Enhancement of insulin's action by PLGA – Cyclodextrin esters nanoparticles

Soares A.F. [1 2 #], Gèze A. [3], Choisnard L[3]. Ribuot C. [4], Faure P.[4], Carvalho R.A. [1], Jones J.G. [1], Veiga F.V. [2] and Wouessidjewe D. [3 *]

[1]Department of Biochemistry, Rua dos estudos, Apartado 3126, 3001-401 Coimbra and [2]Faculty of Pharmacy, University of Coimbra, Portugal [3]Université de Grenoble, CNRS UMR 5063, DPM-Pharmacotechnie, FR 2607, [4]Université de Grenoble, HP2 ERI 0017, France, *supervisor #franciscasoares@ci.uc.pt

INTRODUCTION

Nanoparticles are promising tools in the biomedical field, namely regarding the administration of peptide drugs such as insulin, for they are able to facilitate the passage of drugs through biological membranes, protect them against degradation, control their release and provide targeting to specific sites of action (Pinto Reis C. 2006). Several methods and polymeric materials are available for the preparation of those systems. Poly(D,L-lactide-co-glycolide) (PLGA) presents the advantage of being a polymer that, once in the body, is hydrolized to biologically compatible and metabolizable moieties, lactic and glycolic acids. As to methodology, the nanoprecipitation technique (Fessi H. 1992) provides mild conditions that are unlikely to disturb insulin active structure.

Natural occurring α-, β- and γ-cyclodextrins (CD) are cyclic oligosaccharides consisting of 6, 7 and 8 glucopyranose units, respectively and can be chemically modified by the grafting of diverse functional groups. For the past decades CD and their derivatives have been used in drug delivery to improve drugs' solubility, bioavailability, and stability (Loftsson T. 1996). About 15 years ago, the preparation of amphiphilic CD derivatives expanded CD pharmaceutical applications to the domain of the nanocarrier systems, since these amphiphiles are able to self associate, in conditions of nanoprecipitation, into nanoaggregates. Both hydrophilic and hydrophobic drugs can be loaded into CD-based nanospheres (Duchene D. 1999). The biodistribution profile for its amphiphilic cyclodextrin-based nanoparticles resembles the one for polymeric nanoparticles with liver and spleen accumulation (Gèze A. 2007). The recent thermolysin catalyzed transesterification of cyclodextrins with vinyl esters (Pedersen N.R. 2005) allowed obtaining amphiphilic CD derivatives with variable chain lengths, in a simple-step procedure (Choisnard L. 2006, 2007).

In the present work we report the preparation of nanoparticles from PLGA and a mixture of CD esters obtained by enzyme catalysis. The aims were to associate insulin to the nanocarrier and investigate the effect on the drug's bioactivity. It may be hypothesized that the CD derivative has a role upon in vivo administration of such a system since CD are known to enhance drug absorption by several mechanisms (Soares A.F. 2007).

MATERIAL AND METHODS

Natural occurring γ-CD (Roquette, Lestrem, France) was grafted C_{10} alkyl chains by a thermolysin-based catalysis as previously described elsewhere (Choisnard L. 2006, Gèze A. 2009). The product was characterized by mass spectrometry MALDI/TOF and ^{13}C and ^{1}H NMR.

Nanoparticles were prepared from either, PLGA 48:52 to 52:48 molar ratio D,L-lactide : glycolide (Boehringer Ingelheim, Germany), or a mixture of the previous polymer and γ-CDC_{10} by nanoprecipitation (Fessi H. 1992). Solutions were prepared in acetone at 1 mg/mL for γ-CDC_{10} and 5 mg/mL for Poly(D,L-lactide-co-glycolide). Typically, 5 mL of the organic solution were poured over 6.5 mL of a Polysorbate 80 aqueous solution (0.015% m/v) under magnetic stirring, in a closed

system thermostatized at 25 ºC. Nanoparticles were spontaneously formed, the organic solvent was removed by evaporation under vacuum and particles were recovered as an aqueous colloidal suspension. To prepare insulin-loaded nanoparticles, 300 μL of Actrapid (100 UI of insulin per mL) (Novo Nordisk, Chartres, France) was added to the aqueous phase prior nanoprecipitation to prevent possible denaturation resulting from contact with acetone.

Particle size was determined in triplicate by quasi-elastic light scattering (QELS) using a Zetasizer 3000 instrument (10 mW HeNe laser at 632.8 nm, K7132 correlator, Malvern). Nanosphere samples were diluted (approximately 2 times) in distilled water in order to obtained adequate Kcount readings for size measurements. The analyses took place at 25ºC, at a reference angle of 90°, viscosity of 0.899×10^{-3} Pa.s, refractive index of 1.330. The polydispersity index (PI), which is a dimensionless measure of the broadness of the size distribution, and Z-average mean hydrodynamic diameter (Dh) of the particles were calculated using a cumulant algorithm with Zetasizer 3000 software version V. 1.51.

Insulin was quantified by HPLC as described. The samples were centrifuged at 15000 rpm (17000 g) for 30 min at 20 ºC. The supernatants were diluted in HCl 0.01M and filtered through 0.22 µm PVDF filters prior to HPLC analysis. The chromatographic equipment consisted of a Prostar 230 ternary solvent delivery module (Varian, CA, USA) equipped with a reverse phase X-Terra C-18 column, 5 μm, 4.6 mm × 250 mm (Waters, Saint-Quentin-En-Yvelines, France) and a Interchrom C-18, 5 μm precolumn (Interchim, Montluçon, France) maintained at 31ºC. A Prostar autosampler 400 (Varian, CA, USA) fitted a 50 μL loop was used for injections. Mobile phase A consisted of tri-distilled water with 0.1% TFA and mobile phase B of Acetonitrile with 0.1% TFA. Each analysis was performed over 18 min with a flow rate of 1 mL/min, a linear gradient from 70:30 (A:B) to 60:40 (A:B) was applied in the first five minutes, the 60:40 (A:B) was maintained for another 8 minutes; system returned to the initial conditions in the final 5 minutes. Insulin was detected at 210 nm with a Varian 9050 variable wavelength UV/Vis detector (Varian, CA, USA). A calibration curve for insulin was set to determine insulin concentration in the supernatant. Standard solutions were prepared by dilution of Actrapid commercial formulation in HCl 0.01 M. Insulin Association Efficacy (AE) was calculated indirectly as a difference of the amount found in the supernatant from the theoretical amount initially added as shown in equation 1.

All animal protocols were approved by the institutional animal care and use committee. Male Wistar rats (245 ± 9 g) were housed under 12-h light/12-h dark cycle with free access to standard chow diet and tap water during acclimatization. Animals were fasted overnight prior the experiments and randomized into six groups (N = 5 per group) receiving the following treatments: CTRL – no treatment; ACTR – subcutaneous injection of Actrapid solution in Polysorbate 80 (0.015% m/v); PL-I – subcutaneous injection of insulin loaded nanoparticles from PLGA; PL – subcutaneous injection of nanoparticles from PLGA; CDPL-I – subcutaneous injection of insulin loaded nanoparticles from γ-CDC10-PLGA blend; CDPL – subcutaneous injection of nanoparticles from γ-CDC10-PLGA blend. The formulations were administered at an insulin dosage of 2.3 UI/Kg or equivalent volume of placebo. Glycemia was monitored from the tail vein with a glucometer (Accu Check Performa, Roche Diagnostics, Meylan, France) before treatment and henceforward at 0.25, 0.5, 1, 2, 4, 6 and 8 hours.

All data are expressed as mean ± standard error. In animal data, two-way ANOVA with Bonferroni post tests was used to analyze glycemia profiles. The Area Above the curve (AAC) was calculated by setting a cutoff at 100% and a one way analysis of variance with Newman-Keuls multiple comparison test was applied to determine statistical significance.

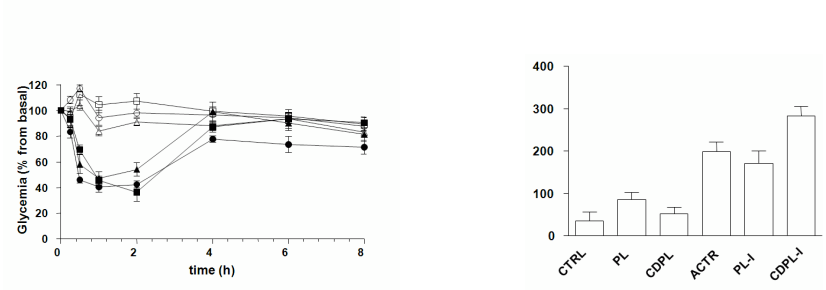
RESULTS AND DISCUSSION

Mass spectrometry revealed that cyclodextrin esters were obtained by enzymatic synthesis as a mixture of over- and under-acylated species. The most frequent acylation numbers were 7 and 8.

Particles in the nano-size range were successfully prepared by our method and excellent percents of insulin association were obtained for both systems (Table 1).

	size (nm)	PI	% IA
PL	117.4 ± 3.3	0.1 ± 0.01	
PL-I	112.4 ± 5.8	0.1 ± 0.001	88.1 ± 5.1
CDPL	122.7 ± 3.5	0.09 ± 0.007	
CDPL-I	115.8 ± 5.4	0.1 ± 0.007	92.0 ± 1.0

Table 1: Sizes (mean diameter) and Insulin association efficacies (%IA) for PLGA (PL) and γ-CDC10-PLGA (CDPL) nanoparticles



A **B**

Figure 1A: Glycemia curves following the subcutaneous injection of formulations at 2.3 IU of insulin per Kg. Open squares: CRTL; open circles: CDPL; open triangles: PL; black squares: ACTR; black circles: CDPL-I; black triangles: PL-I. B: Graph representing the AAC for the glycemia curves. See text for details.

Figure 1A shows glycemia curves (expressed as percent from the initial) for all experimental groups. Animals that were injected with placebo formulations (PL and CDPL groups) presented profiles similar to controls, maintaining the initial glycemia throughout the experimental period with a slight initial increment attributed to manipulation-induced stress. This observation demonstrates that the formulations *per se* don't elicit hypoglycemic response. When insulin nanoparticle formulations were administered to the animals, a hypoglycemic response was observed indicating the drug activity was not compromised. Moreover this effect was immediate, overcoming the initial stress-induced raise in glycemia, although statistically significant differences from control and placebos ($P<0.001$) were only attained from 0.5h on. Glycemia curve for ACTR group is

characterized by a peak-like shape with a minimum at 2h. In contrast, for both nanoparticle formulations the negative peak is smoothen and the curves assume a rounder shape, enlarging the interval for the minimum, which in these cases ranges from 0.5 to 2 h. Furthermore, an earlier onset on the hypoglycemic effect was observed for the formulation containing γ-CDC_{10} ($P<0.01$, Vs ACTR at 0.5 h) as well as a prolonged effect since it is still detected 6h past the injection, unlike other formulations ($P<0.05$ Vs all other groups). The overall hypoglycemic effect was further assessed by calculating AAC for all groups (Figure 1B). Once again, all the insulin formulations presented AACs significantly higher ($P<0.01$) than controls and placebo formulations. In addition, AAC for CDPL-I was significantly higher than PL-I ($P<0.01$) and ACTR ($P<0.05$). On the other hand, the AAC for PL-I was not statistically different from the ACTR one.

CONCLUSIONS

Both the formulations studied showed monodisperse size distributions with mean particle diameters around 115nm suggesting that the two material co-nanoprecipitated.

Assessment of biological activity on rats demonstrated that insulin action was not compromised when the drug was formulated in either system. Also a sustained hypoglycemic effect was observed for both formulations, which was maintained up until 6h past administration when γ-CDC10 was present. Thus, adding γ-CDC10 to the system was advantageous regarding the *in vivo* performance of the nanoparticles. An enhancement of insulin and/or particle absorption from the injection site could be responsible for such observation.

The γ-CDC10-PLGA system for insulin delivery was capable of providing a rapid onset on the drug's hypoglycemic action, as well as a sustained and delayed effect. These properties make it a promising candidate for the development of new insulin formulations destined to the subcutaneous route, that optimize the drug's effect in agreement with the patient's needs, reducing the number of daily injections.

BIBLIOGRAPHY

Choisnard L. et al. (2006). *Nanoparticles of β-Cyclodextrin Esters Obtained by Self-Assembling of Biotransesterified β-Cyclodextrins* Biomacromolecules 7(2) 515-520.

Duchene D. et al. (1999). *Cyclodextrins in targeting: Application to nanoparticles* Adv. Drug Deliv. Rev. 36(1) 29-40.

Fessi H. et al. (1992). *Process for the preparation of dispersible colloidal systems of a substance in the form of nanoparticles* US Patent 5118528.

Gèze A. et al. (2007). *Biodistribution of intravenously administered amphiphilic b-cyclodextrin nanospheres* International Journal of Pharmaceutics 344 135–142.

Gèze A. et al. (2009). *Colloidal systems made of biotransesterified α, β, γ cyclodextrins grafted with C10 alkyl chains* Materials Science & Engineering C 29 (2009) 458–462.

Loftsson T. et al. (1996). *Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization* J. Pharm. Sci. 85(10) 1017-1025.

Pedersen N.R. et al. (2005). *Thermolysin catalyses the synthesis of cyclodextrin esters in DMSO* Tetrahedron: Asymmetry 16(3) 615-622.

Pinto Reis C. et al. (2006). *Nanoencapsulation II. Biomedical applications and current status of peptide and protein nanoparticulate delivery systems* Nanomedicine 2(2) 53-65.

Soares A.F. et al. (2007). *Oral administration of peptides and proteins: nanoparticles and cyclodextrins as biocompatible delivery systems* Nanomedicine 2(2) 183-202.