International Journal of Pharmaceutics 428 (2012) 143–151

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Development and characterisation of chitosan films impregnated with insulin loaded PEG-*b*-PLA nanoparticles (NPs): A potential approach for buccal delivery of macromolecules

Concetta Giovino, Isaac Ayensu, John Tetteh, Joshua S. Boateng*

Department of Pharmaceutical, Chemical and Environmental Sciences, School of Science, University of Greenwich at Medway, Chatham Maritime, Kent ME4 4TB, UK

ARTICLE INFO

Article history:
Received 14 December 2011
Received in revised form 16 February 2012
Accepted 21 February 2012
Available online 1 March 2012

Keywords:
Buccal delivery
Chitosan films
Insulin
Nanoparticles
Poly(ethylene glycol)methyl ether-block-polylactide

ABSTRACT

Mucoadhesive chitosan based films, incorporated with insulin loaded nanoparticles (NPs) made of poly(ethylene glycol)methyl ether-block-polylactide (PEG-*b*-PLA) have been developed and characterised. Blank-NPs were prepared by double emulsion solvent evaporation technique with varying concentrations of the copolymer (5 and 10%, w/v). The optimised formulation was loaded with insulin (model protein) at initial loadings of 2, 5 and 10% with respect to copolymer weight. The developed NPs were analysed for size, size distribution, surface charge, morphology, encapsulation efficiency and drug release. NPs showing negative (ζ)-potential (<−6 mV) with average diameter > 300 nm and a polydispersity index (P.I.) of ≈0.2, irrespective of formulation process, were achieved. Insulin encapsulation efficiencies of 70% and 30% for NPs-Insulin-2 and NPs-Insulin-5 were obtained, respectively. The *in vitro* release behaviour of both formulations showed a classic biphasic sustained release of protein over 5 weeks which was influenced by pH of the release medium. Optimised chitosan films embedded with 3 mg of insulin loaded NPs were produced by solvent casting with homogeneous distribution of NPs in the mucoadhesive matrix, which displayed excellent physico-mechanical properties. The drug delivery system has been designed as a novel platform for potential buccal delivery of macromolecules.

1. Introduction

In the last decade, protein drug delivery has become an important area of research due to the large number of recombinant proteins being investigated for therapeutic applications (Kim et al., 2001). However, the therapeutic effects of these macromolecules are limited by their poor stability, low bioavailability and short half-lives. As a result, most of these proteins are only therapeutically useful following multiple injections, which present patient compliance challenges. The design and development of colloidal systems such as nanoparticle (NP) carriers (Crommelin et al., 2003) and microspheres (Mundargi et al., 2011a,b) represent a valid approach to overcome these drawbacks. It is well known that NPs are colloidal-sized particles, possessing diameter less than 1 μm where the drug may be encapsulated, adsorbed or dispersed within them. The popularity of these systems in the pharmaceutical arena is due to their many advantages including improved drug efficiency, enhanced drug protection from *in vivo* enzymatic degradation, controlled release of drug payload, prolonged biological effect and enhanced patient compliance (Stark, 2011; Soppimath et al., 2001). Holpuch et al. (2010) have recently demonstrated the feasibility of using NP formulations for local oral mucosal delivery. This study was performed on human oral explants and the results showed that NPs, with size of about 200 nm and negatively charged, penetrate through the epithelium and basement membrane into the underlying connective tissue suggesting the possibility of oral transmucosal NP delivery for systemic therapeutics.

In vivo experiments performed by Mundargi et al., have demonstrated that insulin loaded NPs administered through the oral route decreased the glucose level from 219 to 86 mg/dL on alloxan-induced diabetic rats (Mundargi et al., 2011a,b). The limitations of oral transmucosal drug delivery have been described by Madhar et al. (2009), and there is therefore scope to stabilise proteins by encapsulating into NPs and subsequently incorporating into mucoadhesive films, intended for mucosal (local) and transmucosal (systemic) delivery of such therapeutic macromolecules.

Abbreviations: ASTM, American Standard Testing Methods; Ch, chitosan; D_H, hydrodynamic diameter; DLS, dynamic laser scattering; NP, nanoparticle; PBS, phosphate buffered saline; PEG-*b*-PLA, poly(ethylene glycol)methyl ether-block-polylactide; PEG, polyethylene glycol; P.I., polydispersity index; PVA, polyvinylalcohol; PVP, polyvinyl pyrrolidone; RP-HPLC, reversed-phase High-Performance Liquid Chromatography; SEM, scanning electron microscopy; TFA, trifluoro acetic acid; TH, trehalose.

* Corresponding author. Tel.: +44 0208 331 8980; fax: +44 0208 331 9805.
E-mail addresses: j.s.boateng@gre.ac.uk, joshboat@hotmail.com (J.S. Boateng).

0378-5173/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2012.02.035

Polymers such as poly-(lactic acid) (PLA) or poly-(lactide-co-glycolide) (PLGA) are commonly used for fabricating NPs due to their excellent biocompatibility and biodegradability (Mundargi et al., 2008). However, despite their versatility, a major drawback relating to their applicability is rapid clearance from the bloodstream by the mononuclear phagocytic system (MPS), following direct administration to the blood circulation, resulting in reduced drug therapeutic effect (Grislain et al., 1983; Moghimi et al., 2001). As a consequence, the concept of long-circulating NPs using amphiphilic copolymers has emerged. Vesicles made of amphiphilic copolymers are the most promising polymeric devices for oral insulin delivery, since they are able to maintain a prolonged hypoglycaemic effect after oral administration (Babu et al., 2008). In the design of an amphiphilic copolymer, the introduction of PEG was conceived with the intention of making NPs coated with PEG-chains more stable when in contact with physiological fluids. The literature widely reports on the ability of PEG to repel opsonin proteins leading to the formation of long circulating NPs (Shan et al., 2009; Essa et al., 2011). Nevertheless, in a recent review, several theories have been proposed to describe the effects of PEGylation (Karakoti et al., 2011). In addition, it is also well established that PEG has a mucoadhesion promoting effect (Peppas, 2004). PEG-*b*-PLA was therefore selected as an appropriate amphiphilic copolymer, to fabricate biodegradable NPs for the incorporation of insulin, chosen as model protein (insulin loaded PEG-*b*-PLA NPs).

Apart from the development of these NP carriers, recent efforts have targeted the use of the buccal route for mucosal (local) and transmucosal (systemic) delivery of therapeutic macromolecules. Considering the ease of buccal administration, the absence of first-pass hepatic metabolism, the lower concentration of extracellular enzymes, its relative permeability to many therapeutic agents as well as the high vascularisation with respect to other non-parenteral sites (nasal, rectal, and intestinal mucosa), the buccal mucosa represents an interesting non-invasive alternative route for protein delivery (Rossi et al., 2005). However, a number of factors limit the absorption of drugs via the buccal mucosa including the limited absorption area and the barrier properties as well as involuntary swallowing of dosage forms and continuous dilution of dissolved drugs by saliva. A strategy that could overcome these obstacles is the employment of polymer based mucoadhesive buccal films. Mucoadhesive films are very important dosage forms produced for buccal delivery of drugs (Boateng et al., 2009), owing to their small size and thickness that promotes patient compliance compared to tablets (Lee and Chien, 1995). A good polymer candidate for buccal delivery is chitosan (Ch) which is a polysaccharide derived from deacetylation of chitin containing amino groups and is soluble in acidic media. Ch is biocompatible and well accepted as a mucoadhesive polymer due to its ability to form ionic interactions with mucin (pH-dependent) (Sudhakar et al., 2006). Ch has been found to enhance drug absorption through mucosae without damaging the biological system (Agnihotri et al., 2004). Interestingly, Cui et al. (2009) have also demonstrated that Ch films offer a unique possibility to administer insulin through the transmucosal route, with pharmacological availability reaching 17% in 5 h as compared with subcutaneous insulin.

This paper reports the development and characterisation of Ch films (Ch-films) containing insulin loaded PEG-*b*-PLA NPs. The innovative approach employed focused on combining NP carriers and mucoadhesive films for the potential administration of proteins across the buccal mucosa. The proposed hypothesis was that the mucoadhesive films administered to mucosal surface could represent not only a delivery device with functionalities such as mucoadhesion, targeted unidirectional release and drug protection by avoiding passage through the GI tract, but also a "depot" of NPs. Furthermore, NPs could protect drugs from degradation, enhance uptake across the epithelium, and act as a controlled release system resulting in therapeutic blood concentrations over a prolonged time period. A physico-chemical study to achieve a formulation of NPs optimised in terms of drug loading and release properties was performed in the first phase. In the second phase, production of optimised mucoadhesive films in which the optimised formulation of NPs was integrated was carried out. To the best of our knowledge, this is the first time that Ch-films impregnated with insulin loaded PEG-*b*-PLA NPs have been investigated.

2. Experimental

2.1. Materials

Poly(ethylene glycol)methyl ether-block-polylactide (PEG average Mn ~ 5000, polylactide average Mn ~ 5000) (PLA-*b*-PEG), insulin (from porcine pancreas, Mw 5777.54 Da), chitosan (medium molecular weight, 75–85% deacetylated), polyvinylalcohol (PVA, Mowiol® 40-88), polyvinyl pyrrolidone (PVP), trehalose (TH), sodium azide, glycerol, sodium chloride, potassium chloride, sodium phosphate dibasic anhydrous, sodium hydroxide, dichloromethane, acetic acid, acetonitrile (HPLC grade) and trifluoro acetic acid (TFA) were supplied by Sigma–Aldrich (UK). Ultrapure water was used to carry out the study.

2.2. Formulation development

2.2.1. Preparation and characterisation of PEG-b-PLA NPs

Blank-NPs made of PEG-*b*-PLA at two different polymer concentrations (5 and 10%, w/v) (Blank-NPs-5 and Blank-NPs-10) were fabricated by double emulsion ($w_1/o/w_2$)-solvent evaporation technique. Briefly, 0.5 mL of water (w_1) was emulsified in 1 mL of dichloromethane containing PEG-*b*-PLA (o) by sonication for 20 s (amplitude 20%) (Ultrasonic Processor VC750, Sonics & Materials, Newtown, CT, USA). The primary emulsion (w_1/o) was immediately added to 2 mL of PVA (Mowiol® 40-88) solution (1%, w/v) and the resulting emulsion ($w_1/o/w_2$) was sonicated for 20 s (amplitude 20%). The double emulsion was diluted in 100 mL of PVA solution (0.3%, w/v) followed by solvent evaporation and subsequent particle hardening under magnetic stirring at room temperature for 3 h. The NPs were then isolated by ultra-centrifugation (13,600 rpm, 4 °C, 30 min) (Sorvall RC 6 Plus, Thermo Electron Corporation, UK) and lyophilised for 24 h with Virtis Advantage Freeze Dryer (Biopharma Process Systems, Winchester, UK). The freeze-drying conditions were as follows: frozen at −50 °C, annealed at −15 °C, primary dried at −20 °C and secondary dried at +20 °C with vacuum of 20 mTorr. Finally the NPs were stored at 4 °C until further use. Each batch was prepared in triplicate.

Insulin loaded PEG-*b*-PLA NPs (NPs-Insulin) were prepared in a similar manner using initial drug loadings of 2, 5 and 10% (w/w, with respect to copolymer weight) and designated NPs-Insulin-2, NPs-Insulin-5 and NPs-Insulin-10, respectively. Insulin was dissolved in the internal water phase (w_1) of the double emulsion. The emulsification and purification steps were repeated as before. Each insulin loaded batch was prepared in triplicate.

2.2.2. Physicochemical and morphological characterisation

The hydrodynamic diameter (D_H) and polydispersity index (P.I.) of NPs were determined by dynamic laser scattering (DLS) (Zetasizer Nano ZS, Malvern, UK). For the particle size analysis, suspended freeze-dried NPs in ultrapure water were diluted to the appropriate concentration, filtered (RC 0.45 μm) and measured at 25 °C at 90° angle. Zeta (ζ) potential was determined by analysing NPs suspended in 10^{-3} M NaCl solution on a Zetasizer Nano ZS (Zetasizer Nano ZS, Malvern, UK). The D_H distribution, P.I. and ζ potential distributions were expressed as average value ± SD for three batches. Particle shape and morphology were analysed by

scanning electron microscopy (SEM). 10 μL of NPs aqueous suspension (0.3 mg/mL) on a cover glass was placed on a metal stub and dried in a desiccator for 30 min. The samples were subsequently sputter coated with gold under vacuum (Edwards S150B Sputter Coater) for 120 s and images acquired on a Cambridge Stereoscan S-360 SEM (Class one equipment, London, UK) and processed with *i-scan2000* software.

2.2.3. Determination of the effect of cryoprotectants

Lyophilised Blank-NPs were suspended in ultrapure water at varying concentrations (1–2 mg/mL) and placed in vials containing either of two cryoprotectants [polyvinyl pyrrolidone (PVP) or trehalose (TH)] at a final concentration of 5% (w/v). The mixture was stirred for several minutes and lyophilised as already described for the preparation of NPs. After lyophilisation, dried NPs were suspended in a known volume of ultrapure water and analysed for particle size and P.I. as previously discussed in Section 2.2.2. The experiments were repeated three times.

2.2.4. Determination of encapsulation efficiency

A solvent extraction method was used to evaluate the encapsulation efficiency of NPs. A known amount of NPs-Insulin (1.5 mg) was dissolved in 0.4 mL of dichloromethane and 1.2 mL of TFA solution (0.05%, v/v) added to the organic phase. The two-phase mixture was stirred for 1 h and the suspension centrifuged (5000 rpm, room temperature, 15 min). The amount of insulin was determined by reversed-phase High-Performance Liquid Chromatography (RP-HPLC) (Agilent Technologies, Santa Clara, CA, USA) with a Jupiter 5-μm C18 column (250 mm × 4.6 mm, 300 Å) (Phenomenex, USA) with the following conditions: mobile phase—water:acetonitrile (67:33, v/v) containing 0.1% (v/v) of TFA; flow rate—1 mL/min; detection wavelength—220 nm and injection volume—20 μL. All samples were analysed in duplicate and the protein concentration was determined based on interpolation from a calibration curve with linearity verified over concentration range of 1–100 μg/mL ($R^2 > 0.99$). The reliability of the method was verified as follows: 1.5 mg of Blank-NPs was dissolved in 0.4 mL of dichloromethane and then mixed with 1.2 mL of TFA (0.05%, v/v) containing a known amount of protein. Insulin was extracted and analysed using the same extraction and RP-HPLC procedure described above. Results of three different batches were expressed as the mean ± SD. Actual percent drug loading (ADL) and encapsulation efficiency (EE) were calculated using the following equations:

$$\text{ADL}\ (\%) = \frac{\text{amount of drug entrapped in NPs}}{\text{amount of NPs}} \times 100 \quad (1)$$

$$\text{EE}\ (\%) = \frac{\text{amount of drug entrapped in NPs}}{\text{amount of drug added}} \times 100 \quad (2)$$

2.2.5. In vitro insulin release studies

The *in vitro* release of insulin from the NPs was monitored by membrane dialysis in phosphate buffered saline (PBS) at 37 °C. The PBS was prepared with 120 mM NaCl, 2.7 mM KCl, 10 mM phosphate salts and sodium azide (NaN_3) 0.05% (w/v) as preservative. 3 mg of NPs was suspended in 0.7 mL of PBS and placed in a dialysis membrane tube (MWCO: 8–10 kDa, Spectra/Por® Float-A-Lyzer, Sigma–Aldrich, UK). The sample was dropped into 4.5 mL of PBS (sink condition) and kept at 37 °C with gentle mixing. At specific time points, 0.5 mL aliquots were withdrawn and replaced with the same volume of fresh buffer. The insulin content in the releasing buffer was determined by RP-HPLC as previously described. The release profile of insulin from NPs with different initial drug loading was performed and evaluated at two physiological pH values of 6.8 and 7.4. The results were expressed as mean ± SD percent drug release of three different samples of the same NP preparation.

2.3. Preparation and characterisation of Ch-films containing insulin loaded PEG-b-PLA NPs

2.3.1. Film preparation and insulin loaded PEG-b-PLA NPs incorporation

The Ch-films were prepared by solvent casting method. Ch gel at a concentration of 1.25% (w/v) was produced by dissolving the required amount of Ch in an aqueous solution of acetic acid 1% (v/v) at room temperature under magnetic stirring. Glycerol (GLY) as a plasticiser was added to the gel at a ratio of 1:0.25 (w/w) Ch:GLY and stirred for 30 min. A known amount of insulin loaded PEG-*b*-PLA NPs (1, 2 and 3 mg of NPs *per* film) suspended in ultrapure water was added to the resultant gel, mixed under sonication (Fisher Scientific, Surrey, UK) for 15 min in order to uniformly disperse NPs and then dried in an oven (GenLab, Oven) at 37 °C overnight. The dried films were neutralised by immersing samples in sodium hydroxide 1% (w/v) and washing with water several times. The samples were further dried in an oven for 15 min to obtain the final product. The Ch-films-NPs prepared were designated: Ch-films-NPs-1, Ch-films-NPs-3 and Ch-films-NPs-5, containing 1, 3 and 5 mg of NPs per films, respectively. Chitosan films without NPs (Blank Ch-films) were also prepared as a positive control, following the same procedure described above. Each formulated film was prepared in triplicate.

2.3.2. Determination of morphological properties of Ch-films

To investigate the microstructure of Ch-films and the uniformity of NPs dispersed in the Ch-films, peripheral and middle portions of films were coated with a thin layer of gold and analysed using the SEM.

2.3.3. Determination of mechanical properties of Ch-films

Ch-film thickness was determined by a micrometre screw gauge (0–0.25 mm) at five different locations on the same film prior to mechanical tensile characterisation. The mechanical properties of the Ch-films were investigated based on American Standard Testing Methods (ASTM) D882 (1991) and measured using Texture Analyzer (TA.HD. *plus*) (Stable Micro Systems Ltd., Surrey, UK). The elongation at break, tensile strength and the Young's modulus were estimated according to Eqs. (3)–(5). The time and work at break were also investigated. Results of three films for each formulation were expressed as the mean values ± SD:

$$\text{elongation at break} = \frac{\text{increase in length at break}}{\text{initial film length}} \times 100 \quad (3)$$

$$\text{tensile strength} = \frac{\text{force at failure}}{\text{cross sectional area of the film}} \quad (4)$$

$$\text{Young's modulus} = \frac{\text{slope of stress–strain curve}}{\text{film thickness} \times \text{cross-head speed}} \quad (5)$$

2.4. Statistical data analysis

A two tailed Student's *t*-test at 95% confidence interval (*p*-value < 0.05) as the minimal level of significance was used to evaluate the data statistically.

3. Results and discussion

3.1. Preparation and characterisation of PEG-b-PLA NPs

3.1.1. Blank PEG-b-PLA NPs

NPs made of PEG-*b*-PLA were successfully prepared using a modified double emulsion solvent evaporation technique by emulsifying a dichloromethane solution of PEG-*b*-PLA in an aqueous phase and precipitating the copolymer into NPs in another aqueous

Table 1
Composition and properties of Blank-NPs.

Formulation	PEG-*b*-PLA (%, w/v)	Hydrodynamic diameter (D_H)[a] (nm ± SD[b])	Polydispersity index (P.I.) (±SD[b])	Zeta potential (ζ)[c] (mV ± SD[b])
Blank-NPs-5	5	266.9 ± 22.9	0.244 ± 0.046	−5.91 ± 2.54
Blank-NPs-10	10	313.3 ± N/C	0.577 ± N/C	−0.571 ± N/C

N/C: not calculated because of poor technological properties of the NPs produced.
[a] Average particle size as determined by dynamic laser scattering.
[b] Standard deviation of values calculated for three different batches of the same NPs formulation.
[c] Average ζ potential as determined by electrophoretic light scattering.

Table 2
Size distribution of Blank-NPs-5 after different stages of preparation.

Formulation	Hydrodynamic diameter (D_H)[a] before freeze drying (nm ± SD[b])	Polydispersity index (P.I.) (±SD[b])[c]	Hydrodynamic diameter (D_H)[a] after freeze drying (nm ± SD[b])	Polydispersity index (P.I.) (±SD[b])[d]
Blank-NPs-5	251.0 ± 20.0	0.227 ± 0.063	266.9 ± 22.9	0.244 ± 0.046

[a] Average particle size as determined by dynamic laser scattering.
[b] Standard deviation of values calculated for three different batches of the same NPs formulation.
[c] Average P.I. as determined by dynamic laser scattering before freeze-drying.
[d] Average P.I. as determined by dynamic laser scattering after freeze-drying.

phase having 0.3% PVA as stabiliser after organic solvent evaporation (Tobío et al., 2000). Influence of formulation conditions on NPs size, polydispersity index and morphology were investigated with Blank-NPs in which the copolymer concentration in the organic phase (o) of the double emulsion varied between 5 and 10% (w/v). Table 1 summarises the composition and some characteristics of Blank-NPs. PEG-*b*-PLA concentration plays a crucial role in determining the physico-chemical properties of the NPs. For Blank-NPs-5 and Blank-NPs-10 formulations, an increase in the copolymer concentration led to an increase in the particle average diameter and P.I. The high value of P.I. (≈0.6) observed in Blank-NPs-10 indicates the presence of large aggregates. These results are consistent with those previously described by Lamprecht et al. (2000) and Ogawa et al. (1988). The authors demonstrated that increasing the polymer concentration in the organic phase (o) of the double emulsion increases the viscosity of the primary emulsion (w_1/o) resulting in an unstable dispersion of the aqueous phase droplets in the organic phase. The instability of the first emulsion (w_1/o) reduces the efficiency of the emulsion droplet size during the second emulsification step (w_1/o/w_2). Consequently, the final product is characterised by irregular and large particle population.

Zeta (ζ) potential measurement is crucial in defining the localisation of the different ingredients on the surface of the NPs. According to Tobío et al. (1998), the low negative charge observed (Table 1) for both Blank-NPs formulations could be explained by the presence of PEG chains that tend to spread on the surface of the NPs. In their report, the authors using hydrophobic interaction chromatography (HIC), efficiently demonstrated that the high negative value of ζ obtained for PLA-NPs (−50 mV) reflects the more hydrophobic surface of these NPs as compared to that of PLA-PEG NPs (−30 mV). From Table 1, further reduction of the ζ potential values (−6 mV) was observed. A similar observation was recently reported by Essa et al. (2010a) showing that this reduction could be attributed to the small fraction of PVA on the surface of NPs which could mask their actual surface charge.

Furthermore, the flexibility of PEG chains which strongly depends on the concentration of the copolymer may also affect the surface characteristics of the NPs. By increasing the PEG-*b*-PLA concentration in the organic phase in Blank-NPs-10, a significant decrease in the ζ potential was observed. Theoretically, aggregation of NPs easily occurs when ζ potential value is low, leading to the formation of large NPs (Yasugi et al., 1999). Accordingly, the aggregation observed in the Blank-NPs-10 formulation resulted from its lower ζ potential value, confirming the results previously described.

The SEM micrograph (Fig. 1A) shows that Blank-NPs-5 formulation is characterised by spherical and regular particles without aggregation or adhesion among the NPs. On the other hand, Fig. 1B demonstrates clearly the presence of large aggregates in the Blank-NPs-10 formulation. Furthermore, the particle size, observed in

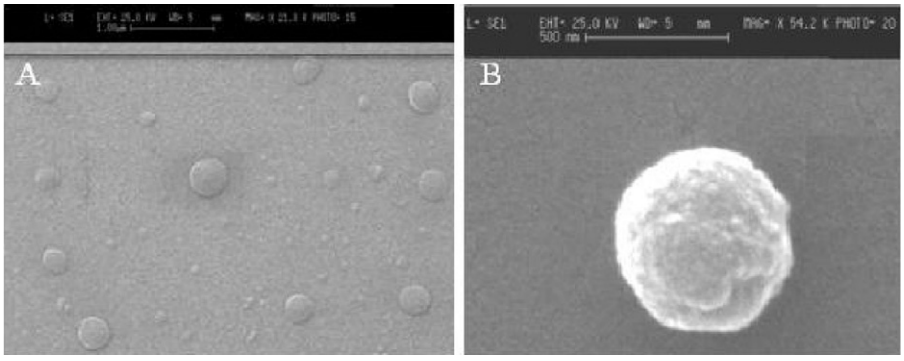

Fig. 1. Representative SEM micrographs of Blank-NPs prepared at different concentrations of PEG-*b*-PLA: (A) 5% (w/v) (Blank-NPs-5) and (B) 10% (w/v) (Blank-NPs-10).

the images, was in good agreement with the result measured by particle size analyser.

3.1.2. Freeze-drying of PEG-b-PLA NPs: effect of cryoprotectants

The last stage in the preparation of the PEG-*b*-PLA NPs involved lyophilisation of the NP suspension to obtain a final dry powder. The storage of NPs in a lyophilised form normally ensures long-term stability (Verdun et al., 1986). A typical freeze-drying cycle which did not have any significant effect on NPs size and P.I. was employed. The NPs suspension (before lyophilisation) showed a uni-modal distribution and size of 251.0 ± 20.0 nm with a very low P.I. (0.227 ± 0.063). These are consistent with those reported for the lyophilised NPs (Table 2). To complete the formulation development work, the protective effect of two cryoprotectants on Blank-NPs-5 formulation was studied. Polyvinylpyrrolidone (PVP) or trehalose (TH) at a final concentration of 5% (w/v) was added to the NPs suspension (1 or 2 mg/mL) evaluating the average size and P.I. before and after the addition of cryoprotectants. The graphs presented in Fig. 2A and B, show that size and P.I. of the NPs remained unchanged before and after the addition of TH at different NP concentrations studied. The differences were not statistically significant ($p = 0.59$). However the addition of PVP led to slightly smaller diameters when 2 mg/mL of NPs were tested (Fig. 2B) and the formation of large aggregates with very high P.I. (≈ 1) in both cases (Fig. 2A and B). From these results, the TH proved to be an effective cryoprotectant, independent of NP concentration as previously reported for PLGA NPs (d'Angelo et al., 2010).

3.1.3. Insulin loaded PEG-b-PLA NPs

It has been established that the double emulsion evaporation process tends to be superior to other methods for encapsulating macromolecules, preserving their stability as well as active conformational structure (Tabata et al., 1993). Insulin loaded PEG-*b*-PLA NPs (NPs-Insulin) were prepared following all process parameters adopted to obtain Blank-NPs-5. Moreover, to elucidate physico-chemical features such as encapsulation efficiency and release profile, insulin loaded PEG-*b*-PLA NPs were prepared at varying initial drug loadings (2, 5 and 10%, w/w) to determine the effect of drug concentration on the formulation characteristics. The composition and characteristics of the particles such as size, encapsulation efficiency and final drug loading are shown in Table 3. As expected, the physico-chemical properties of NPs were strongly affected by the concentration of insulin added in the internal aqueous phase of the double emulsion. This exerts a critical effect upon particle morphology. The formulations NPs-Insulin-2 (Fig. 3A) and NPs-Insulin-5 (Fig. 3B), appeared spherical, with relatively mono-dispersed size distribution. As previously discussed (Section 3.1.1), the control of particle formation performed by the double emulsion technique is due to the stability of the primary emulsion. This stability could be affected by (i) polymer concentration in the organic phase of the double emulsion, as discussed above and (ii) the amount of drug initially added to the formulation. An increase in initial insulin concentration resulted in the formation of large aggregates (Fig. 3C). This effect is due to the fact that insulin, when used at high initial concentration, exerts a destabilising effect on the emulsion. Based on these preliminary results, further development and characterisation of the NPs-Insulin-10 formulation was discontinued.

The particle size, P.I. and ζ potential of NPs-Insulin-2 and NPs-Insulin-5 (Table 2) were comparable to those obtained for corresponding Blank-NPs with no significant statistical differences ($p = 0.33$). This might indicate that drug loading had no observable effect on physical features of NPs, suggesting that size, P.I. and ζ potential were mainly controlled by homogenisation parameters during NPs preparation.

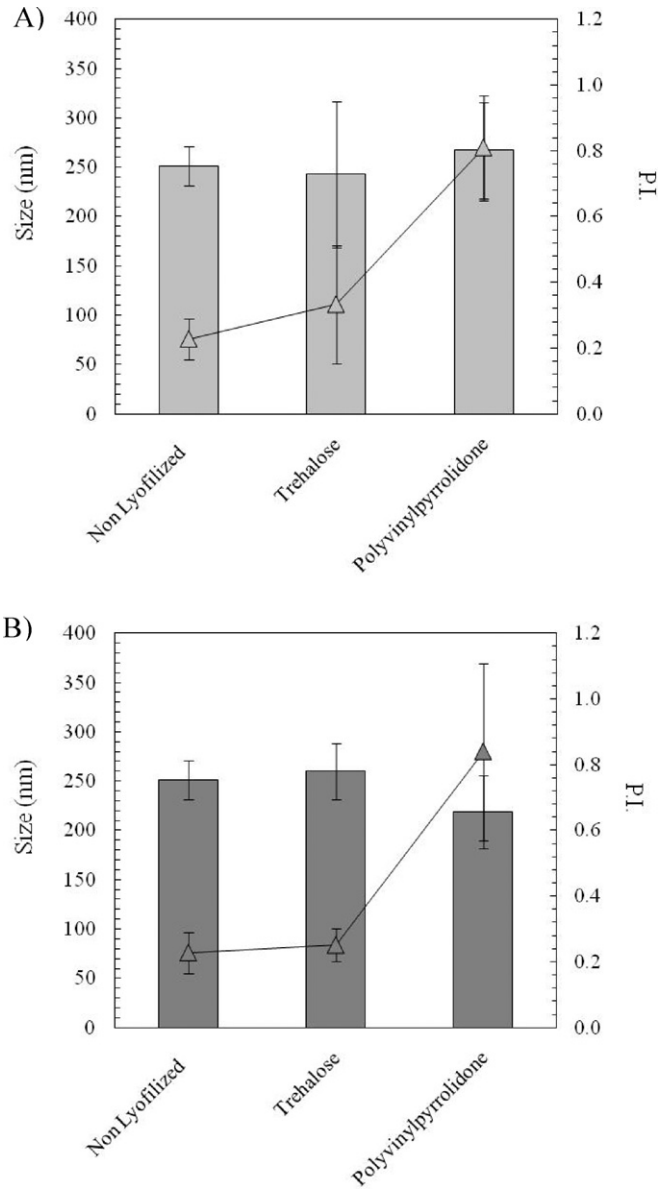

Fig. 2. Effect of cryoprotectants on the physical properties of (A) 1 mg/mL or (B) 2 mg/mL of Blank-NPs-5 lyophilised by adding TH or PVP at a concentration of 5% (w/v). Each bar is the mean of three measurements (±SD).

3.1.4. Encapsulation efficiency (EE)

An insulin recovery of $76 \pm 4.6\%$ with respect to the initial loading values was obtained. From Table 2, EE (%) was found to be 71% and 27% for NPs-Insulin-2 and NPs-Insulin-5, respectively. The high EE values observed are due to the amphiphilic di-block copolymer (PEG-*b*-PLA) employed. As described by Essa et al. (2010b), NPs prepared using a linear copolymer, exhibit higher EE than those made of multi-block copolymer. This effect could be reasonably attributed to the fact that in a linear copolymer, such as PEG-*b*-PLA, the PEG chains have more flexibility to form a stearic barrier, preventing the diffusion of drug into the external aqueous phase during solidification of NPs, thus the amount of drug encapsulated is increased. A significant difference in EE (%) between the two formulations was observed which reflects the variations in the amount of protein dissolved in the internal water phase of the double emulsion. When a high amount of insulin was added to the preparation, a loss in initial drug employed was observed. This might be due to the difference in the osmotic pressure between the internal aqueous phase (w_1) containing insulin and the external aqueous phase (w_2)

Table 3
Composition and characteristics of NPs-Insulin.

	NPs-Insulin-2	NPs-Insulin-5
PEG-*b*-PLA (%, w/v)	5	5
Insulin concentration (%, w/w)	2	5
Hydrodynamic diameter (D_H)[a] (nm ± SD[b])	215.5 ± 2.5	231.9 ± 16.2
Polydispersity index (P.I. ± SD[b])	0.169 ± 0.025	0.242 ± 0.114
Zeta potential (ζ)[c] (mV ± SD[b])	−5.71 ± 3.17	−4.86 ± 3.81
Insulin loading (ADL% ± SD[b])[d]	1.47 ± 0.07	1.19 ± 0.42
Insulin encapsulation efficiency (EE% ± SD[b])	71 ± 3.4	27 ± 9.3

[a] Average particle size as determined by dynamic laser scattering.
[b] Standard deviation of values calculated for three different batches of the same NPs formulation.
[c] Average ζ potential as determined by electrophoretic light scattering.
[d] Insulin (mg) encapsulated per 100 mg of NPs. The theoretical loading was 2.08 and 4.45 mg of insulin per 100 mg of NPs-Insulin-2 and NPs-Insulin-5, respectively.

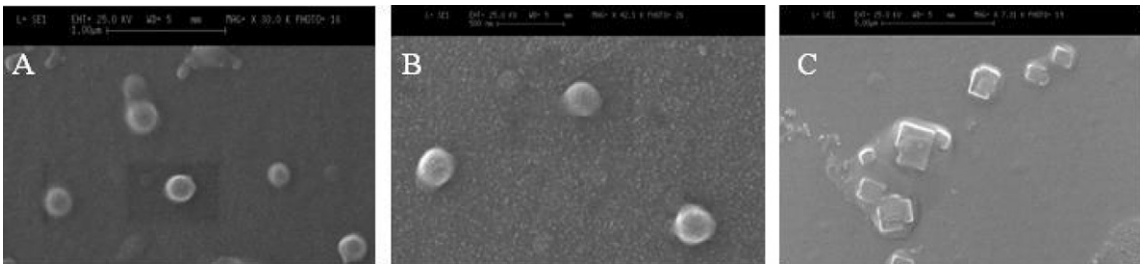

Fig. 3. Representative SEM micrographs of insulin loaded PEG-*b*-PLA NPs prepared using different amounts of initial drug: (A) 2% (w/w) (NPs-Insulin-2); (B) 5% (w/w) (NPs-Insulin-5); (C) 10% (w/w) (NPs-Insulin-10).

of the double emulsion. Increase in osmotic pressure with increased insulin loading results in rupture of the lipophilic droplets and an exchange of protein between the two phases, with a consequent decrease in the amount of insulin entrapped.

These results are consistent with those previously reported by Lamprecht et al. (2000) for efficient entrapment of bovine serum albumin in both PLGA and PCL NPs. Therefore these data suggest that it is possible to prepare NPs made of linear amphiphilic copolymers with favourable characteristics for the entrapment of hydrophilic macromolecules, by modulating the process parameters.

3.1.5. *In vitro* insulin release from NPs

The *in vitro* release of insulin from NPs-Insulin-2 and NPs-Insulin-5 formulations is shown in Fig. 4. The effect of pH of the release media on the release characteristics of the NPs was investigated by monitoring insulin release profiles with a dialysis membrane in phosphate buffer (PBS) at pH 6.8 and 7.4 that are associated with the ionic H^+ concentrations of the buccal mucosa and blood circulation, respectively at a temperature of 37 °C. The pH of 6.8 was used because after coming in contact with dissolution medium, the films swell and begin a first release of NPs and then when the Ch-film dissolves the NPs escape from the polymeric network to be completely released into the buffer solution. This hypothesis could imply that there is a period in which the NPs, before reaching the blood circulation, could be in contact with the buccal mucosa environment. Therefore the release profile of insulin from insulin loaded *PEG-b-PLA NPs* was monitored utilising buffer solution with pH 6.8, simulating salivary pH. As can be seen from Fig. 4 the results are encouraging, because at pH 6.8 a lower amount of drug was released in the initial hydration and diffusive phases for both formulations studied. Therefore, the insulin content is expected to be preserved even if the NPs remained in the buccal environment (pH 6.8) for a short period of time.

Each formulation showed a classic biphasic release characterised by a first initial phase (burst effect) in which approximately 40% of encapsulated drug was released in the first 6 h. This initial release phase could be attributed to the amount of drug adsorbed on the surface of the NPs (Magenheima et al., 1993). This was then followed by a constant sustained release of insulin for 5 weeks. As regards the second phase, release could be mainly attributed to diffusion of protein through the polymer matrix and the erosion of polymer matrix overtime (Panyam et al., 2003; Mittal et al., 2007). Another factor to be considered is the type of polymer employed. For an amphiphilic copolymer, the architecture and the hydrophilicity markedly influence the ability of the polymer to be wetted and hence its drug release profile (Dipasree and Bhagwan, 2002; Sung et al., 1998). The linear amphiphilic copolymer PEG-*b*-PLA used to prepare the NPs exhibited a slower release of encapsulated drugs as compared to multi-block copolymer (Yasugi et al., 1999). This may be explained by the PEG chains tending to orient mainly towards the external aqueous medium

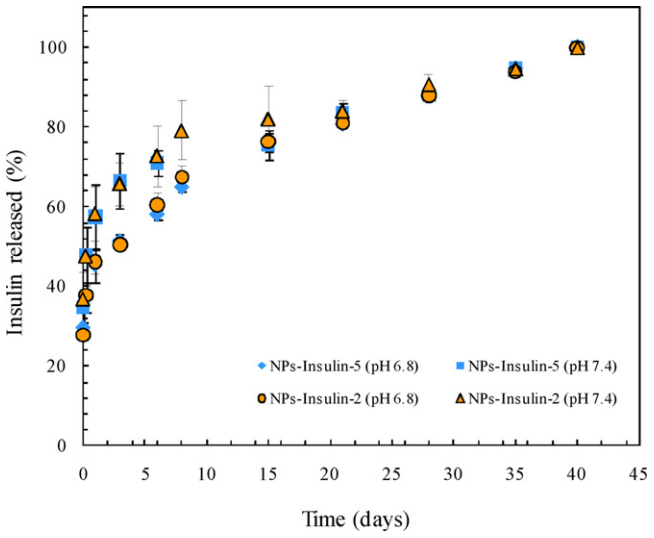

Fig. 4. Release profiles of insulin in phosphate buffer at physiological conditions from: NPs-Insulin-2 at pH 6.8 (●) and pH 7.4 (▲); NPs-Insulin-5 at pH 6.8 (♦) and pH 7.4 (■). Each point is the mean of three different measurements (±SD).

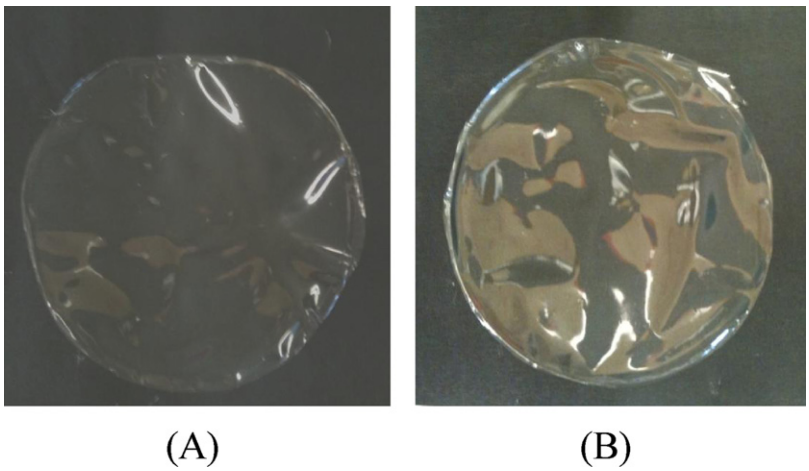

Fig. 5. Representative digital images of Ch-films-NPs-3 (A) before and (B) after neutralisation procedure performed by immersing dried films in 1% (w/v) solution of NaOH. Similar images were obtained for all formulated Ch-films.

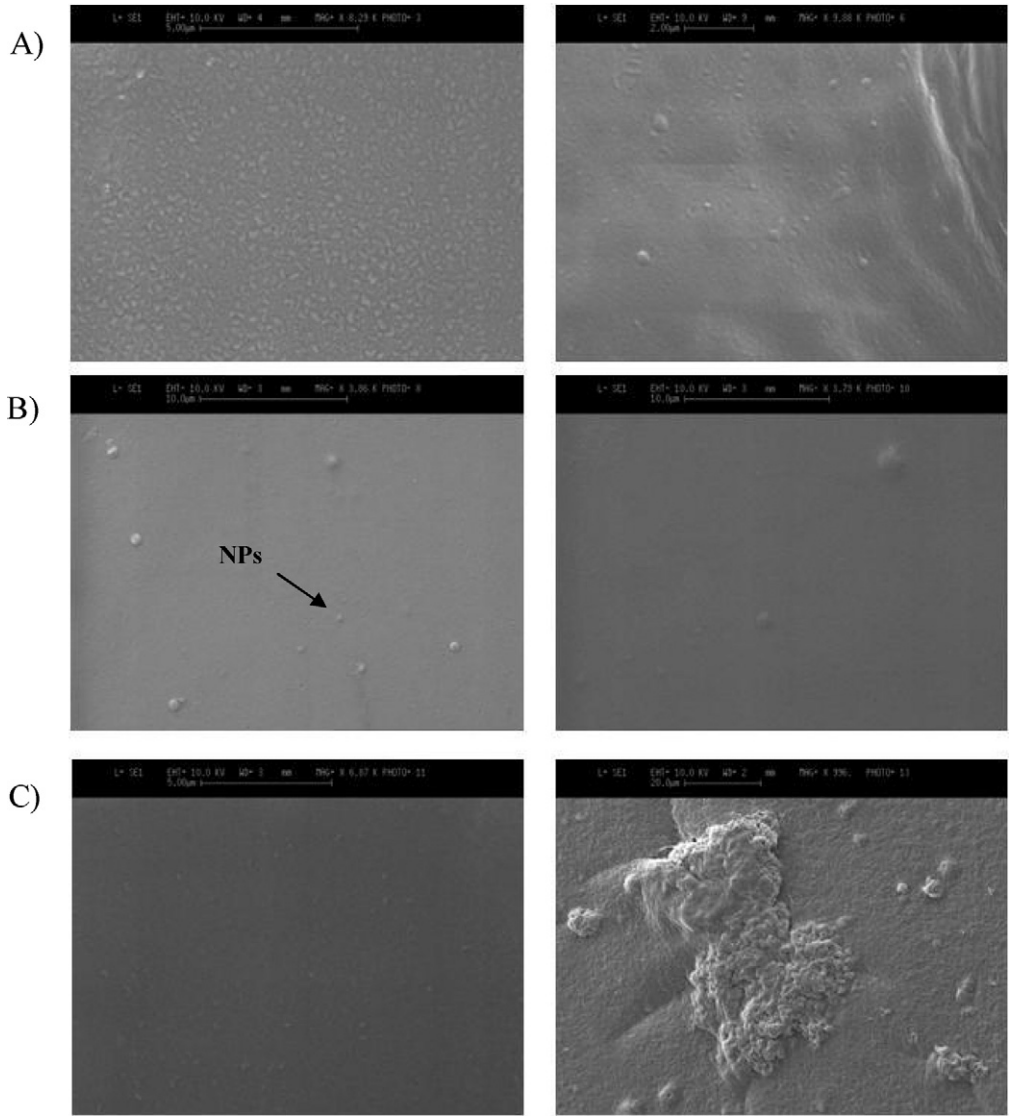

Fig. 6. SEM micrographs showing the differences in surface distribution of NPs in the Ch-films: (A) Ch-films-NPs-1, (B) Ch-films-NPs-3 and (C) Ch-films-NPS-5. The images of the peripheral and middle regions of formulated films are represented on the left and right side, respectively.

Table 4
Mechanical properties of formulated Ch-films.

Formulation	Time to break (s ± SD[a])	Elongation at break (% ± SD[a])	Tensile strength (N ± SD[a])	Young's modulus (N/mm²/% ± SD[a])	Work done to break (J ± SD[a])
Blank Ch-films	2.7 ± 3.8	36 ± 12.9	36.3 ± 19.6	46.6 ± 0.04	3.7 ± 0.1
Ch-films-NPs-1	3.3 ± 0.4	158 ± 29.0	36.3 ± 35.3	13.6 ± 8.8	60.6 ± 58.7
Ch-films-NPs-3	4.6 ± 2.2	174 ± 14.9	55.9 ± 5.4	67.6 ± 5.9	131.0 ± 15.7
Ch-films-NPs-5	6.6 ± 3.7	171 ± 14.5	78.2 ± 6.7	115.4 ± 53.6	359.6 ± 297.7

[a] Standard deviation of values calculated for three different batches of the same formulated film.

while the core remained predominantly hydrophobic, resulting in a very slow wetting of the core when the PEG-*b*-PLA NPs were suspended in the release medium. In addition, the presence of PEG on the polymeric structure could have a stabilising effect on the encapsulated protein (insulin). It is possible for the PEG chains to migrate towards the inner aqueous phase of the double emulsion that contains the protein, creating a theoretical stearic barrier that would reduce the interaction of protein with PLA matrix. From Fig. 4, the difference between the release profile of insulin for NPs-Insulin-2 and NPs-Insulin-5 formulations was not statistically significant ($p = 0.11$). The results obtained are consistent with another study by Cohen et al. (2000) which indicated that the amount of protein initially added to the preparation did not significantly influence the release pattern of the NPs. However, different release profiles were observed when the value of pH of the release medium was modified. In fact, by reducing the pH to 6.8, it was observed that a lower amount of drug was released in the initial hydration and diffusive phases for both formulations (Fig. 4) although the difference was not statistically significant ($p = 0.08$). These results suggest the possibility of the use of PEG-*b*-PLA NPs as drug delivery system for macromolecules for different therapeutic goals in which release profiles and rates are modulated by the ionic H^+ concentration of the target site.

3.2. Preparation and characterisation of Ch-films containing insulin loaded PEG-b-PLA NPs

3.2.1. Processing characteristics

As established in the NP formulation work, NPs-Insulin-2 was the optimised formulation in terms of physico-chemical properties with insulin encapsulation efficiency at about 70%. In the light of these promising results, it was selected for the second part of the work to develop a novel system consisting of Ch-films containing insulin loaded PEG-*b*-PLA NPs (Ch-films-NPs), which has been characterised in the proceeding sections. The Ch-films dispersed with different concentrations of NPs (1, 3, and 5 mg of NPs per film) were prepared by solvent casting method and plasticised with glycerol to obtain elegant films (namely Ch-films-NPs-1, Ch-films-NPs-3 and Ch-films-NPs-5). Additionally, Ch-films without NPs were prepared, as control for all experiments. Finally, the dried films were neutralised with 1% (w/v) solution of NaOH and then carefully washed with ultrapure water (Gartner et al., 2011). No heterogeneities or phase separations in the Ch-films were observed during processing and treatment with NaOH. On visual inspection, highly uniform and optically transparent dried films (average thickness range of 0.70–1.11 mm) were obtained with NaOH treatment having no significant effect on film properties (Fig. 5).

3.2.2. Structural morphology by SEM

For a good representation of NPs in the Ch-films as well as to examine their distribution within the films, SEM studies were performed on the peripheral and middle portions of each formulated film. The SEM images of Ch-films-NPs-1, Ch-films-NPs-3 and Ch-films-NP-5 are shown in Fig. 6. In all formulated films, the Ch network formed a dense continuous sheet with no fractures. Ch-films-NPs-3 (Fig. 6B) exhibited an unequivocally uniform distribution of NPs in both peripheral and middle portions. However in Ch-films-NPs-1 (Fig. 6A), the NPs were located only at the peripheral region of the film. Finally, large particle aggregates were found in the middle region of Ch-films-NPs-5 (Fig. 6C). These results suggest that NP concentration plays a significant role in determining the films morphology.

3.2.3. Mechanical properties

The tensile properties of the Ch-films in the present study are summarised in Table 4. In previous work (Boateng et al., 2009), it was demonstrated that the addition of glycerol imparts flexibility to films with increased thickness, which makes for easy removal without damage to film configuration. Thus, in all formulated films, glycerol was used, resulting in optimised plasticised films. The glycerol molecules form H-bonds with Ch and interrupts inter and intra-molecular H-bonds between the polymer chains with consequent partial disintegration of the H-bonded network structure (Bajdik et al., 2009). Blank Ch-films (without NPs) were compared with Ch-NPs-films containing 1, 3 and 5 mg of NPs *per* film. Notably, increasing the concentration of NPs per film led to a gradual increase in 'time to break' as well as tensile strength obtained. On the other hand, the 'elongation at break' increased with the incorporation of 3 mg of NPs per film (Ch-films-NPs-3) to a maximum value and then decreased again (Ch-films-NPs-5) but remained higher than Blank Ch-films. The Young's modulus is a measure of film stiffness and rigidity, and it was calculated from the slope of the initial linear portion (region of linear elastic deformation) of a stress–strain curve. Interestingly, the Young's modulus value for Ch-films-NPs-3 was very close to that of Blank Ch-films, whereas it was lower or higher in the case of Ch-films-NPs-1 and Ch-films-NPs-5, respectively. These may be attributed to the non homogeneous distribution of the NPs in these formulated films confirming the data presented by SEM images (Fig. 5A–C). Marked differences were observed in terms of 'work done to break' (area under the force–time curve) the films which is an indication of film toughness. A regular increase in the 'work done to break' from 60 to 359 J was observed with increasing NPs content (Table 4). Nevertheless, there was a sharp decrease of this value in the case of Blank Ch-films (3.7 J), considering that this mechanical feature is strongly affected by elongation as well as stress.

4. Conclusions

NPs made of PEG-*b*-PLA loaded with insulin have been successfully prepared by double emulsion solvent evaporation technique. Optimisation of NPs in terms of EE and release characteristics was established. The physico-chemical properties were strongly influenced by the initial amount of drug loaded. A lower concentration of insulin resulted in a higher EE while varying concentration of insulin did not significantly affect the release profile. Ch-films embedded with varying concentrations of insulin loaded NPs were produced by solvent casting method. The formulation with 3 mg of NPs per film was deemed optimised based on the homogeneous distribution of NPs in the polymeric matrix, resulting in films with excellent physico-mechanical properties. In the light of these observations, Ch-films integrated with insulin loaded NPs could

represent a novel platform for the controlled delivery of proteins via the buccal mucosa. Work is underway to evaluate the novel drug delivery system in an *ex vivo* environment.

Acknowledgements

The authors would like to acknowledge the Greenwich Research and Enterprise (GRE) of the University of Greenwich for sponsoring this research work. We also extend our appreciation to Dr. Ian Slipper for his assistance with SEM imaging.

References

ASTM, 1991. Standard Test Methods for Tensile Properties of Thin Plastic Sheeting—D 882. American Standard Testing Methods.

Agnihotri, S.A., Mallikarjuna, N.N., Aminabhavi, T.M., 2004. Recent advances on chitosan-based micro- and nanoparticles. J. Control. Release 100, 5–28.

Babu, V.R., Patel, P., Mundargi, R.C., Rangaswamy, V., Aminabhavi, T.M., 2008. Developments in polymeric devices for oral insulin delivery. Expert Opin. Drug Deliv. 5, 403–415.

Bajdik, J., Marciello, M., Caramella, C., Domjánc, A., Süveghd, K., Mareke, T., Pintye-Hód, K., 2009. Evaluation of surface and microstructure of differently plasticized chitosan films. J. Pharm. Biomed. Anal. 49, 655–659.

Boateng, J.S., Stevens, H.N.E., Eccleston, G.M., Auffret, A.D., Humphrey, M.J., Matthews, K.H., 2009. Development and mechanical characterization of solvent-cast polymeric films as potential drug delivery systems to mucosal surfaces. Drug Dev. Ind. Pharm. 35, 986–996.

Cohen, H., Levy, R.J., Gao, J., Fishbein, I., Kousaev, V., Sosnowski, S., Slomkowski, S., Golomb, G., 2000. Sustained delivery and expression of DNA encapsulated in polymeric nanoparticles. Gene Ther. 7, 1896–1905.

Crommelin, D.J.A., Storm, G., Jiskoot, W., Stenekes, R., Mastrobattista, E., Hennink, W.E., 2003. Nanotechnological approaches for the delivery of macromolecules. J. Control. Release 87, 81–88.

Cui, F., He, C., He, M., Tang, C., Yin, L., Qian, F., Yin, C., 2009. Preparation and evaluation of chitosan–ethylenediaminetetraacetic acid hydrogel films for the mucoadhesive transbuccal delivery of insulin. J. Biomed. Mater. Res. A 89, 1063–1071.

d'Angelo, I., Parajo, Y., Horvath, A., Keri, G., La Rotonda, M.I., Jose, A.M., 2010. Improved delivery of angiogenesis inhibitors from PLGA: poloxamer blend micro- and nanoparticles. J. Microencapsul. 27, 57–66.

Dipasree, S.R., Bhagwan, D.R., 2002. Comparative evaluation of rate of hydration and matrix erosion of HEC and HPC and study of drug release from their matrices. Eur. J. Pharm. Sci. 16, 193–199.

Essa, S., Rabanel, J.M., Hildgen, P., 2011. Characterization of rhodamine loaded PEG-g-PLA nanoparticles (NPs): effect of poly(ethyleneglycol) grafting density. Int. J. Pharm. 411, 178–187.

Essa, S., Rabanel, J.M., Hildgen, P., 2010a. Effect of aqueous solubility of grafted moiety on the physicochemical properties of poly(D,L-lactide) (PLA) based nanoparticles. Int. J. Pharm. 388, 2632–2673.

Essa, S., Rabanel, J.M., Hildgen, P., 2010b. Effect of polyethylene glycol (PEG) chain organization on the physicochemical properties of poly(D,L-lactide) (PLA) based nanoparticles. Eur. J. Pharm. Biopharm. 75, 96–106.

Gartner, C., Lopez, L.B., Sierra, L., Graf, R., Spies, H.W., Gaborieau, M., 2011. Interplay between structure and dynamics in chitosan films investigated with solid-state NMR, dynamic mechanical analysis, and X-ray diffraction. Biomacromolecules 12, 1380–1386.

Grislain, L., Couvreur, P., Lenaerts, V., Roland, M., Deprez-Decampeneere, D., Speiser, P., 1983. Pharmacokinetics and distribution of a biodegradable drug-carrier. Int. J. Pharm. 15, 335–345.

Holpuch, A.S., Hummel, G.J., Tong, M., Seghi, G.A., Pei, P., Russell, P.M., Mumper, J., Mallery, S.R., 2010. Nanoparticles for local drug delivery to the oral mucosa. Pharm. Res. 27, 1224–1236.

Karakoti, A.S., Das, S., Thevuthasan, S., Seal, S., 2011. PEGylated inorganic nanoparticles. Angew. Chem. Int. Ed. 50, 1980–1994.

Kim, Y.J., Choi, S., Koh, J.J., Lee, M., Ko, K.S., Kim, S.W., 2001. Controlled release of insulin from injectable biodegradable triblock copolymer. Pharm. Res. 18, 548–550.

Lamprecht, A., Ubrich, N., Hombreiro, P.M., Lehr, C.M., Hoffman, M.M.P., 2000. Influences of process parameters on nanoparticle preparation performed by a double emulsion pressure homogenization technique. Int. J. Pharm. 196, 177–182.

Lee, Y., Chien, Y.W., 1995. Oral mucosa controlled delivery of LHRH by bilayer mucoadhesive polymer systems. J. Control. Release 37, 251–261.

Madhar, S.N.V., Shakya, A.K., Shakya, P., Singh, K., 2009. Orotransmucosal drug delivery systems: a review. J. Control. Release 140, 2–11.

Magenheima, B., Levya, M.Y., Benita, S., 1993. A new in vitro technique for the evaluation of drug release profile from colloidal carriers-ultrafiltration technique at low pressure. Int. J. Pharm. 94, 115–123.

Moghimi, S.M., Hunter, A.C., Murray, J.C., 2001. Long-circulating and target-specific nanoparticles: theory to practice. Pharmacol. Rev. 53, 283–318.

Mittal, G., Sahana, D.K., Bhardwaj, V., Kumar, M.N.V.R., 2007. Estradiol loaded PLGA nanoparticles for oral administration: effect of polymer molecular weight and copolymer composition on release behavior in vitro and in vivo. J. Control. Release 119, 77–85.

Mundargi, R.C., Rangaswamy, V., Aminabhavi, T.M., 2011a. Poly(N-vinylcaprolactam-co-methacrylic acid) hydrogel microparticles for oral insulin delivery. J. Microencapsul. 28, 384–394.

Mundargi, R.C., Babu, V.R., Rangaswamy, V., Patel, P., Aminabhavi, T.M., 2008. Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives. J. Control. Release 125, 193–209.

Mundargi, R.C., Vidhya, R., Aminabhavi, T.M., 2011b. pH-sensitive oral insulin delivery systems using Eudragit microspheres. Drug Dev. Ind. Pharm. 37, 977–985.

Ogawa, Y., Yamamoto, M., Okada, H., Takatsuka, Y., Tsugio, S., 1988. A new technique to efficiently entrap leuprolide acetate into microcapsules of polylactic acid or copoly(lactic/glycolic) acid. Chem. Pharm. Bull. 36, 1095–1103.

Panyam, J., Dali, M.M., Saho, S.K., Ma, W., Sudhir, C.S., Amidon, G.L., Levy, R.J., Labhasetwar, V., 2003. Polymer degradation and in vitro release of a model protein from poly(-lactide-co-glycolide) nano- and microparticles. J. Control. Release 92, 173–187.

Peppas, N.A., 2004. Devices based on intelligent biopolymers for oral protein delivery. Int. J. Pharm. 277, 11–17.

Rossi, S., Sandri, G., Caramella, C.M., 2005. Buccal drug delivery: a challenge already won? Drug Discov. Today: Technol. 2, 59–65.

Shan, X., Liu, C., Yuan, Y., Xu, F., Tao, X., Sheng, Y., Zhou, H., 2009. In vitro macrophage uptake and in vivo biodistribution of long-circulation nanoparticles with poly(ethylene-glycol)-modified PLA(BAB type) triblock copolymer. Colloids Surf. B: Biointerfaces 72, 303–311.

Soppimath, K.S., Aminabhavi, T.M., Kulkarni, A.R., Rudzinski, W.E., 2001. Biodegradable polymeric nanoparticles as drug delivery devices. J. Control. Release 70, 1–20.

Stark, W.J., 2011. Nanoparticles in biological systems. Angew. Chem. Int. Ed. 50, 1242–1258.

Sudhakar, Y., Kuotsua, K., Bandyopadhyay, A.K., 2006. Buccal bioadhesive drug delivery: a promising option for orally less efficient drugs. J. Control. Release 114, 15–40.

Sung, K.C., Han, R., Hub, O.Y.P., Hsu, L., 1998. Controlled release of nalbuphine prodrugs from biodegradable polymeric matrices: influence of prodrug hydrophilicity and polymer composition. Int. J. Pharm. 172, 17–25.

Tabata, Y., Takebayashi, Y., Ueda, T., Ikad, Y., 1993. A formulation method using D, L-lactic acid oligomer for protein release with reduced initial burst. J. Control. Release 23, 55–63.

Tobío, M., Gref, R., Sánchez, A., Langer, R., Alonso, M.J., 1998. Stealth PLA-PEG nanoparticles as protein carriers for nasal administration. Pharm. Res. 15, 270–275.

Tobío, M., Sanchez, A., Vila, A., Soriano, I., Evora, C., Vila-Jato, J.L., Alonso, M.J., 2000. The role of PEG on the stability in digestive fluids and in the vivo fate of PEG-PLA nanoparticles following oral administration. Colloids Surf. B: Biointerfaces 18, 315–323.

Verdun, C., Couvreur, P., Vranckx, H., Lenaerts, V., Roland, M., 1986. Development of a nanoparticle controlled-release formulation for human use. J. Control. Release 3, 205–210.

Yasugi, K., Nagasaki, Y., Kato, M., Kataoka, K., 1999. Preparation and characterization of polymer micelles from poly(ethylene glycol)–poly(D,L-lactide) block copolymers as potential drug carrier. J. Control. Release 62, 89–100.