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Effect of chitosan coating in overcoming the phagocytosis of insulin loaded solid lipid nanoparticles by mononuclear phagocyte system

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ABSTRACT

In this work, RAW 264.7 cell line was used to investigate the macrophage internalization of insulin-loaded SLN coated with chitosan. SLN were prepared by the w/o/w double emulsion method with different lipids and two different surfactants, Pluronic and Tween 80, following chitosan coating. Witepsol 85E based SLN were suitable for dimensions, sizing between 200 and 400 nm and insulin association efficiency was around 60% for both surfactants. Fluorescence microscopy confirmed that chitosan coated SLN were neglectfully internalized within RAW 264.7 cells as compared with uncoated SLN and with polystyrene latex nanoparticles as positive control, which were fully taken up. These results were quantitatively confirmed by flow cytometry assay. Chitosan-coated SLN demonstrated to be potentially able to prolong insulin blood levels and to avoid phagocytosis by MPS after intestinal uptake.

1. Introduction

Several attempts have been made to find alternative routes of insulin administration, avoiding injections and improving patient compliance (Cefalu, 2004; Ghilzai, 2003; Owens, 2002). The oral approach remains the most attractive one since it is non-invasive and is also physiologically desirable because the exogenous protein imitates the physiological pathway in which insulin is secreted into blood vessels that lead to hepatic portal circulation (Saffran, Pansky, Budd, & Williams, 1997).

As other peptides, insulin shows a poor physical and chemical stability and relatively short plasma half-time. Low pH and protease hydrolysis have demonstrated to be important limitations to intestinal absorption of intact insulin and transport into systemic circulation that is reduced also for the large molecular weight and the hydrophilicity of the molecule (Carino & Mathiowitz, 1999).

Among nanoparticulate systems, solid lipid nanoparticles (SLN) gained increased attention during the last years for their good tolerability and biocompatibility, biodegradation and improved bioavailability of protein drugs (Almeida & Souto, 2007; Sarmento, Martins, Ferreira, & Souto, 2007). SLN are able to improve protein stability against enzymatic degradation in the intestinal environment, to enhance their selective uptake, resulting in the increase of the duration of the therapeutic effect (Sarmento et al., 2007). SLN have been therefore proposed for the delivery of insulin in the treatment of Diabetes (Liu, Gong, Wang, Zhong, & Zhang, 2007; Sarmento et al., 2007; Trotta, Cavalli, Carlotti, Battaglia, & Debernardi, 2005; Zhang et al., 2006). Lipid-based drug delivery systems, such as SLN, are in fact known to facilitate oral absorption of many drugs (Charman, 2000; Charman, Porter, Mithani, & Dressman, 1997). They also allow administration through non-parenteral routes (Almeida & Souto, 2007). Orally administered SLN, like other nanoparticles, can be absorbed both transcellularly, and through the membranous epithelial cells (M-cells) of the Peyer's patches in the gut-associated lymphoid tissue (GALT) (Damge, Reis, & Maincent, 2008).

In order to improve the gastrointestinal uptake of insulin contained into nanoparticles, a promising strategy is the use of multifunctional polymers, such as chitosan, which exhibit permeation enhancing and mucoadhesive properties. Chitosan has been described as biocompatible, biodegradable and mucoadhesive (Muzzarelli, 2009; Muzzarelli & Muzzarelli, 2006; Muzzarelli et al., 2006), enabling numerous pharmaceutical and biomedical applications. Chitosan increases the stability of nanospheres, minimizes

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the loss of encapsulated material, exerts mucoadhesive properties and facilitates drug absorption by localizing drug residence in the gut. Mucoadhesion properties can promote drug adsorption because the contact with intestinal epithelium is kept for extended periods and the penetration of active drug through and between cells is improved due to the prolonged concentration gradient between nanoparticles and intestinal membrane. Chitosan is moreover an effective permeability enhancer because it reversibly alters the tight junctions (Cano-Cebrian, Zornoza, Granero, & Polache, 2005; Smith, Dornish, & Wood, 2005).

In our group, we have recently demonstrated the feasibility of chitosan-coating SLN encapsulating insulin to improve the intestinal insulin absorption through the confirmation of its physiologic effect after oral administration (Fonte, Nogueira, Gehm, Ferreira, & Sarmento, submitted for publication; Teixeira et al., 2009). Compared to uncoated SLN, we were able to reach a significant improvement of hypoglycemic effect, mostly due to the mucoadhesive properties of chitosan, promoting the intestinal insulin permeation, as well as overcoming insulin degradation in the gastrointestinal tract. Moreover, it is expected that chitosan-coated SLN can also be taken up by Payer's patches and conducted to the circulation, releasing insulin in a sustained manner, thus justifying the prolonged effect of insulin. However, a major limitation facing the oral delivery of nanoparticles carrying insulin, besides the rate of nanoparticles passing through the gastrointestinal tract, is the elimination of nanoparticles by the mononuclear phagocyte system (MPS) (Champion, Walker, & Mitragotri, 2008; Yin, Chen, Qiao, Wei, & Hu, 2007). Additional elimination could also be performed by macrophages founded in tissues such as bone marrow, liver, spleen, and lymph nodes.

The role of PEG coating (Owens & Peppas, 2006) and of polysaccharide coating (Lemarchand, Gref, & Couvreur, 2004) in the nanoparticle phagocytosis by macrophages are reported in the literature, although little specific evidence is given concerning chitosan and in particular chitosan coating of SLN.

After demonstrating that chitosan-coating SLN could improve absorption of insulin entrapped into SLN (Fonte et al., submitted for publication; Teixeira et al., 2009), the specific aim of the study presented in this article was to investigate the ability of chitosan-coated SLN to overcome phagocytosis by the cells of the mononuclear phagocytic system (MPS) after intestinal uptake, using *in vitro* the RAW 264.7 macrophage cell line.

The method of w/o/w double emulsion technique was selected to prepare SLN because it is mild and avoids possible degradation of insulin due to high temperatures. Among the formulative parameters evaluated to prepare SLN with suitable diameter, zeta potential and association efficiency, ultra-turrax and sonication have been compared as dispersion methods, Witepsol 85E, Compritol 888 ATO and Precirol ATO 5 have been considered as bases of lipid matrix, Pluronic and Tween 80 have been compared as stabilizing surfactants. SLN have been further coated with chitosan. Fluorescent-labeled SLN were prepared either by loading with Fluorescein-isothiocyanate (FITC)–insulin or by coating with FITC–chitosan. Their uptake by RAW 264.7 macrophage cell line was assessed by fluorescence microscopy and flow cytometry.

2. Materials and methods

Witepsol 85E (Tri-glycerides of C 10–C 18 saturated fatty acids), Compritol 888 ATO (glycerol dibehenate EP) and Precirol ATO 5 (glyceryl mono-/di-/tri-palmitate/stearate) lipid matrixes were a gift from Gattefossé (France). Tween 80 and Pluronic F127 used as stabilizing surfactants were from Sigma (Portugal). RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection (ATCC, USA). Low molecular weight chitosan, fluorescein 5(6)-isothiocyanate (FITC) and FITC–insulin from bovine pancreas were purchased from Sigma (Portugal). Alexa Fluor 594 conjugate of WGA was obtained from Molecular Probes (Oregon, USA). FluoSpheres® carboxylated nanospheres (200 nm) were from Invitrogen (Spain).

2.1. Preparation of FITC-labeled chitosan

Chitosan (50 kDa, 100 mg) was dissolved in 10 mL of acetic acid 1% (w/v) overnight with constant stirring. Methanol (10 mL) was added to the chitosan solution after vacuum filtration. FITC (7 mg) was dissolved in methanol (to a concentration of 2.0 mg/mL) and added to the filtrate. After stirring at room temperature for 3 h, in the dark, the mixture was precipitated with NaOH 1 M, centrifuged and washed with methanol until the free FITC could not be detected in the supernatant. The resulting FITC-labeled chitosan was then lyophilized.

2.2. Preparation and characterization of nanoparticles

Nanoparticles were prepared by an adapted w/o/w double emulsion technique (Sarmento et al., 2007). 200 mg of lipid matrix (either Witepsol 85E, Compritol 888 ATO or Precirol ATO 5) was dissolved in 2 mL of dichloromethane, 1 mg of insulin was dissolved in 0.2 mL of HCL 0.1 M and then the insulin solution was added to the lipid solution and homogenized for 30 s in ultra-turrax T25 at 20,000 rpm (IKA-Labortechnik, Germany) or by sonication at 70% output intensity (VibraCell model VCX 130W equipped with a 6 mm probe, Sonics & Materials, Inc., Newtown, CT, USA). The primary emulsion was poured into 20 mL of 2% Pluronic or Tween 80 solution and homogenized for additional 30 s. The dichloromethane was removed by natural evaporation under magnetic stirring. To coat SLN with chitosan, SLN were transferred to 20 mL of a 0.5% (w/w) chitosan solution in acetic acid 1% (w/v) containing 2% surfactant as secondary aqueous solution and physical adsorption of chitosan promoted under magnetic stirring until solvent removal, resulting in a final colloidal dispersion of pH around 4.

Measurements of particle size were performed by photon correlation spectroscopy (PCS). Samples were diluted with Milli-Q-water to suitable concentration and the size was measured with using a ZetaSizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). All measurements were performed in triplicate.

The electrophoretic mobility was measured by Laser Doppler Anemometry (LDA) using a ZetaSizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Samples were diluted with Milli-Q-water having a conductivity adjusted to 50 μS/cm by addition of a 0.9% NaCl solution.

2.3. HPLC insulin analysis

Insulin concentration was determined by HPLC (Sarmento, Ribeiro, Veiga, & Ferreira, 2006). Briefly, the mobile phase of the chromatographic method consisted of acetonitrile and 0.1% TFA aqueous solution initially set in the ratio 30:70 (v/v), which was linearly changed to 40:60 (v/v) over 5 min. From 5 to 10 min the ratio 40:60 (v/v) was kept constant. Eluent was pumped at a flow rate of 1 mL/min, the injection volume was 20 μL and detection wavelength was 214 nm. The HPLC system was equipped with an XTerra RP 18 column, 5 μm particle size, 4.6 mm internal diameter × 250 mm length (Waters®, USA) and a LiChrospher® 100 RP-18, 5 μm particle size guard column (Merck, Germany). All experiments occurred at room temperature and the total area of peak was used to quantify the insulin.

For the preparation of the calibration curves, five human insulin standard solutions were prepared at concentrations of 3, 6, 12.5, 25, and 100 μg/mL in USP XXVI acetate buffer pH 4.7.

2.4. Insulin association efficiency

The association efficiency (AE) was determined indirectly. The amount of insulin entrapped into SLN was calculated by the difference between the total amount used to prepare the systems and the amount of insulin remained in the aqueous phase after SLN isolation. After the preparation, aqueous SLN dispersion was centrifuged (UL 80 ultracentrifuge, rotor type 55-S, Beckman Instruments, German) for 45 min at 50,000 rpm (corresponding to approx. 200,000 × *g*). Insulin concentration in the supernatant was determined by HPLC as described before. Association efficiency was calculated according to the formula:

$$AE = \frac{(\text{total amount of insulin} - \text{free insulin in supernatant}) \times 100}{\text{total amount of insulin}}$$

2.5. Cell culture

RAW 264.7 macrophage cells were maintained in complete Dulbecco's Modified Eagle's Medium (DMEM, Gibco™, Invitrogen Corporation) supplemented with 10% fetal bovine serum (FBS), 1% glutamine (2 mM) and 1% antibiotic-antimycotic (Gibco™, Invitrogen Corporation: 10,000 units/mL penicillin G sodium, 10,000 mg/mL streptomycin sulfate and 25 mg/mL amphotericin B as Fungizone™, in 0.85% saline) in a humidified incubator (Heraeus Hera Cell incubator) at 37 °C under 5% CO_2 atmosphere. Cells were subcultured every 3–4 days using a rubber cell scrapper.

2.6. Cellular uptake assays

For experiments, 1 mL of cell suspension (1×10^5 cells/mL) was seeded onto coverslips (22 mm × 22 mm), whereas a cell suspension of 5×10^5 cells/well was seeded in 6 well culture plates and grown overnight in complete culture medium.

Uptake experiments were initiated by removing the medium, replacing it by nanoparticle solution followed by incubation at 37 °C. Nanoparticles suspension was diluted 10× in complete culture medium before incubation with macrophages. At predetermined times, the cells were washed three times with PBS pH 7.4 and samples were taken.

To control the phagocytic capacity of the cells, 1 mL of FluoSpheres® carboxylated nanospheres, in a 0.2% solids concentration in complete culture medium, was added to each well and incubated at 37 °C. In order to avoid nanospheres adsorption to the proteins of the cellular membrane before incubation they were pretreated with bovine serum albumine (BSA) 1% for 1 h, then centrifuged at 13,000 rpm for 10 min and washed with PBS. After exposing the cells to the nanospheres, the same washing procedure was followed.

2.7. Fluorescence microscopy and flow cytometry

For cellular membrane labelling, after the uptake assay, 1 mL of a 1 μg/mL solution of Alexa Fluor 594 conjugate of WGA was added to each well and incubated for 10 min.

The coverslips were then washed with PBS and loaded onto microscope slides. Cellular imaging was captured under a Nikon Eclipse TE 2000 U inverted fluorescence microscope (60× oil immersion lens) equipped by digital camera and controlled through Nikon ACT-1 software.

After the uptake assay, cells from each sample were scraped from the wells and collected in PBS solution (1 mL/sample) for flow cytometry assays (Beckman Coulter Epics XL). At least 5000 cells were counted for each sample.

3. Results and discussion

3.1. SLN characterization

Different formulations were prepared regarding the most suitable size and zeta potential properties. In general, the sonication originated SLN with lower mean particle size compared to ultra-turrax methodology, ranging from 150 nm for Witepsol/Pluronic SLN to 412 nm to Compritol/Pluronic SLN. The same formulations prepared by ultra-turrax double emulsion ranged from 210 nm for Witepsol/Pluronic SLN to 712 nm to Precirol/Tween SLN. On the other hand, Pluronic seems more efficient in stabilizing the systems that have lower particle size with respect to those obtained with Tween. In all cases, the obtained SLN were negatively charged, with zeta potential values between −15 and −25 mV.

A second step was to introduce chitosan to SLN to improve the interaction (mucoadhesion and internalization) across mucus epithelia and to avoid the internalization by macrophage cells. The coating with chitosan is clearly identified due to the increase of mean particle size and reversion of negative to positive zeta potential values for all developed formulations, as summarized in Table 1. Mean particle dimensions increased, probably due to a certain degree of aggregation, following the presence of polymer chains at the surface. So, it is possible to state that when SLN are coated with chitosan, nanoparticles tend to agglomerate each other due to the high density of chitosan that may coalesce with more than one lipid nanoparticle, and the mean size of SLN increases. The dimensions are still, in most cases, in the nanometer range with values compatible with possible positive effect on gastrointestinal absorption (Damge et al., 2008).

The microscopic appearance and the structural characterization of SLN showed before that these particles exhibited spherical shape and dense lipid matrix (Teixeira et al., 2009). After coating, fluffy surface layer due to chitosan deposition was noted and some aggregation (Teixeira et al., 2009), which may justify the increase of mean particle size for chitosan-coated SLN. Nevertheless, chotosan-coated SLN were easily resuspended, indicating that partial aggregation is reversible.

The effect of surfactant varied according to the lipid used. In the case of Precirol-based SLN, a smaller mean diameter is shown when the sonication stirring method is used. The best surfactant in terms of particle size resulted to be Pluronic for each method of homogenization. The size of SLN prepared with Compritol, and based on Tween rather than Pluronic as surfactant, is around 500 nm when ultra-turrax T25 is used as high-speed stirring, but higher than 1000 nm for sonication. Witepsol-based SLN resulted in the smallest particle size formulation, irrespective of the emulsion methodology. The mean particle size of these SLN was smaller in the presence of Pluronic rather than Tween. Nevertheless, the surfactant is believed not to be present on the final formulation, instead mostly removed due to chitosan coating at the final stage.

Insulin association efficiency was significantly high for all the systems, ranging between 39 and 66%, resulting in final loading between 0.3 and 0.5% (w/w). The Witepsol-based SLN are in particular characterized by association efficiency among the highest under all the experimental set conditions.

After nanoparticles characterization and optimization, Witepsol-based SLN prepared by the sonication stirring method, were chosen due to their mean particle size (<400 nm; 200 nm for SLN prepared with Pluronic and 400 for SLN prepared with Tween), to perform the studies on cell substrates RAW 264.7 by fluorescence microscopy and flow cytometry uptake assays. SLN were all fluorescent labeled by using alternatively FITC–insulin and FITC–chitosan.

Table 1
Characterization of insulin-loaded chitosan-coated SLN obtained with ultra-turrax T25 homogenization and sonication methods.

Matrix/surfactant	Homogenization			Sonication		
	Diameter (nm ± SD)	Zeta potential (mV ± SD)	AE (% ± SD)	Diameter (nm ± SD)	Zeta potential (mV ± SD)	AE (% ± SD)
Witepsol/Pluronic	240 ± 11	25.0 ± 1.0	52.4 ± 2.5	191 ± 24	28.2 ± 1.0	57.3 ± 0.2
Witepsol/Tween	470 ± 18	11.0 ± 0.9	66.2 ± 0.5	404 ± 7	37.5 ± 1.0	52.4 ± 0.4
Compritol/Pluronic	483 ± 315	27.2 ± 1.1	42.5 ± 1.4	454 ± 52	35 ± 1.2	49 ± 2.5
Compritol/Tween	520 ± 8	25.0 ± 1.5	39.1 ± 1.9	538 ± 169	17.1 ± 2.4	61.6 ± 0.9
Precirol/Pluronic	791 ± 100	22.6 ± 2.6	40 ± 0.2	446 ± 49	39.5 ± 1.0	41.4 ± 2.2
Precirol/Tween	2200 ± 243	17.4 ± 1.6	39 ± 1.6	1100 ± 61	19.3 ± 1.2	48.9 ± 1.3

3.2. Fluorescence microscopy

In order to confirm the presence of phagocytosed nanoparticles, during uptake assays, macrophage cells were incubated with SLN and were analyzed by fluorescence microscopy (Fig. 1a). After 4 h of incubation it was not seen green fluorescence inside and outside the cells, while the macrophage cell membrane appears in red. These SLN were used as a negative control of the phagocytosis process to confirm that the lipid matrix does not present native fluorescence that potentially could interfere with fluorescein. FluoSpheres® carboxylated nanospheres sizing 200 nm with green fluorescence were used as a positive control of the phagocytosis process. (Fig. 1b). After 4 h of incubation a strong green fluorescence was observed inside the cells, corresponding to a large number of nanospheres that were internalized. FITC–insulin in solution was also slightly internalized by macrophages after 4 h (Fig. 1c), and such internalization was observed immediately after incubation, after 30 min. The internalization of FITC–insulin had also been demonstrated before (Wang, Wang, Liu, & Barrett, 2008) in bovine aortic endothelial cells, although in that case insulin uptake required intact insulin signaling via both the PI 3-kinase and MEK signaling cascades and the cSrc-family tyrosine kinases, in a sensitive-based uptake to cytokine-induced insulin resistance. For macrophages uptake, it is more likely to be related with a non specific phagocytosis due to FITC moiety.

Nanoparticles with FITC–insulin or FITC–chitosan have green fluorescence, while the macrophage cell membrane appears in red, since it was stained with Alexa Fluor WGA.

As it is shown in Figs. 2 and 3, after 4 h of incubation, macrophages did not internalize any of the chitosan coated SLN formulations. In particular for insulin-loaded FITC–chitosan coated Witepsol/Tween SLN, some green fluorescence was visible outside the cells or close to the apical membrane, but not inside.

On the other hand, uncoated SLN were taken up by macrophages, as can be detected in Figs. 2a and 3a. This indicates that the presence of chitosan is important to avoid the uptake of SLN by macrophages camouflaging nanoparticles from the MPS. It is well known that a method of imparting stealth, or sterically stabilized properties to nanoparticles is through the hydrophilization of the surface of nanoparticles (Owens & Peppas, 2006), which is provided by chitosan.

Physical and chemical properties of the nanoparticles, including particle size and surface hydrophilicity, are important parameters to determine the long-term circulation on the blood. Particles sizing in the range of 200–500 nm has been reported as potentially more long-circulating (Gaumet, Vargas, Gurny, & Delie, 2008). Particles smaller than 100 nm are eliminated by renal excretion, while larger particles can be rapidly taken up by the MPS cells present in the liver, the spleen, and to a lesser extent, in the bone marrow (Gaumet et al., 2008). Moreover, the opsonization of hydrophobic, negative nanoparticles, as compared to hydrophilic, neutral nanoparticles, may occur more quickly due to the enhanced adsorption of opsonins on their surfaces (Sheng et al., 2009). Negative charged nanoparticles with lower zeta potential values are more prone to phagocytosis (Gbadamosi, Hunter, & Moghimi, 2002). Surface hydrophilicity of nanoparticles may also be responsible for suppressing plasma proteins approach. The opsonization existing between the complement proteins involved on the opsonization and the nanoparticles are more favorable to occur on the hydrophobic surfaces than on the hydrophilic ones (Vonarbourg, Passirani, Saulnier, & Benoit, 2006). SLN when coated with chitosan possess surface with high affinity to water due to the hydrophily of chitosan, helping to introduce hydrophilic moieties that camouflage SLN and make them invisible to phagocytic. Thus, it would be expected, as founded in the present work that chitosan coated SLN, less hydrophobic and positively charged, could be taken up less efficiently that uncoated SLN.

3.3. Flow cytometry and uptake assays

Since cells do not exhibit significant fluorescence on their own, it is assumed that the fluorescence levels measured in cells are due to fluorescence taken up.

In Fig. 4 the internalization of FluoSpheres® carboxylated nanospheres in macrophages is quantified by means of flow cytometry assay, serving as positive control.

Fig. 5 presents histograms of flow cytometry assays in macrophage cells after 1 h incubation with, on the left, SLN pre-

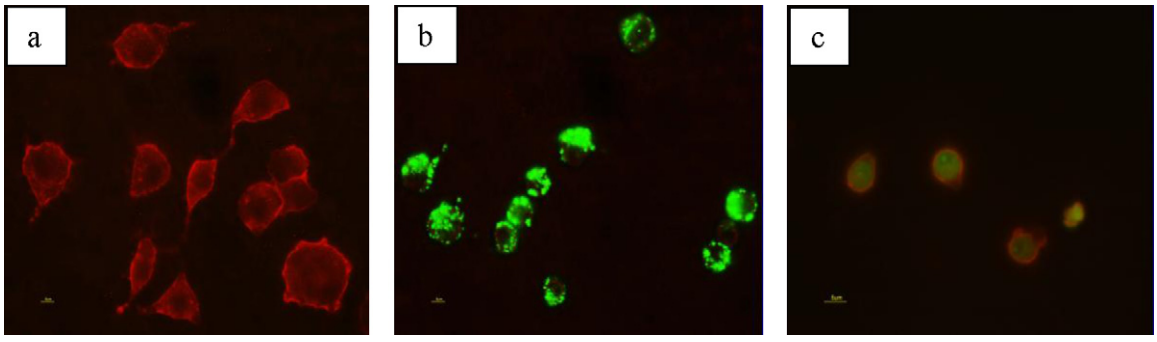

Fig. 1. Fluorescence microscopy images of macrophage cells incubated with SLN (a), polystyrene latex nanoparticles (b) and FITC–insulin in solution after 4 h of incubation (c) (magnification 60×).

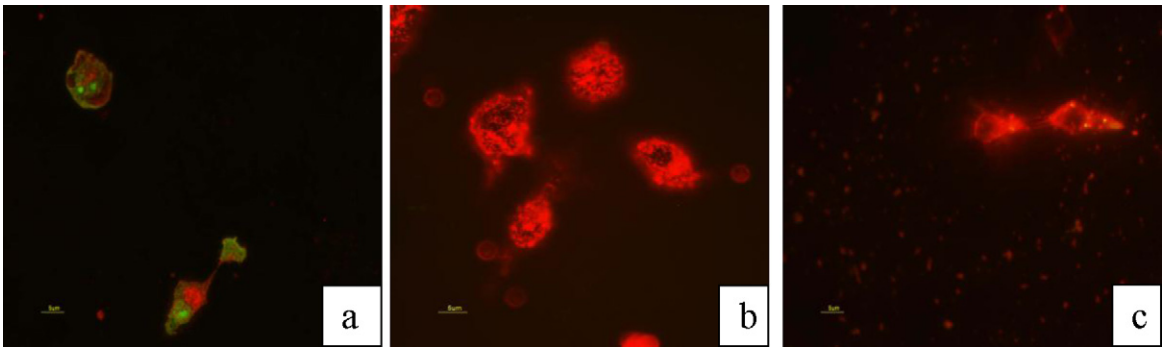

Fig. 2. Fluorescence microscopy images of macrophage cells incubated with FITC–insulin loaded Witepsol/Pluronic SLN (a), FITC–insulin loaded chitosan coated Witepsol/Pluronic SLN (b) and insulin-loaded FITC–chitosan coated Witepsol/Pluronic SLN (c) (magnification 60×).

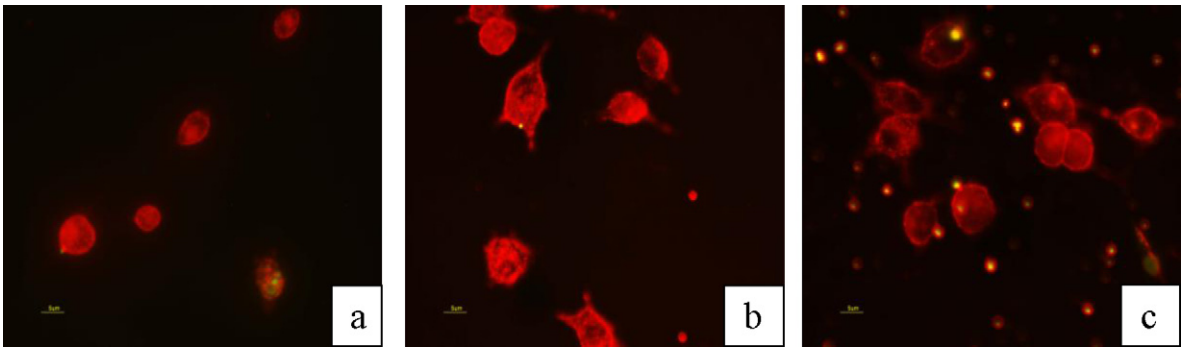

Fig. 3. Fluorescence microscopy images of macrophage cells incubated with FITC–insulin loaded Witepsol/Tween SLN (a), FITC–insulin loaded chitosan coated Witepsol/Tween SLN (b) and insulin-loaded FITC–chitosan coated Witepsol/Tween SLN (c) (magnification 60×).

pared with Pluronic, and on the right, SLN prepared with Tween as surfactant. It is possible to appreciate, with respect to the samples without chitosan (Fig. 5c and f), a shift of the peak towards higher FL1 values, indicating higher amount of fluorescent cells counted and therefore higher internalization. These results further confirmed what previously observed in the fluorescence microscopy observations, as for both the samples coated with chitosan the internalization is lower than for the corresponding uncoated nanoparticles.

To better compare these data, the SLN uptake values expressed as relative fluorescence, that is the total fluorescence in the cells after incubation with the FITC–insulin samples calculated as relative percentage of the positive control (fluorescence of the cells incubated with FluoSpheres® carboxylated nanospheres) assumed 100%. For both the tested systems, with Pluronic and with Tween, the relative fluorescence results of less than 1 and 2%, respectively, clearly indicated that the SLN coated with chitosan are less easily internalized in macrophage cell line. All the experimental results in

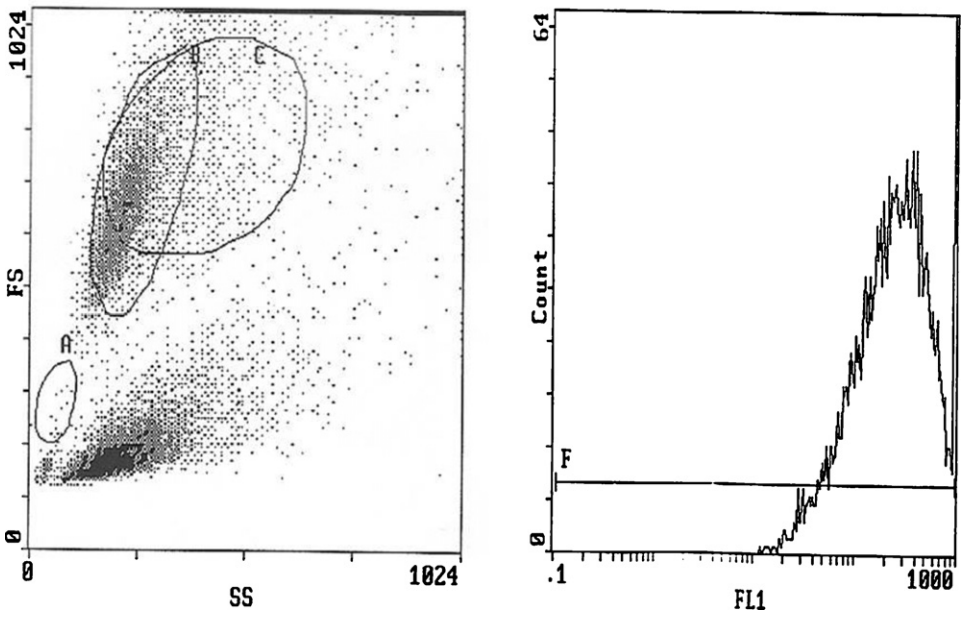

Fig. 4. Histogram of flow cytometry assays in macrophage cells after 1 h incubation with FluoSpheres® carboxylated nanospheres. The histogram on the left represents side (SS) versus size of the cell (forward scatter, FS) and on the right represents fluorescence intensity (FL1) versus cell count (Count).

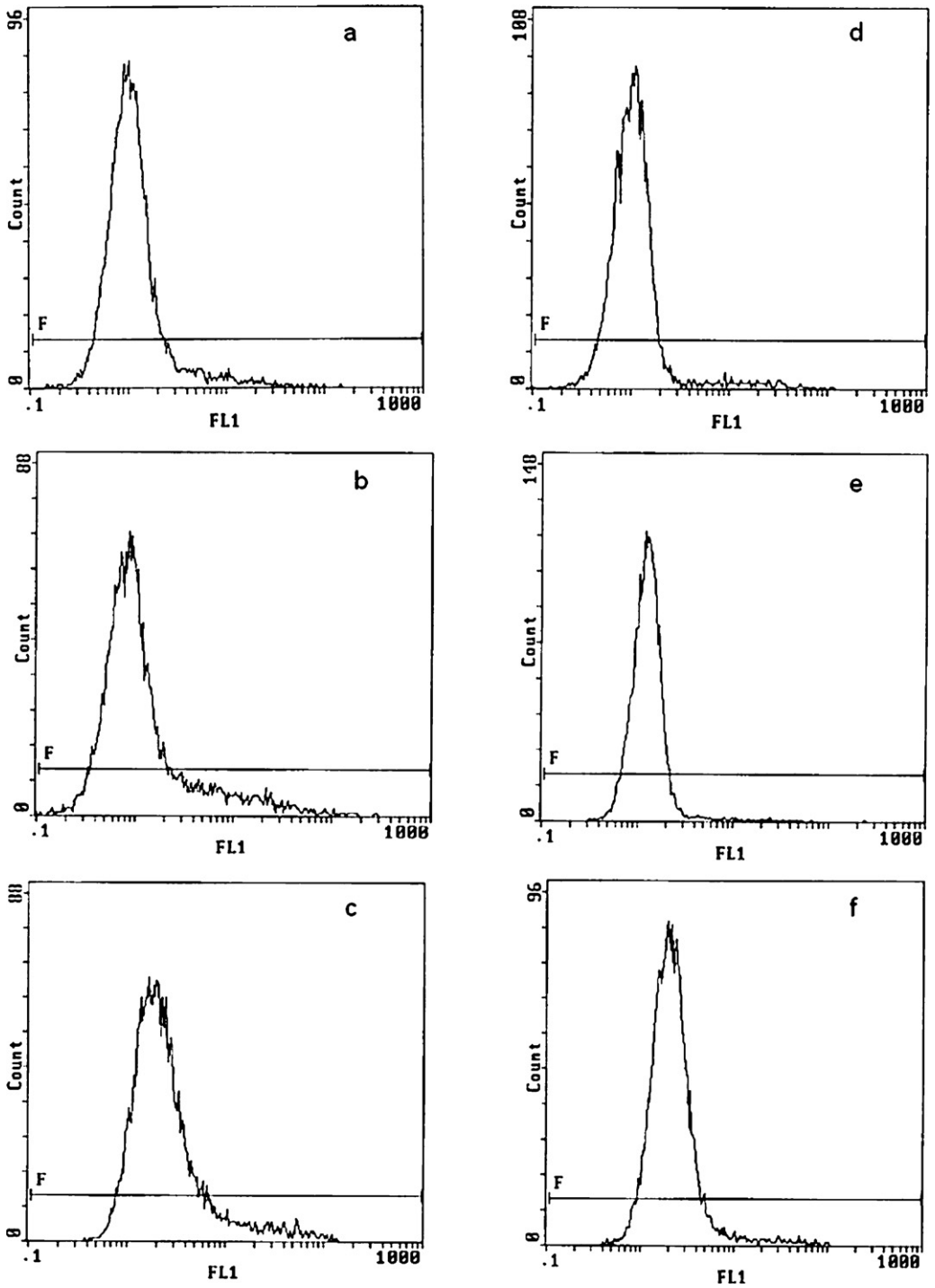

Fig. 5. Histograms of flow cytometry assays in macrophage cells after 1 h incubation with FITC–insulin loaded chitosan coated Witepsol/Pluronic SLN (a), insulin-loaded FITC–chitosan coated Witepsol/Pluronic SLN (b) and FITC–insulin loaded Witepsol/Pluronic SLN (c), FITC–insulin loaded chitosan coated Witepsol/Tween SLN (d), insulin-loaded FITC–chitosan coated Witepsol/Tween SLN (e) and FITC-insulin loaded Witepsol/Tween SLN (f). The histograms represent fluorescence intensity (FL1) versus cell count (Count).

RAW 264.7 cell line indicated therefore that chitosan coating was effective in reducing macrophage uptake of SLN.

4. Conclusion

Solid lipid nanoparticles prepared by Witepsol as lipid core and by Pluronic or Tween as surfactant in the second aqueous phase, resulted suitable for particle size and insulin association efficiency to gastrointestinal absorption. SLN with mean particle size ranging between 200 and 400 nm, with insulin AE up to 66% were prepared and further coated with chitosan. Zeta potential values after chitosan adsorption on the nanoparticles indicated effective coating by the polymer. Results from uptake assays in a macrophage cell line RAW 264.7 showed that the chitosan-coated SLN were not internalized by these cells, in contrast with uncoated SLN. Chitosan was able to provide stealth properties to SLN resulting in the

absence of phagocytosis by escaping to macrophage uptake. Such findings open perspectives for the optimization of long-time blood circulation chitosan-based nanoparticles.

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References

Almeida, A. J., & Souto, E. (2007). Solid lipid nanoparticles as a drug delivery system for peptides and proteins. *Advanced Drug Delivery Reviews*, *59*(6), 478–490.

Cano-Cebrian, M. J., Zornoza, T., Granero, L., & Polache, A. (2005). Intestinal absorption enhancement via the paracellular route by fatty acids, chitosans and others: A target for drug delivery. *Current Drug Delivery*, *2*(1), 9–22.

Carino, G. P., & Mathiowitz, E. (1999). Oral insulin delivery. *Advanced Drug Delivery Reviews*, *35*, 249–257.

Cefalu, W. T. (2004). Concept strategies, and feasibility of noninvasive insulin delivery. *Diabetes Care*, *27*, 239–246.

Champion, J., Walker, A., & Mitragotri, S. (2008). Role of particle size in phagocytosis of polymeric microspheres. *Pharmaceutical Research*, *25*(8), 1815–1821.

Charman, W. N. (2000). Lipids, lipophilic drugs, and oral drug delivery—Some emerging concepts. *Journal of Pharmaceutical Sciences*, *89*(8), 967–978.

Charman, W. N, Porter, C. J. H., Mithani, S., & Dressman, J. B. (1997). Physicochemical and physiological mechanisms for the effects of food on drug absorption: The role of lipids and pH. *Journal of Pharmaceutical Sciences*, *86*(3), 269–282.

Damge, C., Reis, C. P., & Maincent, P. (2008). Nanoparticle strategies for the oral delivery of insulin. *Expert Opinion on Drug Delivery*, *5*(1), 45–68.

Fonte, P., Nogueira, T., Gehm, C., Ferreira, D., & Sarmento, B. Chitosan as mucoadhesive agent to enhance oral absorption of insulin entrapped into solid lipid nanoparticles, submitted for publication.

Gaumet, M., Vargas, A., Gurny, R., & Delie, F. (2008). Nanoparticles for drug delivery: The need for precision in reporting particle size parameters. *European Journal of Pharmaceutics and Biopharmaceutics*, *69*(1), 1–9.

Gbadamosi, J. K., Hunter, A. C., & Moghimi, S. M. (2002). PEGylation of microspheres generates a heterogeneous population of particles with differential surface characteristics and biological performance. *FEBS Letters*, *532*(3), 338–344.

Ghilzai, N. M. K. (2003). New developments in insulin delivery. *Drug Development and Industrial Pharmacy*, *29*, 253–265.

Lemarchand, C., Gref, R., & Couvreur, P. (2004). Polysaccharide-decorated nanoparticles. *European Journal of Pharmaceutics and Biopharmaceutics*, *58*, 327–341.

Liu, J., Gong, T., Wang, C., Zhong, Z., & Zhang, Z. (2007). Solid lipid nanoparticles loaded with insulin by sodium cholate-phosphatidylcholine-based mixed micelles: Preparation and characterization. *International Journal of Pharmaceutics*, *340*(1–2), 153–162.

Muzzarelli, R., & Muzzarelli, C. (2006). Chitosan as a dietary supplement and a food technology agent. In *Functional food carbohydrates*. CRC Press.

Muzzarelli, R. A. A. (2009). Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. *Carbohydrate Polymers*, *76*(2), 167–182.

Muzzarelli, R. A. A., Orlandini, F., Pacetti, D., Boselli, E., Frega, N. G., Tosi, G., et al. (2006). Chitosan taurocholate capacity to bind lipids and to undergo enzymatic hydrolysis: An in vitro model. *Carbohydrate Polymers*, *66*(3), 363–371.

Owens, D. E., & Peppas, N. A. (2006). Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. *International Journal of Pharmaceutics*, *307*(1), 93–102.

Owens, D. R. (2002). New horizons—Alternative routes for insulin therapy. *Nature Reviews Drug Discovery*, *1*, 529–540.

Saffran, M., Pansky, B., Budd, G. C., & Williams, F. E. (1997). Insulin and the gastrointestinal tract. *Journal of Controlled Release*, *46*(1–2), 89–98.

Sarmento, B., Martins, S., Ferreira, D., & Souto, E. B. (2007). Oral insulin delivery by means of solid lipid nanoparticles. *International Journal of Nanomedicine*, *2*(4), 743–749.

Sarmento, B., Ribeiro, A., Veiga, F., & Ferreira, D. (2006). Development and validation of a rapid reversed-phase HPLC method for the determination of insulin from nanoparticulate systems. *Biomedical Chromatography*, *20*(9), 898–903.

Sheng, Y., Yuan, Y., Liu, C., Tao, X., Shan, X., & Xu, F. (2009). In vitro macrophage uptake and in vivo biodistribution of PLA-PEG nanoparticles loaded with hemoglobin as blood substitutes: Effect of PEG content. *Journal of Materials Science: Materials in Medicine*, *20*(9), 1881–1891.

Smith, J. M., Dornish, M., & Wood, E. J. (2005). Involvement of protein kinase C in chitosan glutamate-mediated tight junction disruption. *Biomaterials*, *26*(16), 3269–3276.

Teixeira, J., Gehm, C., Julio, A., Castro, C., Fonseca, J., Martins, S., et al. (2009). Chitosan as mucoadhesive agent to enhance absorption of therapeutic proteins encapsulated into solid lipid nanoparticles. In *Technical Proceedings, Nanotech Conference & Expo 2009, Vol. 2* (pp. 111–114).

Trotta, M., Cavalli, R., Carlotti, M. E., Battaglia, L., & Debernardi, F. (2005). Solid lipid micro-particles carrying insulin formed by solvent-in-water emulsion/diffusion technique. *International Journal of Pharmaceutics*, *288*(2), 281–288.

Vonarbourg, A., Passirani, C., Saulnier, P., & Benoit, J.-P. (2006). Parameters influencing the stealthiness of colloidal drug delivery systems. *Biomaterials*, *27*(24), 4356–4373.

Wang, H., Wang, A. X., Liu, Z., & Barrett, E. J. (2008). Insulin signaling stimulates insulin transport by bovine aortic endothelial cells. *Diabetes*, *57*(3), 540–547.

Yin, Y., Chen, D., Qiao, M., Wei, X., & Hu, H. (2007). Lectin-conjugated PLGA nanoparticles loaded with thymopentin: Ex vivo bioadhesion and in vivo biodistribution. *Journal of Controlled Release*, *123*(1), 27–38.

Zhang, N., Ping, Q., Huang, G., Xu, W., Cheng, Y., & Han, X. (2006). Lectin-modified solid lipid nanoparticles as carriers for oral administration of insulin. *International Journal of Pharmaceutics*, *327*, 153–159.