Fabrication and characterization of core-shell microspheres composed of pectin and arabinoxylans as controlled release systems for insulin

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ABSTRACT

Core-shell microspheres were manufactured by the method of coaxial electrospray. The microspheres designed are intended as colon-targeted oral insulin delivery system. The fabrication materials were polysaccharides such as low degree of esterification pectin (shell) and ferulated arabinoxylans (core). Microspheres were analyzed by dynamic light scattering to determine the average particle size. Structure and morphology was characterized by scanning electron microscopy, transmission electron microscopy, and confocal microscopy, in order to observe and verify the presence of the core-shell structures and determine the distribution of insulin in the microspheres. Core-shell arrangement was observed for all particle sizes. In 50% of the experimental trials 3 μm and lower average diameters were obtained. At the moment of this work the authors had not found previous reports on electrospray fabrication of particles with these biopolymers nor core-shell arrangement for oral insulin delivery matrices.

Keywords: pectin, arabixylan, microspheres, electrospray, core-shell

1 INTRODUCTION

WHO classifies diabetes as an epidemic [1], and in Mexico is the second leading cause of death [2]. The traditional application of insulin subcutaneously, is difficult, painful, and tedious and sometimes causes withdrawal. Therefore, it is necessary to develop new forms of administration, such as oral administration targeting colon. Encapsulation of bioactive compounds such as insulin, using biopolymers based matrices, stability and protection against pH, and enzyme activity [3], are provided, thus avoiding the loss of activity [4].

Polysaccharides are widely used as carriers, as they are considered non-toxic and biocompatible materials obtained from renewable sources (agro-industrial waste) and are the most abundant polymer on earth [5-9].

Pectins of low degree of esterification (LDE) gel in the presence of divalent ions such as calcium (Ca^{+2}) [10], the formation of strongly linked dimmer associations is followed by the formation of weak inter-dimer associations mainly governed by electrostatic interactions [11]. Ferulated arabinoxylans (AXF) consist of xylose in β-(1, 4) with ramifications of α-L-arabinofuranose in α-(1, 3) and α-(1, 2) [12]. AXF gels are formed by oxidative coupling of ferulic residues [13].

Pectins and AXF have already been proposed and studied, individually, as matrices for the release of drugs and bioactive compounds, by virtue of their biodegradability in the colon, and gelling capacity [14-18]. The gelation process of AXF is slow, and is a disadvantage which makes difficult the stabilization of the structures. Conversely, pectins (LDE) gels quickly. Therefore, it was sought to complement with rapid gelling LDE pectins, the slow formation of the pH resistant AXF gels. For fabrication purposes, the former aimed to stabilize the particles and prevent the formation of aggregates.

Electrospray involves the application of an electrodynamic force on a controlled drip flow [19,20]. Insulin nanospheres for subcutaneous application, with no effect on insulin biological activity [21].

The joint application of these polysaccharides can overcome the respective disadvantages of their gels, allowing the manufacture by electrospray in a fast way and without aggregation, of insulin-loaded spherical structures. The aim of this study was to fabricate by coaxialelectrospray, core-shell microspheres based on low methoxyl pectin and ferulated arabinoxylans as an insulin carrier system.

2 EXPERIMENT

For the manufacture of the matrix, pectin extracted from thinning apple fruit (*Malus domestica* Borkh) was used, with a degree of esterification of 41%. A desesterification chemical treatment was applied to this pectin to reduce the degree of esterification to 6%. The AXF used were from the pericarp of maize (*Zea mays*) [22]. The carried molecule was insulin (Insulin human recombinant dry powder, SAFC-91077C-SIGMA), and laccase enzyme (*Trametes versicolor*, SIGMA 53739 powder, 15 U/mg) and calcium chloride (Sigma-Aldrich, C50080) as cross-linking agents

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2.1 Pectin degree of esterification modification

Chemical deesterification of the pectin from apple thinning was performed. Pectin underwent a basic treatment with $NaOH_2$ 2M (Chemicals Monterrey SA, 36904) for a period of 2h, then cold HCl 4M was added (Sigma-Aldrich, 258148) (4 °C) and adjusted to pH 2-2.2. Subsequently, cold ethanol (4 °C) was added to obtain a final ratio of 70% ethanol, mixed and left in the fridge for 24h. Finally the mixture was filtered using vacuum filtration equipment on glass fiber filters (Whatman™ 1825-047). The pellets were dried in an oven at 60 °C. Determining the final degree of esterification was performed by Infrared spectroscopy on a FT-IR Nicolet Protégé 460® (Madison, Wl ,USA) according to the technique reported by Urias-Orona *et al*. [23].

2.2 Solutions preparation

The pectin and AXF solutions were prepared separately. LDE pectin solution (6% DE) was prepared at 1 % (w/v) in double distilled water, and left under constant stirring for a period of 24 hours to achieve dispersion. AXF dispersion was prepared at 6% (w /v) in an acetic acid/sodium acetate 300 mM buffer (pH5.5) with constant stirring for a period of 24h. Both solutions were filtered through a syringe filter with a steel mesh (diameter 350 µm). Insulin solubilization was performed according to Martinez-Lopez *et al*. [24].

2.3 Spheres manufacture

The spheres were produced by the elesctrospray method, with coaxial flow. A Spraybase system (Profector ™, Dublin Ireland) was used to control the applied voltage. As flow control two syringe pumps (World Precision Instruments, AL-1000) independently connected to the coaxial needle were used, as shown in Figure 1. To receive the drops of spray, a cross-linking solution with ethanol and $CaCl_2$ was prepared at different concentrations, as stated in the experimental design (Table 1). 100 µL of each polysaccharide were injected; in the outer syringe 1% pectin solution, in the internal syringe AXF-Insulin solution which had added 1.25 U/mL of laccase. The reception of the spray was performed in a volume of 10 mL of cross-linking solution ($CaCl_2$/ethanol, Table 1), with constant stirring at 200 rpm (Corning ®, PC-420D) during and after manufacture. All samples were stored in the cross-linking solution at 4 °C.

2.4 Size and distribution of the spheres by DLS

A study of size and population distribution of the spheres in different manufacturing runs, using a process called dynamic light scattering (DLS) was made. The measurements were performed in a sieve analyzer (Delsa™ NanoC Particle analizer, BeckmanCoulter), 2 mL of samples were deposited within the rectangular disposable cell The incidence of the laser light was at 658 nm. The microspheres were analyzed triplicate.

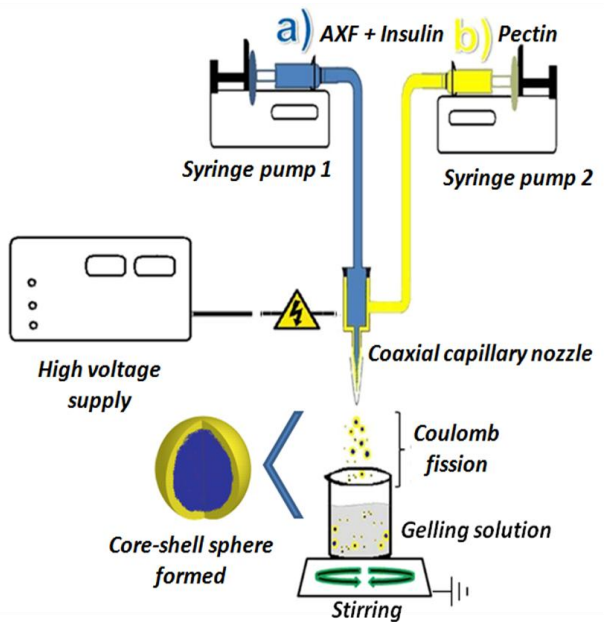

Figure 1: Schematic depiction of the electrospray device arrangement. Spraybase system (Profector ™, Dublin Ireland) was used for the set up of nanoparticle fabrication.

2.5 Analysis by microscopy techniques

Scanning electronic microscopy (SEM): SEM technique was used to observe the morphology and geometry of spheres and corroborate their size and distribution. The measurements were performed on a JEOL USA JSM-6610/LV scanning electron microscopy, JEOL USA, Inc. Elemental analysis was performed using the technique of energy dispersive spectrometry (EDS) at an angle of 35 º.

Transmission electronic microscopy (MET): A transmission electron microscope (JEOL JEM-1230 electron microscope, JEOL USA, Inc.). The analysis by EDS was obtained with an angle of 45 °.

Confocal laser scanning microscopy (CLSM). Was used a high-resolution confocal Leica TCS Spectral SPE (Leica Microsystems) by means of fluorescence, distribution of protein (mainly, insulin) inside the spheres was observed. (635 nm for excitation and 620-680 nm for the emission, with a magnification of 40x)

2.6 Experimental Design

For optimization of the spheres production conditions, a central composite rotary design (CCR) was used with a 24

factorial arrangement (Table 1). Response variables were: average diameter of the spheres, and variation coefficient (CV). Experimental design and data analysis were performed using JMP ® Statistical Software 11.1.0 2013 SAS Institute Inc. Note: Results of the optimization of the fabrication process do not appear in this publication

Table 1: Experimental design with different assayed levels for each factor.

Arrangement	***Factors***	***Levels***				
		- α	***-1***	***0***	***1***	***α***
2^4 factorial	Flow (mL/h)	0.05	0.163	0.275	0.387	0.5
	Voltage (kV)	8	10	12	14	16
	Conc. ethanol (%)	50	62,5	75	87,5	100
	Conc. $CaCl_2$ (%)	2	6,5	11	15,5	20

3 RESULTS

It was possible to reduce the degree of esterification of the apple thinning fruit pectin from 41 down to 6%, a pectin of very low degree of esterification (VLDE). With these pectins and AXF-insulin, polydisperse microspheres by coaxial electrospray were obtained.

The spheres' diameter mean values of the for each experimental set of conditions determined by DLS assays, were in the range of 0.58-28.89 microns, and 16 of the 18 runs from the 28 experimental runs have an average < 3 microns diameter. 4 experimental runs showed average diameters <1 microns, these were taken as representative microscopy images (Figure2). Moreover, the samples showed high dispersal indices diameters, with values in the range of 3% to 55% CV, varying in each experimental run.

In the SEM image (Figure1a) only spheres larger than 1 micron were observed. In these images the spherical geometry and symmetry of the structures is highlighted as well as the stability of the same, as there was no evidence of aggregation.

In addition, TEM images for structures smaller than 1 micrometer were observed. Geometry and symmetry results are consistent. The most remarkable results in these images are that no aggregation is present, and the core-shell type estructure is distinguished. Also, it appears to show an interface region between pectin shell and AXF-insulin core.

Finally, in the CLSM image (Figure2d), it is confirmed by means of the observed fluorescence, that the spheres core only, contains all the protein (mostly insulin, with laccase traces). Also, image analysis with the equipment software, revealed that the structures are not flat, but mostly spherical as they show depth (data not shown).

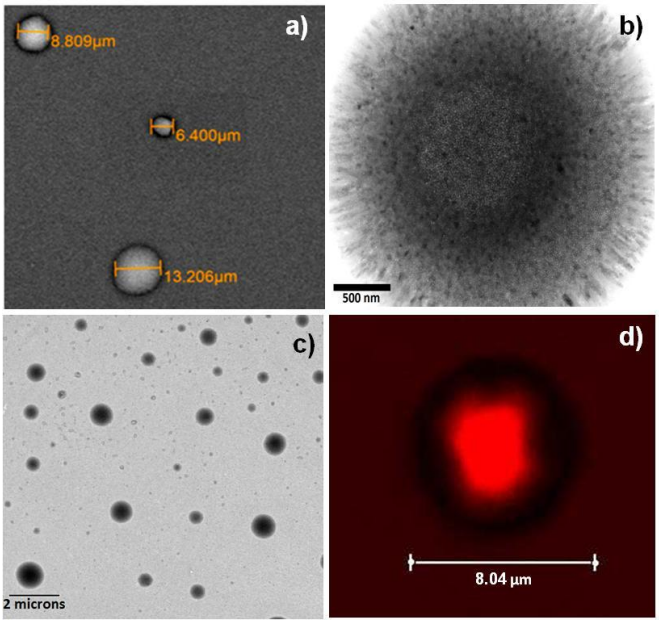

Figure 2: SEM images: a) (*0.388 mL/h, 14 kV, 87.5 % ethanol, 6.5% $CaCl_2$*); TEM images: b) (*0.275 mL/h, 12 kV, 75 % ethanol, 2% $CaCl_2$*)**;** c) (*0.275 mL/h, 12 kV, 100% ethanol, 11%$CaCl_2$*); CLSM images: d) (*0.5 mL/h, 14 kV, 87.5 % ethanol, 15.5 % $CaCl_2$*).

4 DISCUSSION

The combination of VLDE pectin and AXF in a coaxial system, allowed the formation and stabilization of the hydrogels, in order to obtain stable microspheres. On one hand the VLDE pectin, due to its almost instantaneous gelation mechanism with Ca^{+2}, allowed preserving the spherical structure of the microdroplets formed in the electrospray, and avoided aggregation of the AXF-insulin gels; secondly, when AXF formed chemical gels, insulin remained trapped in the center of the sphere in a pH resistant proved structure.

Accordingly, Ghayempour *et al*. in 2013 [25] also worked with a coaxial electrospray system, with alginate (having a similar gelling mechanism as pectin), they found formation of symmetrical and spherical structures, with no evidence of aggregation. This indicates that the nucleation of calcium on the surface of the spheres, helps stabilize the particles and their charges, likewise in the present work

Regarding the size of the spheres, both the diameter and the dispersion ranged in each experimental run. In a previous work [26], similar average diameter of about 3µm, were found in the manufacture of spheres with a core-shell structure by coaxial electrospray technique. Our evidence shows that coaxial electrospray is an effective method for the manufacture of biopolymer spheres in a core-shell structure type.

Unlike other studies [27, 28], with this manufacturing method, it was possible to locate the insulin in the core of the sphere, and not dispersed throughout the structure. This

arrangement benefits the conservation of the properties of the insulin, to have a protective shell of pectin, and be trapped within a network of covalent arabinoxylans. Overall, this "armor" is intended to prevent premature release and subsequent degradation of insulin in upper digestive tract conditions. Hopefully, colon microflora may degrade these particle polymers for insulin release. Further studies in this regard are in process

5 CONCLUSIONS

VLDE pectin and AXF based microspheres loaded with insulin were manufactured by coaxial electrospray method. Average size less than 3 microns in 50% of the conditions sets tested were obtained. It was shown that the microspheres are core-shell structured, with insulin occupying mainly the core. Structures formed showed to be spherical and symmetric regardless of theexperimental run, and no evidence of aggregation was found. Electrospray method for microspheres fabrication meets the design intended. Due to the structures and arrangement, these microspheres are promising for future degradation tests, tests of insulin release and subsequent evaluation *in vivo* glicemic control in animal models, and hopefully, an oral insulin for diabetes in the midterm.

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