy; and the EE was defined by the following expression:

$$\textit{Entrapment Efficiency} = \frac{\textit{mg Entrapped insulin}}{\textit{mg insulin (initially added)}} \times 100$$

where *mg Entrapped insulin* was calculated from the difference between the initial amount of protein and the free insulin in the supernatant after the entrapment step. The last parameter was measured by a UV/visible method, using a calibration curve relating absorbance (*A*) and concentration of insulin (mg mL^{-1}). The standards were prepared using aqueous insulin commercial solution and ethanol as diluting solvent, the water/ethanol ratio was 1 : 3 and the resultant standard solutions were stirred vigorously for 45 min to homogenize. The following concentrations were employed to perform the calibration curve: 0.019, 0.040, 0.080, 0.10, 0.16, 0.22 and 0.24 mg insulin mL^{-1} solution.

Several difficulties emerged at this step of the experimental work, especially regarding construction of the calibration curve. Such difficulties and the strategies implemented to solve them and to avoid possible erroneous results, are detailed later in the discussion section.

Release *in vitro*

About 15–25 mg of PLGA/insulin complex was incubated in 3 mL of PBS in a 10 mL vial at 37 °C in a water bath under continuous stirring. Two replicate tubes were employed for each time point and the vials were sampled at various time points. The release tests were designed for a period of 24 h.

Protein liberated was measured by withdrawing 0.25 mL of supernatant at different intervals of time and diluting to 3 mL with distilled water. The samples were centrifuged and analyzed by UV/visible spectroscopy using a calibration curve relating absorbance (*A*) and concentration of released insulin (mg mL^{-1} solution of supernatant). The standards concentrations used in the calibration curve were the same as those used to determine EE.

Characterization techniques

Fourier transform infrared (FTIR) spectroscopy was employed to analyze the structure of the protein and PLGA after entrapment and after *in vitro* release tests. A Nicolet• FTIR 520 spectrometer was used for recording transmission spectra in the range 4000–400 cm^{-1}. The spectra of PLGA and PLGA/insulin complex were obtained by casting a CH_2Cl_2 solution onto a KBr window; the assays were performed after solvent evaporation. To record the insulin spectra, a few drops of commercial solution were dispersed on a KBr window and the spectra recorded after solvent evaporation. The supernatant of the entrapment/release experiments were allowed to evaporate completely. The solid obtained was recovered with acetone and dispersed on a KBr window. Spectra of the insulin were collected after entrapment/release processes.

UV/visible analyses were performed using a double beam spectrophotometer Shimadzu• 160, equipped with a computer-assisted system for data acquisition.

SEM (detalles del equipo de SEM del CRIBABB•) was used to evaluate the morphology of the solid complexes and to estimate the particle size and shape in the recovered solid.

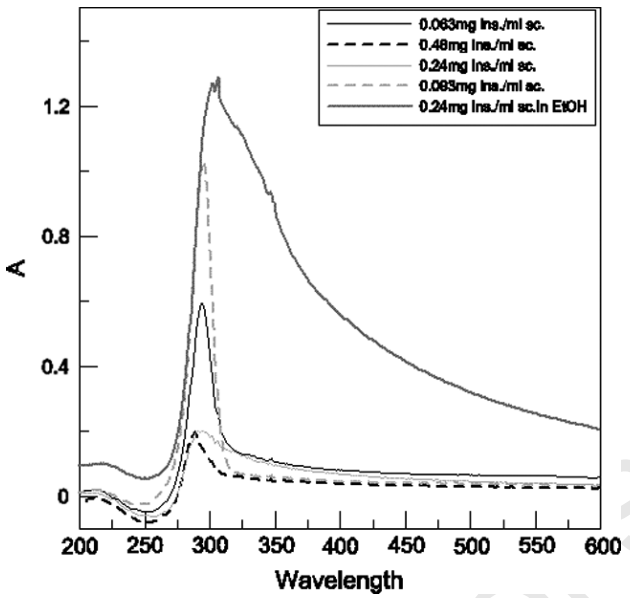

Figure 2. UV/visible spectra of insulin commercial solutions diluted in water and in ethanol.

RESULTS AND DISCUSSION

Experimental/analytical problems found during entrapment

Quantification of the entrapped insulin: background of the construction of the calibration curve

Confusing results were obtained in the determination of protein content for the entrapment efficiency when commercial insulin solutions of different concentrations were analyzed by UV/visible in order to perform the calibration curve. Figure 2 contains the complete UV/visible spectra of commercial insulin solution diluted in water to different concentrations. The lack of linearity between the absorbance (*A*) and the concentration of insulin (expressed as mg insulin mL^{-1} solution) is evident. Spectra corresponding to solutions with lower protein content (0.063 and 0.093 mg insulin mL^{-1} solution) show an almost linear trend between *A* and concentration, while this is not observed when higher insulin concentrations were employed. It was thus impossible to construct a calibration curve from the data of Fig. 2. The different association states of the protein molecules was the cause of these observations. Insulin exists primarily as a monomer but there are several factors that may induce physical or chemical interactions leading to aggregation. In the presence of Zn, natural insulin associates to a hexamer with two Zn atoms coordinated octahedrally to each monomer, and three water molecules. Phenolic ligands or certain salts are capable of promoting similar conformational transitions. Concentrations higher than $\sim 10^{-6}$ mol L^{-1}, neutral pH and aqueous media also favour the association of insulin in dimers, tetramers and hexamers.[11,12]

Considering that the commercial solution of insulin employed in this work contains phenolic moieties (10% v/v) and aqueous media, it is expected that the protein would be in the hexameric form. Dilution of the original solution (0.063 and 0.093 mg insulin mL^{-1} solution) induced disagreggation leading to monomeric moieties. In the more concentrated solutions (0.24 and 0.48 mg insulin mL^{-1} solution), the protein retains the associated state, leading to a fall in the absorbance (Fig. 2). To avoid mistakes during quantification by UV/visible, insulin should be present in solution in its monomeric state. This structure is also preferred from the therapeutic point of view.

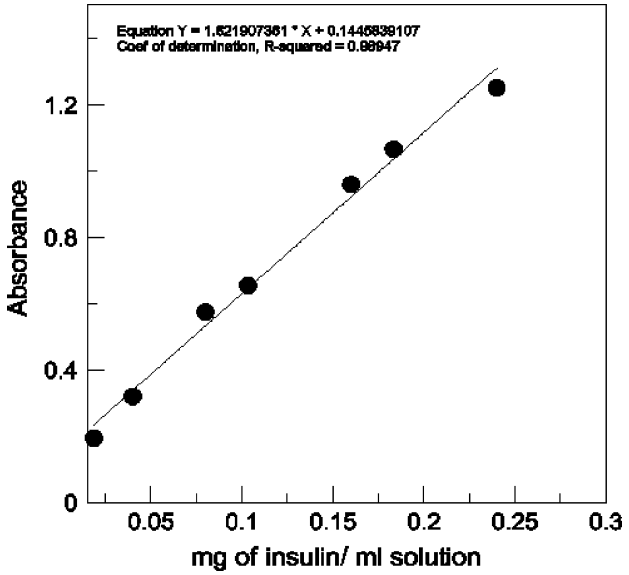

Figure 3. Calibration curve relating absorbance (*A*) and the concentration of insulin (mg insulin mL^{-1} solution) in ethanol.

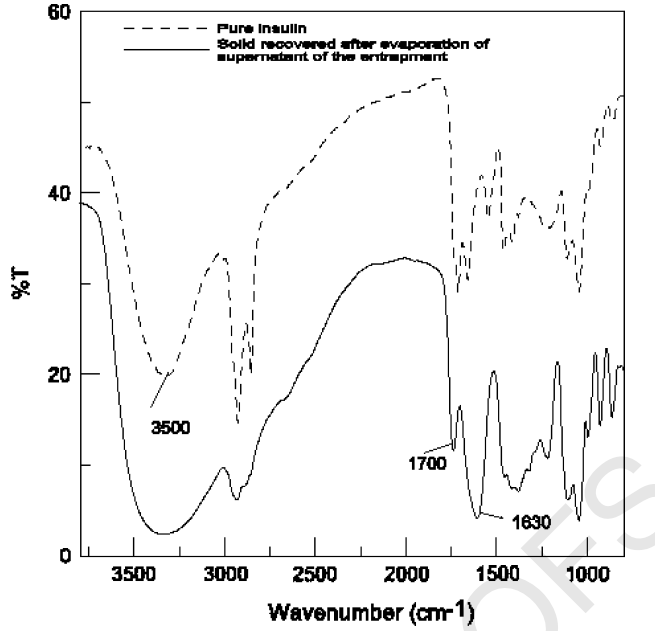

Figure 4. Region between 3600 and 900 cm^{-1} of FTIR spectra for pure insulin and the residue from evaporation of the entrapment supernatant.

The effect of aqueous media on insulin aggregation was evaluated using ethanol instead of water as diluting solvent. The differences in the UV/visible spectra are shown in Fig. 2, where the spectrum of the solution containing 0.24 mg insulin mL^{-1} solution diluted in ethanol is included. Comparing the spectra of aqueous and ethanolic solutions containing identical insulin concentrations (0.24 mg mL^{-1}), it is noted that the first displays a maximum at 292 nm with an absorbance of 0.20. The spectrum of the ethanolic insulin solution exhibits a maximum at 305nm and the band is considerably more intense, with an absorbance of 1.18. These differences can be attributed to the level of association of the protein in each solution. Aggregation produces not only a reduction in the absorbance but also a shift of the signal to lower wavelengths.

The benefits of certain co-solvents such as ethanol or acetic acid, to render insulin predominantly monomeric have already been addressed in the open literature. Thus, the use of ethanol instead of water to dilute the commercial insulin solution promoted dissociation of the aggregates. It is worth noting that according to reported articles (and by FTIR evidences showed later in this work) protein stability remained unaltered after ethanolic treatment.[13–15]

The standards used to obtain the calibration curve were diluted in ethanol and the range of concentrations employed was between 0.019 and 0.24 mg insulin mL^{-1} solution. The calibration curve is presented in Fig. 3, demonstrating a linear change in absorbance with protein concentration, with high correlation factor (R^2).

Entrapment efficiency (EE)

Stability of the insulin during the entrapment process

The stability of the protein is of huge importance when reporting EE, since changes in protein structure contaminate the data, especially when UV/visible based methods are utilized. During the complex formation the protein is exposed to a temperature of 37 °C for a prolonged time (24 h) under stirring and is in contact with organic solvents (ethanol and CH_2Cl_2); these conditions may alter the protein stability and conformation. Consequently, results derived from UV/visible regarding insulin quantification may be erroneous. The supernatant remaining after the complex formation, which contains the non-entrapped insulin, was studied by UV/visible in order to detect potential changes in the insulin structure. The spectra collected were compared with those of the standard solutions used in the construction of the calibration curve (used to calculate EE). The UV spectra of insulin before and after the entrapment process were very similar (data not shown). The accuracy of the UV analysis was useful to validate the calibration curve but more precise information about the structure, stability and conformation of entrapped insulin was provided by FTIR. Figure 4 shows the spectrum of the pure insulin and that of the residue of the supernatant entrapment, recovered after solvent evaporation. The main peaks corresponding to β-sheet (≈1630 cm^{-1}) and especially α-helix (≈1700 cm^{-1}) remained almost unchanged in the spectrum of the residual insulin after entrapment. These results indicate that the protein secondary structure was not significantly altered. Similarly, the wide band located at almost 3500 cm^{-1}, attributed to OH and NH groups, is also observable in the spectrum of pure insulin and in that of the residue entrapment.

The signal located at 1540 cm^{-1}, also attributed to the amide group, is not clearly observed in the spectrum of the residue since it may not be distinguished from the signal located at roughly 1630 cm^{-1}. This is because of the low resolution of the spectrum as a consequence of the low amount of sample employed to perform the assay.

This evidence reveals that the structure and stability of the protein remained almost unaltered after the entrapment process and also after the pre-treatment with ethanol - at least from the FTIR point of view.

Determination of EE

The EE and the entrapment yield (EY) obtained are listed in Table 1 and were calculated from the calibration curve included in Fig. 3. The measurements were performed in duplicate and two different PLGA/insulin initial ratios were employed by changing the amount

Table 1. Entrapment efficiency (EE) and entrapment yield (EY) of insulin in PLGA complexes for two different formulations using UV/visible quantification methods

PLGA/Insulin ratio	Insulin in the supernatant (%)[a]	EE (%)[b]	Entrapment yield (%)[c]
22.43	7.5	92.5	~100
17.36	2.5	97.5	64

[a] Defined as the content of insulin in the supernatant (mg) related to the initial concentration of insulin, that was constant and fixed in 0.411mg mL^{-1} solution.
[b] As defined in the Experimental section.
[c] Defined as the amount (mg) of recovered PLGA-loaded insulin complexes related to the total mass (PLGA + insulin) initially fed to the reaction.
The data presented in the table are the average of two entrapment tests.

of copolyester while the insulin content was kept constant in all tests (0.411 mg insulin mL^{-1} solution). The data in the table reveal that satisfactory EE were reached under the applied experimental conditions. When lower PLGA/insulin ratios were employed, higher EE were achieved. According to the SEM study, the morphology as well as the average size of the nanocomplexes obtained was roughly similar in all the formulations explored and coincide with the images shown in Fig. 1; hence EE and EY results may be interpreted on the basis of the PLGA/insulin interactions. Lower PLGA amounts (meaning lower PLGA/insulin ratio) increase the possibilities that the protein and the polymeric chain are in close contact, promoting the interaction/adsorption of insulin in the polyester moieties. When the concentration of copolyester in the organic solution is higher (higher PLGA/insulin ratio) it is suggested that the possibilities for strong interaction between the protein and the polymeric chains decrease, leading to lower EE values.

In the case of EY, an opposite trend with respect to EE was observed. The reduction of PLGA concentration in the organic solution induces a reduction of EY (and vice versa). This finding can be justified by considering that EY is a gravimetric magnitude related to the recovery of the final solid complex. Therefore, a smaller initial amount of polyester increases the possibilities of the loss of material during the entrapment and purification steps, giving a reduction of EY.

As was stated earlier, the nature of insulin/PLGA interactions appears to determine the efficiency of entrapment of the protein in the polymeric chain; thus an in-depth study of these interactions and the way in which the insulin remains linked to the copolyester is currently under development as a future contribution.

It is important to note that many authors have reported similar EE values for the entrapment of insulin in carriers based on PLA polymers, copolymers and/or other substrates using UV/visible techniques for quantification.[16–18]. However, to the best of our knowledge, in most cases limited (if any) data were provided about the initial protein quality, their properties and the pre-formulation treatment. Thus, it is very difficult to find the tools needed to compare published and own data.

Experimental/analytical problems found during the release *in vitro*

Quantification of released insulin

Releasing a protein from conventional formulations based on biodegradable polymers appears an easy task in most of the

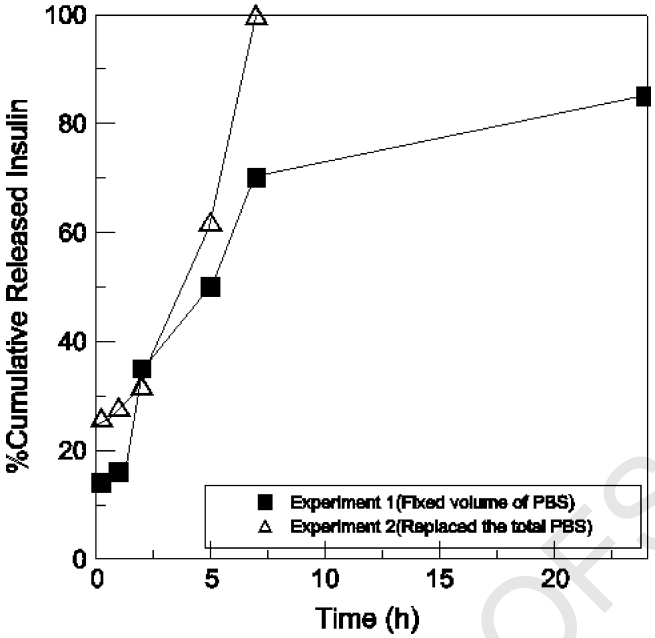

Figure 5. Release profiles of insulin from different release experiments expressed as the percentage of cumulative insulin released (considering the initially present) as a function of time.

published articles. However, the reported results are very different in terms of the maximum percentage of drug delivered during the time of the test, even when identical releasing systems (polymer/protein) were employed. Table 2 exemplifies this and demonstrates that employing the same protein, the same release experiment and the same polymer, the maximum release percentage and release time (meaning the time comprising the release test), are significantly different (see entries 1 and 2). In this case, it is obvious that the kind of carrier, its morphology and particle size directly affect the results obtained. From comparison of the data collected in entries 3 and 4, the causes for the discrepancies in reported release percentages are not so obvious since copolyesters with comparable M_n and a similar kind of carrier and protein were utilized.

Thus, the information in Table 2 strongly suggests that among the parameters commonly studied in the reported literature, such as kind of device, morphology and particle size, there are a number of factors (i.e. design of release experiment, nature and molecular weight of the polymer, method selected to quantify) able to influence the final results (in terms of maximum percentage of drug liberated during the time of incubation) in a significant way depending on the analytical methods used (specially UV/visible based). In this section such unexplored factors involved in the release procedure are examined.

Design of the release experiment

Among other *in vitro* release tests, the separation method is the most widely reported in the open literature (Table 2). It is very simple and consists in the separation of the complex insulin/PLGA (or the nano/microparticles) from the protein-containing release medium at different intervals of time.[31] In several publications phosphate saline buffer (PBS) is used as the release medium and the temperature of the test is generally fixed at 37 °C, with the goal of reproducing the corporal• environment. The supernatant is used to quantify the released drug, and the sampling procedure

Table 2. Comparison between published and own data for the maximum percentage (%) of cumulative release and incubation time in terms of the kind of release test, protein, polymer, polymeric device and quantification method

Entry	Release test [a]	Polymer and molecular weight of the polymer	Protein	Polymeric device	Quantification method	Maximum %Release	Time (h)[b]	Ref.
1	Partial removal of the PBS volume	PLA/0.19 dl g^{-1} (intrinsic viscosity)	Insulin	*In situ* formed gel	MicroBSA assay	40	2160	2
2	Partial removal of the PBS volume	PLGA/0.15–0.17 dl g^{-1} (intrinsic viscosity)	Insulin	microparticles	RP-HPLC with detector of the eluent at 280 nm	93	480	3
3	Total removal of the PBS volume	PLGA/3480 Da (Mn)	Insulin	microcapsules	Method of Lowry	17	24	4
						60	336	
4	Partial removal of the PBS volume	PLGA/8000 Da (Mw)[c]	Insulin	microcapsules	Peterson-Lowry method	60	600	19
5	Total removal of the PBS volume	PLGA/0.5 dl g^{-1} (intrinsic viscosity)	Insulin	microspheres	HPLC with UV/visible detector	100	216	20
6	Total removal of the PBS volume	PLGA/38000Da (Mw)	Insulin	nanospheres	BCA protein assay	30	6	21
7	Total removal of the PBS volume	PLA/17000 Da (Mw)	Insulin	microspheres	HPLC with UV/visible detector	69	6	22
		PLGA/14100Da (Mw)				80	432	
8	Constant volume of PBS	PLA/120 kDa (Mw)	Insulin	Stereocomplex	HPLC measuring the insulin at $\lambda = 208$ nm	27	360	17
9	Total removal t of the PBS volume	Not specified	myoglobin	microspheres	UV/Visible	90	360	23
10	Not specified	Not specified	β-lactoglobulin	microspheres	Bio red DC protein microassay	14–49[d]	0.033	24
11	Partial removal of the PBS volume	PLA/75000(Mn)	Protein C	nanoparticles	Lowry Peterson method	5	48	25
		PLA/49500(Mn)				50		
		PLA/19000(Mn				70		
12	Constant volume of PBS	PLA/47000 (Mn)	Bovine serum albumin	nanoparticles	Blue Commasie G250 protein assay	60	600	26
13	Total removal of the PBS volume	PLGA/900000 (Mw)	Bovine serum albumin	microparticles	Micro BCA protein assay	90	720	27
14	Partial removal of the PBS volume	PLGA/0.19–0.32 dl g^{-1} (intrinsic viscosity)	Lysozyme	*In situ* forming gel	UV/visible	100	3360	28
15	Constant volume of PBS	PLGA/8400 (Mn)	Insulin	Complex	UV/visible	80	24	This work
16	Total removal of the PBS volume	PLGA/8400 (Mn)	Insulin	Complex	UV/visible	100	24	This work

[a] In all the cases the release experiment was done using the separation method.
[b] Time of incubation.
[c] With addition of oligomers of 325 Da(Mw).
[d] Depending on the protein concentration.

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usually varies between the different papers (Table 2). In some cases the total supernatant is removed and replaced by fresh PBS, while in other cases only aliquots are withdrawn and replaced. Less common is the use of a constant volume of PBS during the entire test.

In this work it was verified that the design of the release experiment, in particular the incubation conditions, strongly affects the release kinetic. To visualize the magnitude of this effect, two different release experiments were performed using the protocol described in the Experimental section:

Experiment 1: A fixed volume of PBS (3 mL) was employed for incubation, withdrawing minimal aliquots (0.25 mL) of the supernatant at different intervals of time and diluting to 3 mL with distilled water.

Experiment 2: 1 mL of PBS was utilized as incubation media and samples (0.25 mL) were withdrawn at different intervals of time and diluted to 3 mL with distilled water. The whole PBS volume was removed after sampling and replaced by fresh buffer. To do this, the sample was centrifuged at 6000 rpm for 30 min and extraction of the buffer was performed with a syringe after a period of 40 min. With this procedure the extraction of solid material was avoided.

In both cases the samples were analyzed by UV/visible using the calibration curve as described in the Experimental section. The release profiles of insulin for both experiments are presented in Fig. 5, and the maximum release percentages after 24 h incubation are listed in Table 2 for comparison. The plots on Fig. 5 indicate that the burst effect is more marked in experiment 2 since between 25 and 35% of the insulin was liberated in the first 2 h of incubation. A more gradual delivery of the protein took place during experiment 1, with 15 to 35% of insulin released in the first 2 h of the test.

Complete liberation of insulin occurred in the first 6 h of the test when the buffer media was completely refreshed (experiment 2); while incomplete release (80% in 24 h) was observed using a fixed volume of PBS (experiment 1).

To further confirm the results of release tests arising from UV/visible quantification and to elucidate the possible causes for the observed release behaviour, FTIR studies were performed. The solids (PLA/insulin complex) recovered after both release tests were analyzed by FTIR, and results are as shown Fig. 6. The data reveal that the spectrum of the solid isolated after release from a fixed PBS volume still presents bands associated with insulin at nearly 1710, 1654 and 1540 cm^{-1}, although their intensity was notably reduced. In the case of the spectrum of the solid arising from the experiment performed with variable PBS, the typical bands overlap with those corresponding to PLGA at 1350, 1400 and 1710 cm^{-1} while bands associated with the protein were not detected. From Fig. 6 it is clear that a certain amount of insulin (or derivatives) remains in the complex after release in experiment 1, while complete liberation of the protein took place during experiment 2. Hence these FTIR results are consistent with those from the UV/visible analysis.

It is believed that when suspending the PLGA/protein complex in buffer using a closed vessel, partial polymeric degradation takes place, leading to acidic water-soluble oligomers. Polyester degradation might affect protein delivery in two ways: (1) favouring its aggregation and/or destabilization; (2) promoting interactions (mainly electrostatic) between the protein and the end groups of the oligomers.[30]

Although these facts may justify the incomplete release, it was demonstrated that they are not valid in this particular case. It was determined that the amount of solid PLGA/insulin complex recovered after the release test remains almost constant, independently of the release experiment. When a fixed PBS volume was employed, 66% of the entire complex mass was recuperated; against 57% that was isolated when the complete volume of PBS was renovated in the release test. As a consequence a minimal and almost similar degradation of PLGA took place during both experiments; with minimal loss of material during extractions

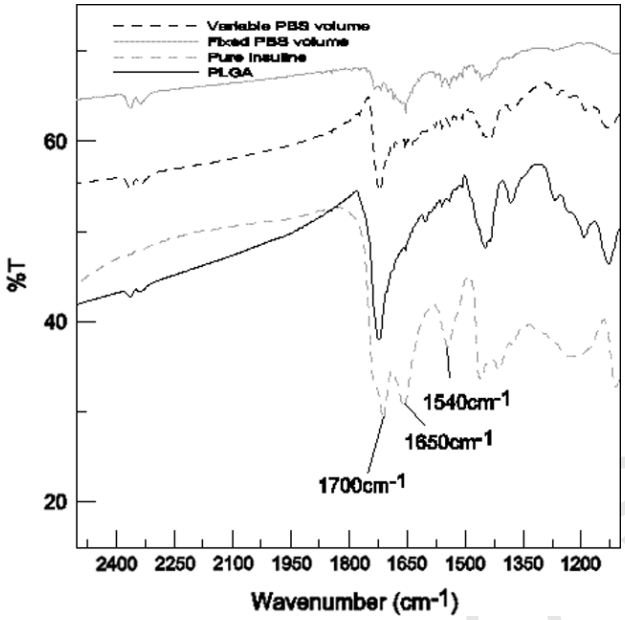

Figure 6. Region between 2600 and 1100 cm^{-1} of FTIR spectra of pure insulin, PLGA and the solids (complexes PLA/insulin) recovered after release from both tests.

in the second case. The published information agrees with this since it establishes that, in general, the decrease in pH of the environment as a consequence of the degradation of polymeric moieties was evidenced after 72 h of incubation. During the first 24 h of treatment, the pH remained almost constant.[6]

It is feasible that factors other than the polymer degradation rate influence the release profile of the insulin. However, some of these factors are related in a complex manner. FTIR was further

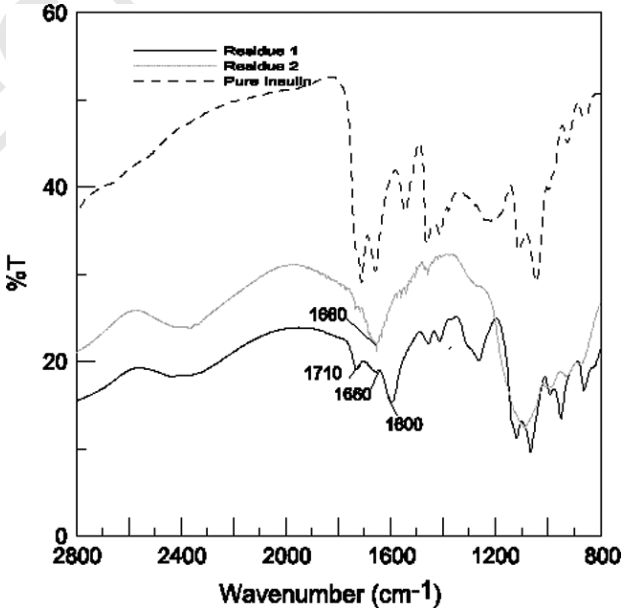

Figure 7. Region between 2800 and 800 cm^{-1} of FTIR spectra of pure insulin and the residues obtained from evaporation of the samples withdrawn during release. Residue 1 is that obtained from experiment 2 (variable PBS volume) and Residue 2 is that obtained in the experiment 1 (with fixed PBS volume).

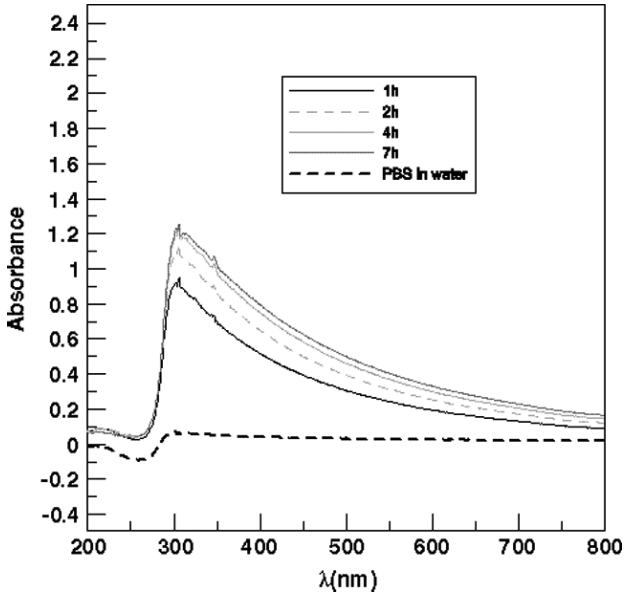

Figure 8. UV/visible spectra of PBS samples withdrawn at different times and diluted in ethanol and in water.

used to determine the causes of the incomplete insulin release. The residues arising from solvent evaporation in the samples withdrawn during the release tests were analyzed, and their spectra were compared with those for pure insulin, and are shown in Fig. 7. The spectrum of pure insulin presents typical protein bands at roughly 1220 (C–O), 1540 (Amide I), 1650 (Amide II) and 1700 cm^{-1} (C═O); the spectrum of the residue recovered after release during experiment 2 (variable PBS volume) identified as Residue 1, shows similar signals but shifted to higher wavenumbers. The shift could be caused by the different state of the samples analyzed since the insulin commercial solution was directly deposited on the KBr window while the solid residue was dissolved in acetone and cast onto the KBr window to perform the assay.

The typical insulin bands are not distinguished in the spectrum of the residue from experiment 1 (fixed PBS volume), identified in the figure as Residue 2. Only a band at 1660 cm^{-1}, assigned to C═O group, is detected in the region of interest. This suggests that association/aggregation and/or the adsorption of insulin molecules took place when the release was performed at constant PBS volume.

The prolonged contact (24 h) between the protein (in the complex) and the PBS media favours the insulin aggregation and/or adsorption on the polymer chains, possibly as a consequence of the generation of saline PBS derivatives induced by the incubation conditions.[31] Some published work supports this hypothesis since it was reported that the *in vitro* release media plays a major role in the release kinetic. It was shown that when PLA microparticles were incubated in PBS, there was a slow and incomplete release of protein (lysozyme), primarily due to adsorption of the protein on the polymer. However there was complete release when the microparticles were incubated in acetate or glycine buffers.[32,33] It is important to note that the articles in the open literature usually refer to longer incubation times than the time employed in this work, thus in such cases the reason for the incomplete release of the insulin (or other proteins) is surely degradation of the polymeric matrix. Therefore, complete removal of the PBS is needed, although in many of the available articles, this is carried out after 3 days of incubation.[6,30] We strongly believe that in such cases, besides degradation of the polymer, the incubation media also contributes to the incomplete release of the protein.

Furthermore, in many of the articles the level of degradation of the polymeric matrix is not well established, so it is feasible that total degradation of PLGA occurs after periods of incubation as long as 100 days, several weeks or even months, under moderate temperatures (37 °C) and in an aqueous environment.[6]

Effect of solvent

The effect of the solvent used to dilute the samples withdrawn during the release tests using PBS as incubation media was investigated. 3 mL of pure buffer solution (PBS) was incubated at 37 °C for 24 h under stirring, and samples (0.25 mL) were withdrawn at different times, diluted (to 3 mL) in ethanol and analyzed by UV/visible spectrophotometry. The data collected are

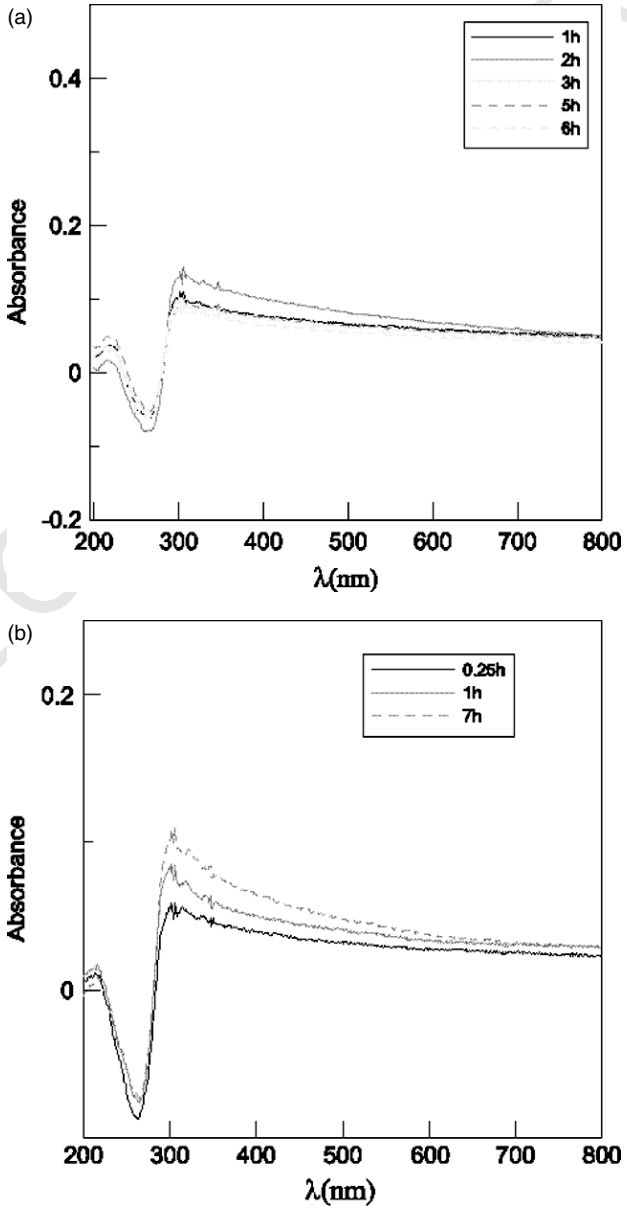

Figure 9. UV/visible spectra of PLGA samples incubated in PBS and withdrawn at different times: (a) PLGA 8400 Da; (b)PLGA 850 Da.

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presented in Fig. 8 and reveal that the PBS samples withdrawn at different incubation times and diluted with ethanol show different UV visible spectra, and the maximum absorbance overlaps with the band from the insulin (≈305 nm). These findings indicate that the PBS composition varies during the incubation period producing species that absorb in the same region as the insulin; thus serious mistakes in the percentage of cumulative insulin release data could be made using UV/visible based methods to quantify the protein liberated.

To solve this problem the incubation of PBS buffer was repeated but the dilution of samples withdrawn was conducted with bidistilled water. In this case a minimal and constant absorbance value was recorded for withdrawn samples at different time points, as shown in Fig. 8 (dashed line). This means than even when variation in the PBS composition took place during the incubation period, it passed unnoticed when the sample withdrawn was diluted with water, and hence it does not interfere with the quantification of protein liberated using UV/visible methods. These data allow one to conclude that the ethanol interacted in a different way with the PBS derivatives formed generating diverse species as demonstrated by the different UV spectra included in Fig. 8.

Based on these results, distilled water, instead of ethanol, was employed to dilute the samples withdrawn in further release tests. It is important to remark that even when water was employed for dilution, no problems concerning insulin aggregation were detected because of the low concentration of protein contained in each sample withdrawn. Note that protein concentration was of the order of 0.0610 mg insulin mL^{-1} solution for the maximum percentage cumulative release values (100%). Insulin at such concentrations still retains its monomeric state, as was demonstrated by the UV visible data included in Fig. 2.

Effect of the PLGA

Although the PLGA has been widely utilized for the encapsulation and posterior sustained release of several therapeutic agents, including proteins, its influence on the release kinetic, in particular when UV/visible based methods are utilized, has been rarely investigated. Here, it was verified that even when a centrifugation process was implemented on each sample withdrawn (the centrifugation procedure will be discussed in the next section), minimal amounts of particulate polymeric moieties or derivatives remain in the supernatant solution. To do so, a certain amount of pure PLGA (≈25 mg, comparable with the amount of PLGA/insulin complex employed in the *in vitro* release) was suspended in 3 mL of PBS at 37 °C under stirring for 24 h. Samples of 0.25 mL were withdrawn periodically, diluted in distilled water (3 mL) and analyzed by UV/visible after centrifugation. The results are presented in Fig. 9(a) where the spectra of samples withdrawn after 1,2,3,5 and 6 h of incubation are included. From the figure it is possible to deduce that the PLGA particles and/or derivatives absorb in the same region as the insulin. The absorption is caused by the end functional groups of the polyester, mainly C═O, COOH and COO^-, which overlap with those present in the insulin molecule.

The data obtained further suggest that roughly identical spectra are obtained independently of the incubation time, which means that there is an almost constant PLGA contribution throughout the release test, and also confirm that no considerable degradation of the polymeric matrix took place during the release experiment since in that case an increment in the absorbance (related to the increment of functional groups) as a function of the release time should be observed. Hence, these results agree with those

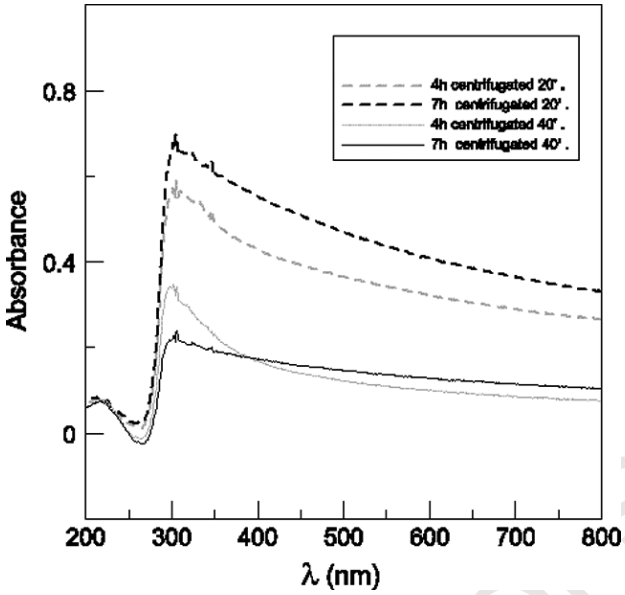


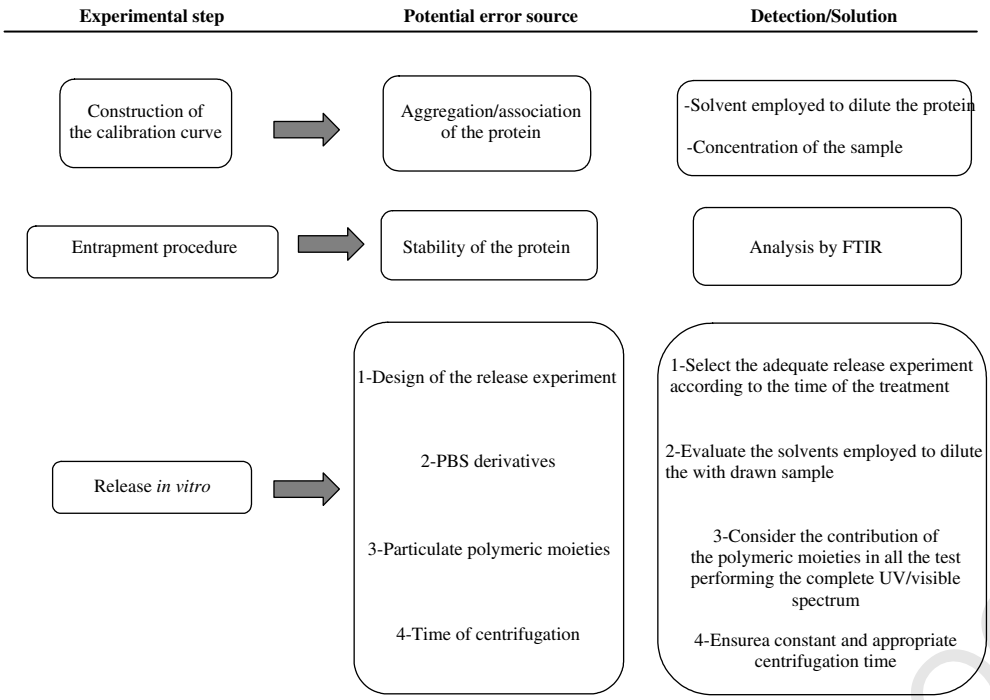
Figure 10. UV/visible spectra of release samples withdrawn at different incubation and centrifugation times.

included in the section 'Design of the release test' in considering that degradation of the PLGA is not significant for the period of the release test.

To complete the analysis, the test was repeated using PLGA with different number average molecular weight; specifically polyesters of 800, 1500 and 8400 Da were employed. The higher molecular weight polymers (1500 and 8400 Da) exhibited almost similar UV/visible spectra (shown in Fig. 9(a)). The spectra of the lower molecular weight polyester present a slight increment in the absorbance over time, as is seen in Fig. 9(b). In this case, partial degradation of the PLGA generated higher concentration of functional groups; the maximum absorbance value increases linearly with the incubation time, indicating a gradual degradation.

Effect of the centrifugation time

It is well known that centrifugation of the samples withdrawn is required when using the separation method for *in vitro* release. Nevertheless the intensity and the time of centrifugation modify the release profile, especially when UV/visible based techniques are employed for quantification. It has been reported that light dispersion affected the values of absorbance of the entire UV spectrum (due to the remaining solid/colloidal particles).[34] In fact serious relative errors may be found if this parameter is not adjusted accurately. Figure 10 shows the influence of centrifugation time on the release procedure. The spectra in the figure correspond to the samples withdrawn from the release media at 4 and 7 h of incubation at a constant centrifugation rate of 6000 rpm. The spectra collected at insufficient centrifugation time (20 min) show a wide dispersion in the region between 800 and 300 nm, affecting the maximum (≈λ = 302 nm) that is the point commonly employed to calculate the amount of delivered insulin. When the centrifugation was extended for 40 min the spectrum shows a good baseline, thus the maximum is not affected. From Fig. 10 it is worth noting that the complete spectrum has to be recorded aiming to avoid this and other mistakes that otherwise may remain unnoticed.



Scheme 1. Summary of the main difficulties reported in the present work and the way to detect/solve them.

A summary of the results achieved within this contribution is presented in Scheme 1. The different experimental steps involved in the global entrapment/release procedure are described with the corresponding difficulties encountered and the way to detect/solve them.

CONCLUDING REMARKS

It was demonstrated that the use of UV/visible methods to quantify both the entrapped and released protein from a biodegradable polymeric carrier might lead to confusing results if certain parameters are not correctly adjusted. Erroneous results were obtained when analyzing standard protein solutions through UV/visible spectrophotometry. The use of ethanol to dilute samples withdrawn instead of water and the use of a concentration range between 0.019 and 0.24 mg insulin mL^{-1} solution reduced the protein aggregation and allowed the construction of an accurate and reproducible calibration curve.

The EE of insulin on PLGA moieties ranged between 92 and 97%; and studies on its stability and conformation during the entrapment procedure revealed that the structure remained almost unalterable – or the changes were not detected by the methods used (FTIR, UV/visible).

Huge discrepancies have been detected in the release results (in terms of the maximum release percentage during the incubation time) by modification of the release procedure. Complete insulin delivery was observed when the release medium (PBS) was continuously refreshed, while 80% of the insulin was liberated using a constant volume of PBS throughout the entire experiment. Apparently, the PBS environment (concentration and composition) was the cause of such differences.

The solvent used to dilute the samples withdrawn was shown to be a source of potential error regarding the quantification of the insulin released in PBS media. Ethanol interacted with PBS derivatives originated during the incubation period leading to chemical species that absorb in the same UV/visible region as the insulin. To solve this, the ethanol was replaced by distilled water to dilute the samples.

Residual particulate PLGAs were present in the samples withdrawn from release tests. An almost constant contribution, in terms of absorbance, of the polyester particles during the release test was found independently of the incubation time. It was further determined that this tendency changed when low molecular weight PLGA (of the order of 853 Da) was employed, since in that case a slight increment of the absorbance as a function of incubation time was noticed and was attributed to partial degradation of the polymeric moieties.

Another source of error found, also concerning the quantification of liberated protein, was the centrifugation time of the sample withdrawn. Wide dispersion of the UV spectra was observed in samples centrifuged for 20 min. This dispersion was eliminated by employing a larger centrifugation time (40 min) at 6000 rpm.

The aspects studied in this work cover an area of the drug delivery field previously unexplored or not reported, which may be very useful for researchers dealing with data collection for entrapment and release of any protein and any polymeric carrier using UV/visible based methods as a quantification tool. Experimental details reported here may help other researchers to obtain accurate and reproducible results.

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