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Title: Oral insulin delivery, the challenge to increase insulin bioavailability: influence of surface charge in nanoparticle system

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Abstract

Oral administration of insulin increases patient comfort and could improve glycemic control thanks to the hepatic first passage. However, challenges remain. The current approach uses poly (D, lactic-co-glycolic) acid (PLGA) nanoparticles (NPs), an effective drug carrier system with a long acting profile. However, this system presents a bioavailability of less than 20% for insulin encapsulation. In this context, physico-chemical parameters like surface charge could play a critical role in NP uptake by the intestinal barrier. Therefore, we developed a simple method to modulate NP surface charge to test its impact on uptake in vitro and finally on NP efficiency in vivo. Various NPs were prepared in the presence (+) or absence (-) of polyvinyl alcohol (PVA), sodium dodecyl sulfate (SDS), and/or coated with chitosan chloride. In vitro internalization was tested using epithelial culture of Caco-2 or using a co-culture (Caco-2/RevHT29MTX) by flow cytometry. NPs were then administered by oral route using a pharmaceutical complex vector (100 or 250 UI/kg) in a diabetic rat model. SDS-NPs (-42 ± 2 mV) were more negatively charged than -PVA-NPs (-22 ± 1 mV) and chitosan-coated NPs were highly positively charged (56 ± 2 mV) compared to +PVA particles (-2 ± 1 mV), which were uncharged. In the Caco-2 model, NP internalization was significantly improved by using negatively charged NPs (SDS NPs) compared to using classical NPs (+PVA NPs) and chitosan-coated NPs. Finally, the efficacy of insulin SDS-NPs was demonstrated in vivo (100 or 250 UI insulin/kg) with a reduction of blood glucose levels in diabetic rats. Formulation of negatively charged NPs represents a promising approach to improve NP uptake and insulin bioavailability for oral delivery.

Key words: Diabetes, Oral administration, Insulin, Nanoparticle, Charge, Bioavailability
1. **Introduction**

In recent years, advances have been made in insulin administration for patients with type 1 diabetes to increase patient compliance. However, trauma and discomfort associated with injections lead to poor adherence to the treatment. Various routes of insulin administration have been developed to improve insulin treatment: nasal (Nazar and Tsibouklis, 2012), ocular (Xuan et al., 2005), buccal (Boateng et al., 2014), and oral (Sheng et al., 2015). Oral administration is the best candidate to increase patient comfort (no injection or no device is needed) and to control glucidic homeostasis with a hepatic first passage (Dal et al., 2015), mimicking the physiological path of endogenous insulin secretion. However, oral delivery presents multiple barriers (physical, chemical, and biological) along the digestive tract such as the stomach and its acid pH, the intestine and its digestive enzymes, and the intestinal barrier with the mucus, which leads to a very low oral bioavailability of proteins such as insulin (Foss and Peppas, 2004).

Many strategies have been developed to increase insulin delivery in the bloodstream after oral administration like chemical structure modification (Naibo Yin, 2014), enzyme inhibition (Agarwal et al., 2001), permeation enhancers (Aungst, 2000), and encapsulation systems (Wu et al., 2012). Polymeric nanoparticle (NP) is a promising system to encapsulate drugs and protect them from pH variations and enzymatic attacks, allowing their direct release in the blood. The first step consists in the choice of polymer used to formulate NPs. Chitosan/tripolyphosphate (Diop et al., 2015), alginate/chitosan (Sarmento et al., 2007), poly(isobutylcyanoacrylate) (Pinto-Alphandary et al., 2003), and poly(lactide-co-glycolic acid) (PLGA) (Cui et al., 2007) are currently used to encapsulate insulin. PLGA has the advantage of being approved by the US Food and Drug Administration (FDA) for drug delivery. However, two major drawbacks remain: PLGA is a non-enteric polymer (Krishnamachari et al., 2007) and the PLGA-NP system presents a low bioavailability, less than 20%, by intraduodenal administration in diabetic rat (Reix et al., 2012), which could be highly influenced by the physicochemical characteristics of NPs.

To solve the gastric environment problem, we developed the double encapsulation principle (patent WO2004096172) based on a gastroresistant carrier protecting insulin in the stomach and containing PLGA NPs. Regarding the first encapsulation level, many carriers have been developed. For example, Yu et al., (2015) and Reix et
al. (2012) developed and validated in vivo gastroresistant capsules covered with eudragit® L100-55, a copolymer commonly used to confer enteric properties to drugs (Li et al., 2006). This copolymer is insoluble in an acid medium and in water, but entero-soluble by deprotonation from a pH of about 6 and above (Hardy et al., 1987). Because of this property, PLGA is degraded as the capsule exits the stomach, at the duodenum level. Similarly, Poncelet et al. (1998) developed alginate beads, produced with non-toxic reagents and with resistance to acid pH due to alginate, which could be used to protect active ingredients in a gastric environment.

Then, carrying insulin, PLGA NPs formulated with double emulsion technic were chosen because this system is simple and proved its efficiency in many systems (Danhier et al., 2012, Sheng, 2015) and presents a low bioavailability in a diabetic rat model (Malathi et al., 2015), (Sonaje et al., 2010). Thus, a fine tuning of NP physicochemical parameters should be considered to resolve the bioavailability problem. Costantino et al. (2006) suggested the modification of surface properties to improve bioavailability of encapsulated insulin. Indeed, particle size, NP stability, and surface charge may affect NP absorption capacities (Bakhru et al., 2013). Regarding NP size, many studies showed various internalization mechanism in function of the NP size (Verma and Stellacci, 2010). However, surface charge studies are less frequent and really controverted. Indeed, various approaches exist, including mucoadhesion or polymer stabilization.

Mucoadhesion is a process by which a coated mucoadhesive polymer (Diop, 2015, Nafee et al., 2007) allows the interaction between particles and the intestinal mucus layer, thereby improving the uptake and the bioavailability of encapsulated insulin (Shakweh et al., 2004). The effects of these polymers are related to their high positive charge density (Plapied et al., 2010). Shakweh et al. (2004) showed an increase in paracellular transport with permeation enhancers such as chitosan because of their positive charge promoting interactions with the negative charge of intestinal mucus cells. Similarly, Diop et al. (2015) demonstrated the ability of chitosan NPs, positively charged, to cross the intestinal barrier in Caco-2 cells.

The second strategy to improve bioavailability with surface charge modification involves polymer stabilization by using surfactants. Grabowski et al. (2013) proved that PLGA negatively charged NPs formulated with a stabilizer, pluronic® F68, influence the uptake, cytotoxicity, and bioavailability profiles of NPs, improving
internalization. Likewise, Yu et al. (2015) demonstrated that PLGA NPs with a negatively charged surface, formulated without any stabilizer provide a good biological efficiency.

The aim of this study was to compare various surface charges of PLGA NPs using various strategies, in a same study to evaluate the impact of this parameter on NP characteristics such as size, stability, uptake in vitro, and efficiency in vivo.

2. Materials and methods

2.1. Materials

PLGA 50:50 Resomer® (RG 502H, Mw 14,000) was purchased from Boehringer Ingelheim (Ingelheim, Germany). SDS, (Mw 288.000) was purchased from Euromedex (Souffleweyersheim, France). Rapid insulin, Umuline® (100 UI/mL), was purchased from Eli Lilly (Indianapolis, IN, USA). Insuman® (400 UI/mL) was generously supplied by Sanofi-Aventis (Paris, France). Polyvinyl alcohol (PVA 18-88, Mw 130.000) was purchased from Fluka (Buchs, Switzerland). Pluronic® F68 was obtained from BASF Corporation (Mount Olive, NC, USA). CL113 (Chitosan chloride Mw 70.000 to 150.000; DD 75 to 90%) was purchased from Nova Matrix (Sandvika, Norway). Human crystallin insulin, FITC labeled insulin, isopropanol, fetal bovine serum (FBS), trypsin, streptozotocin, 96 and 24 wells plates CELLSTAR® (organic Greiner), and ethyl acetate were from Sigma Aldrich (Saint Louis, MO, USA). Acetonitrile was purchased from VWR (Fontenay-sous-Bois, France), anhydrous sodium sulfate was from SDS (Peypin, France). The cellular lineage of human adenocarcinoma (Caco-2) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the lineage RevHT29MTX was supplied by Dr. Thécla Lesuffleur (INSERM U505, Villejuif, France). DMEM (Dulbecco Modified Eagle Medium), antibiotics, antymycotic, no essential amino acids glutamine, phosphate buffered saline (PBS), hank buffered salt solution (HBSS) was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. NP preparation

NPs were prepared by double emulsion and solvent evaporation method as previously described (Vauthier and Bouchemal,2009). PLGA and Pluronic® F68 at
the weight of 200 mg and 100 mg, respectively, were dissolved separately in 500 µL of ethyl acetate and mixed after dissolution. A volume of 400 µL of commercial insulin or crystalline insulin (3.5 µg/mL) was added. FITC-labeled insulin (3.5 mg/mL) or deionized water was added in the organic phase and then emulsified under a 66 Watt sonication over 15 seconds in an ice-cold bath. A volume of 2 mL of aqueous phase with surfactant, PVA (2.5% w/v), SDS (0.01% w/v), or deionized water was added to the resulting water-in-oil (w/o) emulsion. The mixture was then sonicated during 10 seconds. The double emulsion (w/o/w) was transferred into 10 mL of PVA aqueous solution (0.15% w/v) or deionized water and gently stirred overnight. Particles were then coated with chloride chitosan by electrostatic interaction directly after the NP formulation by a simple transfer into 10 mL of a mix water/chitosan at 7 mg/mL (w/v) under gentle stirring. In order to limit NPs loss, no washing step was realized and NPs were lyophilized for a long-term conservation or kept at 4°C.

2.3. NP characterization

Size distribution of NPs was determined by dynamic light scattering using Horiba NanoZS (Horiba, Japan) at 25°C. NP characterization included the measurement of three parameters: NP mean size (diameter is calculated from the cumulative function of particles light scattering intensity); surface charge (Zeta potential), and determination of polydispersity index (PdI). The entrapment efficiency was determined (20 000 × g, 4°C for 1 h) and insulin quantity was measured by using HPLC based on monography of European pharmacopeia method. The system consisted of two Prostar 210 solvent delivery systems, a Prostar 410 autosampler and a Prostar 330 Photodiode Array (PDA) UV/vis detector (Varian, Les Ulis, France) and Water’s Symmetry C18 5 µm, column (Waters Corporation, Milford, MA, USA). Eluent A was composed of 0.05 M Na₂SO₄ in water at pH 2.3 and eluent B was a mix of eluent A/Acetonitrile (55:45, v/v). The mobile phase consisted of a mixture of eluent A/eluent B (42:58, v/v) at a flow rate of 1 mL/min. The column temperature was kept at 40°C and the detection wavelength was 214 nm. The entrapment efficacy (EE) and insulin loading were calculated using the following equations:

\[
EE(\%) = \left(\frac{\text{theoretical total amount of insulin} - \text{free insulin}}{\text{theoretical total amount of insulin}}\right) \times 100
\]
2.4. Scanning Electron Microscopy
NP samples were air dried and carbon-coated prior to SEM examination at 5 kV (Hitachi S800, Tokyo, Japan).

2.5. Nanoparticle stability test
NP size and zeta potential were measured as described above once a week during three weeks. According to European pharmacopeia, the gastric medium contained 35 mM NaCl and 80 mM HCl at pH 1.2 and the intestinal medium was prepared with 50 mM KH₂PO₄ and 15 mM NaOH adjusted at pH 6.8. The simulated mediums are enzyme free to allow the quantification of insulin. Then, NPs were incubated with medium (v/v) at 37°C and, at predetermined time points, samples were centrifuged and the amount of free insulin was measured by HPLC as described above. Results are expressed in percentage of insulin release.

2.6. Models and cell culture
Two cell culture models were used: Caco-2 and co-culture (75% Caco-2 + 25% RevHT29MTX) as described by Nollevaux et al. (2006). Cell culture was performed as described by Reix et al., (2012). Briefly, cells were seeded at a density of 73000 cells/cm² in DMEM containing 4.5 g/L glucose and L-glutamine. The medium was supplemented with 20% FBS, concentration currently used and recommended for Caco-2 culture cell (Yee, 1997), 1% antibiotic–antimitotic solution, and 1% of non-essential amino acids solution at 10 mM. Cells were grown over 21 days (to acquire enterocyte phenotype) at 37°C in an atmosphere of 5% CO₂ before testing and were used from passage 30 to 60.”

2.7. Particle toxicity
Toxicity was determined by measuring mitochondrial activity with CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega Corporation (Madison, WI, USA). Cells (19200 cells/well) were seeded on 96-well plates and cultured during 21 days for differentiation. Various concentrations of NPs were tested in previous study and 5 mg of PLGA/well was selected for in vitro test because first it was not deleterious for the cells and the concentration of insulin contained in 5mg/ml of PLGA
corresponds to 0.5-1UI of insulin per well, which could mimic the local *in vivo* concentration in the intestine.

Then, NPs were incubated with cells for 4 hours and cells were washed three times (10 min) with calcium- and magnesium-free HBSS solution. After treatment, 100 µL of culture medium with 20 µL of 3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) were added. Cells were incubated 1 hour at 37°C, in 5% CO$_2$ and absorbance was measured at 490 nm in Metertech 960 microplate reader (Metertech Inc., Taipei, Taiwan). The quantity of formazan product is directly proportional to mitochondrial activity, which is related to the number of living cells. Results are expressed in percentage of viable cells compared to the negative control (untreated cells).

### 2.8. Transepithelial Electric Resistance (TEER) measurement

Cells (200 000) were seeded on 0.336 cm$^2$ insert and cultured during 21 days. Cells were incubated with NPs and TEER was measured each hour during 4 hours. EGTA was used as positive control of gap junction opening reflecting possible cell toxicity. Results are expressed as a percentage of TEER decrease.

### 2.9. Study of nanoparticle uptake by flow cytometry

To study NP uptake in our 2 cell culture models, flow cytometry was performed as described by Reix et al. (19). Briefly, cells were cultured in 24-well plates for 21 days to allow differentiation. Cells were then incubated with FITC-insulin loaded NPs (5 mg of PLGA/well) in DMEM without FBS for 4 hours. Cells were washed three times (10 min) with calcium- and magnesium-free HBSS solution, detached by trypsinization, centrifuged, and resuspended in HBSS. Samples were analyzed by a flow cytometer BD (Becton Dickinson and Company) LSR II (Franklin Lakes, NJ, USA) in triplicate; 10 000 events were recorded per well. Results are presented as the amount of intracellular fluorescence.

### 2.10. *In vivo* validation

All animal experiments were performed in accordance with European Institutes of Health Guidelines regarding the care and use of animals for experimental procedures, approval AL/60/67/02/13). Male Wistar rats (120–140 g) (Charles River,
Wilmington, MA, USA) were placed in standard collective cages. They were kept under 12 h light/12 h dark cycles and were fed with a standard laboratory rodent diet in pellets form (Safe diets, Augy, France). Food and water were available *ad libitum*. Diabetes was induced by an intraperitoneal injection of streptozotocin at the single dose of 100 mg/kg, leading to a state of hyperglycemia in 3 days. Diabetic rats were selected with c-peptide (<200) and glycemia values (>5 g/L). They were then orally administered the formulations (gastroresistant vehicle containing +PVA lyophilized NPs at the dose of 100 UI/kg of insulin or SDS NPs at the doses of 100 and 250 UI/kg) by gavage. The negative control group was vehicle with empty NPs. The biofunctionality of particles was assessed by following the glycemia under fasting conditions during 18 hours.

2.11. Statistical analysis

Statistical analyses were performed by using Graph Pad Prism 7 software (GraphPad, San Diego, CA, USA). *In vitro* data were analyzed by one-way ANOVA with LSD Fisher's post-hoc test for parametric data or Kruskal-Wallis for non-parametric analysis. *In vivo* data were analyzed by repeated-measures ANOVA or one-way ANOVA with a LSD Fischer’s post-hoc test. Results are presented as mean ± SEM. P values of less than 0.05 were considered significant.

3. Results

3.1. Particle characterization

The mean size of +PVA NPs measured by dynamic light scattering was 188 ± 2 nm with a neutral ζ-potential -2 ± 1. PVA removal (-PVA NPs) decreased the size of NPs (168 ± 8 nm) and a negative charge appeared (-22 ± 1 mV). The size of the NPs was maintained (151 ± 4 nm) after incorporation of SDS-like surfactant with a ζ-potential of -42 ± 2 mV. Coating of the NPs using chitosan had no impact on NP size (162 ± 4 and 184 ± 4 nm) for chitosan and SDS-chitosan NPs, respectively, which were positively charged (56 ± 2 and 40 ± 1 mV) (Table 1).

Encapsulation efficacy (EE) was near 100% for all the formulations except for chitosan NPs with a significant decrease to 34% (P < 0.05) (Table 1).
SEM analysis of NPs (Figure 1) indicated a homogenous size and a round shape for +PVA, -PVA, and SDS particles. In contrast, with chitosan, an aggregation was observed with a network conformation and a polydispersity of NP size confirmed with a Pdi around 0.3. This phenomenon was not observed with SDS-chitosan NPs, which seemed more stable.

3.2. Nanoparticle stability

All the formulations of insulin loaded NPs were stable (size and charge) at 4°C during 21 days (Figure 2A and 2B), except for a decrease in size of chitosan NPs after 7 days (Figure 2A). Stability of NPs was then tested in stimulated medium (Figure 3). +PVA NPs released almost all encapsulated insulin in gastric and intestinal simulated medium after 4 hours of incubation. For –PVA NPs, we observed a decrease of insulin released to around 60–70% in intestinal medium after 4 hours (Figure 3B). SDS NPs presented a low release of insulin in gastric conditions (30–40% of free insulin) compared to other NPs (Figure 3A). However, in intestinal conditions, a burst release was observed after only 30 minutes. For chitosan-coated NPs, around 75% and 50% of insulin was released in gastric and intestinal conditions, respectively, after 4 hours. Chitosan incorporation to SDS NPs provoked a delay in insulin release in gastric medium after 30 minutes compared to chitosan particles alone (25% vs. 60%). In contrast, an increase of insulin released, 100% of insulin release, was observed in intestinal conditions after 4 hours (Figure 3).

3.3. In vitro toxicity

Compared to cells without treatment, no toxicity was observed in the two cell culture models, Caco-2 and Caco-2/HT29MTX co-culture. In fact, addition of NPs to the cell culture during 4 hours did not induce a significant decrease in cell viability regardless of the condition tested (Figure 4A and 4B) except for a significant decrease of viability of co-cultured cells treated with SDS-chitosan NPs (P < 0.05) (Figure 4A).

No significant change was observed in term of TEER at 5 hours after addition of SDS (89 ± 3, 75 ± 7%), +PVA (85 ± 4, 75 ± 6%) NPs, and –PVA (79 ± 6, 65 ± 9%) compared to cells without NPs (88 ± 3, 81 ± 11%) in Caco-2 and co-culture models, respectively (Figure 5A and 5B). In contrast, for chitosan and SDS-chitosan NPs, respectively, a strong and significant decrease of TEER was observed in Caco-2 (32
and Caco-2/HT29-MTX (30 ± 14, 30 ± 13%, P < 0.01) models compared to the negative control. These values obtained with chitosan-coated NPs were comparable to EGTA, indicating the opening of tight junctions (Figure 5).

3.4. Associated fluorescence of PLGA-nanoparticles

An increase of associated fluorescence of all types of NPs was observed compared to negative control in the 2 cell culture models, but it was only significant for SDS (P < 0.01), chitosan (P < 0.05), and SDS-chitosan NPs (P < 0.05) (Figure 6A). Then, all associated fluorescence results of NPs was compared to standard NPs (+PVA NPs). In Caco-2 cells, the highest associated fluorescence of NPs was observed in cells incubated with NPs containing SDS (P < 0.05) (Figure 6A). Contrary to the other types of particles in Caco-2 cells, SDS NPs (1787 ± 407, P < 0.05) showed a significant increase of fluorescence compared to +PVA (1277 ± 225). Likewise, in co-culture, SDS NPs showed an increase of associated fluorescence compared to negative control (P < 0.05) (Figure 6B). Chitosan-SDS NPs uptake (2821 ± 1229) seems to increase, but with a very high standard deviation. Moreover, no statistical difference was observed with these NPs. For –PVA NPs, in the 2 cell culture models, no significant increase of fluorescence was observed compared to +PVA NPs (Figure 6A and 6B). Similarly, in the co-culture model, no significant increase of fluorescence was observed for all types of NPs compared to +PVA NPs, except for a trend towards statistical significance for SDS NPs (P = 0.08).

3.5. In vivo validation using a diabetic rat model

For all conditions, a decrease of glycaemia was observed from 4 hours to 18 hours (Figure 7). Oral administration of the complex vector (SDS NPs with gastroresistant vehicle) induced a significant decrease in blood glucose levels in diabetic rats as compared to +PVA NPs at 12 hours. Compared to free insulin, this decrease was concentration dependent with a higher decrease with a concentration of 250 UI/kg at 12 hours (P < 0.005) than with a concentration of 100 UI/kg at 16 hours (P < 0.01). Gastro-resistant vehicle containing empty NPs had no effect on fasting glycemia in diabetic rat during the follow up compared to free insulin (Figure 8).
4. Discussion

Our study shows that modifying the NP surface charge could increase the bioavailability of insulin. We demonstrated that negative surface charge impact on NP uptake via the intestinal membrane compared to uncharged NPs and mostly positively charged NPs.

We investigated the role of surface charge using several formulations of PLGA NPs, including NPs stabilized with a surfactant, PVA, which presents a neutral surface charge (Reix, 2012) and is considered a reference NP, NPs formulated without PVA with a low negative surface charge, NPs formulated with SDS with a high negative charge, and NPs positively charged covered with or without surfactant. In summary, the physicochemical characteristics of all types of PLGA loaded NPs were stable during 21 days with a mean diameter around 200 nm for all types of NPs, but different surface charges, which correlated with the NP uptake results obtained in 2 in vitro cell culture models.

4.1. Impact of formulation on NP charge

Positively charged NPs were obtained by using a coating method, (13) allowing a mucoadhesive polymer, chitosan, to be positioned around NPs via an electrostatic interaction between the amine group (NH$_3^+$) and carboxylic group (COO$^-$) of PLGA (Zhou et al., 2010). However, chitosan NPs seemed to be unstable. A study reported that this electrostatic interaction could induce swelling or shrinking of NPs (Lopes et al., 2016), which could explain the instability of NPs in term of size. “In fact, chemical properties of chitosan play a major role in this instability because chitosan is a stable polymer, which is positively charged only at low pH (pH < 5.5) (Szymanska and Winnicka, 2015). Consequently, it is unstable in our experimental conditions that are close to pH = 7, confirmed by an aggregation of NPs with a high PdI and a low encapsulation efficiency. Moreover, we could observe that if there is not a strong interaction between the nanoparticles and the chitosan, the NPS were instable and the efficiency of encapsulation were low. In contrast, when a strong interaction was performed between negatively charged NPS and chitosan, (SDS-NPs), the system is more stable and the efficiency of encapsulation increased.
At the opposite, chitosan NPs were stable in gastric medium (pH = 1.2), where it could prevent the release of insulin because of a stable system with electrostatic interactions between positively charged chitosan and hydrolyzed and negatively charged PLGA (Lopes, 2016).

In contrast, negatively charged NPs were formulated without PVA or with negatively charged surfactant (SDS). In absence of stabilizing agent (-PVA NPs), NPs were safer and respected pharmaceutical requirements, which limit the use of surfactant (Sekhon, 2013). The negative charge is provided by PLGA hydrolysis in aqueous medium (Gentile et al., 2014); lactic acid and glycolic acid are metabolized by the Kreb’s cycle (Danhier, 2012), which renders the system biocompatible. In term of stability, surprisingly, the absence of surfactant does not affect physicochemical characteristics of NPs (low size and PdI). An hypothesis could be that pluronic® F68 with good surfactant properties (Santander-Ortega et al., 2009) in suspension and present in excess in the first emulsion allows the stabilization of the second emulsion. This explanation was confirmed when we formulated insulin NPs with a higher concentration of insulin (Insuman® 400 UI/mL, data not shown) where aggregation was observed only for NPS without PVA, indicating that the concentration of Pluronic® F68 was insufficient to stabilize insulin in high concentration. This phenomenon could also be attributed to the electrostatic interaction between insulin and PLGA, which could induce insulin aggregation (Danhier, 2012). Nevertheless, the negative charge of NPs formulated without PVA could be higher. Indeed, Danhier (2012) demonstrated that electrostatic interactions exist between positive charges of insulin (pH < 5.5) and negative charges of hydrolyzed PLGA (Danhier, 2012), which could partially mask negative charges. Therefore, to test the effect of a high negative charge, we used SDS, a negatively charged surfactant commonly used in the pharmaceutical industry (Anderberg et al., 1992). It was adsorbed to the NP surface via a negatively charged hydrophilic head, permitting the stabilization of the system (Gao and Chorover, 2010). Additionally, SDS was used to stabilize chitosan NPs. The idea was to create electrostatic interactions between chitosan (+) and SDS (-) to obtain a stable NPs system positively charged. This system showed stability in terms of size distribution and insulin quantity inside the system compared to the classic chitosan NPs.

4.2. Impact of formulation on biological systems
To study the impact of the charge of the designed NP systems on insulin bioavailability, we assessed *in vitro* uptake in 2 cell culture models. The first model was composed of Caco-2 cells, enterocytes involved in the formation of a brush border and expressing typical metabolic enzymes and efflux transporters. The second model was a co-culture of Caco-2 and HT29 MTX cells, which are goblet cells producing mucus that covers the epithelium and protects it (Schimpel et al., 2014), to mimic the intestinal epithelium model.

In this context, a significant higher uptake was observed with negatively charged NPs. To explain this result, toxicity and internalization mechanism should be discussed for each type of NPs. For negatively charged NPs, Qian et al. showed that surfactants like SDS induce an opening of cell junctions through their detergent properties (Yu Qian, 2013). However, in our study, TEER results showed no effect of SDS NPs on these cell junctions and proved that SDS NPs do not induce a long-term toxicity and do not use a paracellular mechanism to cross the intestinal barrier. However, a higher negative charge could have an effect on NPs uptake through a transcellular mechanism to cross the intestinal barrier via the endocytic pathway (Grabowski, 2013) and more precisely using caveolin dependent pathway to cross intestinal barrier (Bannunah et al, 2014). However, for –PVA NPs, an effect on tight junction was observed on both *in vitro* models, which could be explained by the aggregation of NPs during evaporation or an effect of negative charges which could bind to Ca\(^{2+}\), presents to maintain tight junction integrity (Sajeesh et al., 2009) and induce toxicity and a possible paracellular mechanism.

In the same way, positively charged NPs like chitosan or SDS-chitosan NPs didn’t show toxicity in terms of viability but a real impact on tight junction was observed with a huge decrease of TEER compared to negatively charged NPs (-PVA NPs). Indeed, it is known that mucoadhesive NPs like chitosan NPs use a paracellular mechanism due to the presence of chitosan which have an effect on protein of tight junction through translocation mechanism from the membrane to cytoskeleton (38) and lead an opening which could provoke a long-term toxicity for an oral insulin administration which is a multiple daily treatment.

However, mechanism to cross intestinal barrier is not the only one explanation of a higher uptake of negatively charged NPs. Indeed, mucoadhesive proprieties of chitosan could play a major role. In this way, some studies showed that the
fluorescence signal of positively charged NPs could also be attributed to adhesion of the NP surface with intestinal mucus at the cell surface rather than to internalization. In fact, positive charge with mucoadhesive properties increase the contact time between the intestinal layer covered with mucus and NPs (Lopes et al., 2014), allowing an electrostatic interaction between positive charges and the negatively charged cell membrane due to the presence of the proteoglycan, heparin sulfate, on the cell surface (Boddupalli et al., 2010). This electrostatic interaction could be too strong and could prevent NP internalization (Sheng, 2015). This theory could be checked thanks to use of a molecule to quench external fluorescence like trypan blue (Loike and Silverstein, 1983) and attributed the fluorescence signal to a real internalization (supplemental data S1).

Moreover, these strong interactions could reduce direct contact of NPs with the epithelium contrary to SDS NPs which are not coated and more accessible for mucus and create more interaction with epithelium (Sajeesh, 2010).

The uptake results obtained in the Caco-2 cell culture model were not reproducible in the Caco-2/HT29MTX co-culture model indicating the importance of the cell culture model. Schimpel et al. (2014) showed that the transport of particles increased (50-fold) in M cells compared to pure Caco-2 cells. The uptake difference observed between SDS-NPs and other NPs could be confirmed by using an in vitro triple culture model including lymphocytes (Antunes et al., 2013) or with an ex-vivo system using Ussing chambers (Lundquist and Artursson, 2016).

Based on this increased uptake with highly negatively charged NPs, insulin loaded SDS-NPs were orally administered to hyperglycemic rats to validate our NP system. For positive control, subcutaneous insulin was used and stopped after 4 hours to prevent on severe hypoglycemia due to fasting condition. For oral insulin conditions, the hypoglycemic effect was dose-dependent with a first effect at 100 UI/kg and an increase at 250 UI/kg. The effect was first observed after 12 hours and was still significant at 18 hours. This delay and long-term effect is confirmed with intraduodenal injection of C-peptide NPs (supplemental data S2). The profile obtained is that of a long-acting insulin but with an absorption wave of insulin bound to the passage of the intestinal barrier. The aim of C-peptide encapsulation was to determine bioavailability in encapsulated system. Indeed, it was impossible to dose
insulin in rat blood because of hemolysis of blood sample due to sampling by the rat's tail (Auberval et al., 2014). Moreover, insulin dosage doesn't reflect the total quantity absorbed in intestinal level due to first hepatic pass effect, a higher stability of C-peptide in blood and a low hepatic extraction (Polonsky et al., 1983). This bioavailability showed around 10% with +PVA NPs, which justify the use of 20 or 50UI/rat compare to 2 at 5UI/rat in subcutaneous injections. Moreover, the low bioavailability could be explained with a burst release of insulin in intestinal conditions. This delay observed to obtain a hypoglycemic effect of insulin NPs could be explained two ways: first, insulin NPs take longer to reach the specific region of the intestines favorable for insulin absorption. In fact, it was reported that the jejunum and ileum have an apparent 2- to 15-fold higher permeability than other segments of the intestinal tract, where abundant Payer's Patches exist (Agarwal, 2001). Moreover, the passage of NPs through the intestinal barrier could also delay the hypoglycemic effect. Reix et al. (2012) demonstrated that NPs are still present in Caco-2 cells 4 h after their absorption and co-localized with lateral cell membranes. Moreover, we proved that differences based on mucoadhesive properties link to charge of NPs exist in vitro. Likewise, Iwanaga et al. showed that the hypoglycemic effect of liposomes was prolonged after modification of their surface by poly(ethylene oxide) (PEO). In fact, PEO present a high affinity to the mucous layer of the small intestine, which is present in pluronic® F68, the surfactant used in our formulation. These data could also explain the long acting effect of insulin related to the NP system, (Agarwal, 2001), like a delayed insulin model.

**Conclusion**

This study demonstrated the real impact of physicochemical parameters like surface charge of NPs on in vitro uptake and bioavailability of insulin. Results showed in a same study that formulating negatively charged particles are simplest to stabilize contrary to mucoadhesive NPs, formulated with chitosan and positively charged. Moreover, negatively charged particles are not toxic, more efficient in vitro and showed efficiency in vivo on diabetic rat model compared to positively charged NPs. This formulation is a promising approach for oral insulin delivery.
Acknowledgments

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**Table legend**

**Table 1. Physicochemical characteristics of modified various insulin-loaded PLGA nanoparticles:** +PVA NPs, -PVA NPs, SDS NPs, chitosan NPs, and SDS-chitosan NPs. Dynamic light scattering measurement (size, PDI, and ζ-potential) and encapsulation efficacy data are presented as mean ± SD (n = 3). All datasets were compared to +PVA NPs. The level of significance was set at \(^{a}p < 0.05\) vs. + PVA particles.
<table>
<thead>
<tr>
<th></th>
<th>+ PVA NPs</th>
<th>- PVA NPs</th>
<th>SDS NPs</th>
<th>Chitosan coated NPs</th>
<th>SDS NPs coated with chitosan</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Size (nm)</strong></td>
<td>188 ± 2</td>
<td>168 ± 8</td>
<td>151 ± 4</td>
<td>162 ± 4</td>
<td>184 ± 4</td>
</tr>
<tr>
<td><strong>PdI</strong></td>
<td>0,16 ± 0,01</td>
<td>0,23 ± 0,02</td>
<td>0,19 ± 0,02</td>
<td>0,27 ± 0,01</td>
<td>0,15 ± 0,02</td>
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<tr>
<td><strong>ζ-Potential (mV)</strong></td>
<td>-2 ± 1</td>
<td>-22 ± 1(^a)</td>
<td>-42 ± 2(^a)</td>
<td>56 ± 2(^a)</td>
<td>40 ± 1(^a)</td>
</tr>
<tr>
<td><strong>EE (%)</strong></td>
<td>100 ± 0</td>
<td>100 ± 0</td>
<td>86 ± 6</td>
<td>34 ± 11(^a)</td>
<td>92 ± 10</td>
</tr>
</tbody>
</table>
Figure legend

**Figure 1.** SEM images of various insulin loaded PLGA nanoparticles: +PVA NPs, -PVA NPs, SDS NPs, chitosan NPs, and SDS-chitosan NPs. Scale bars = 2 µm

**Figure 2.** Stability of various insulin loaded PLGA nanoparticles: +PVA NPs, -PVA NPs, SDS NPs, chitosan NPs, and SDS-chitosan NPs. The physicochemical parameters: size (A) and ζ-potential (B) were evaluated over time. Data are presented as mean ± SEM (n = 3)

**Figure 3.** Stability of various insulin loaded PLGA nanoparticles: +PVA NPs, -PVA NPs, SDS NPs, chitosan NPs, and SDS-chitosan NPs in gastric (A) (pH = 1.2) and intestinal medium (B) (pH = 7.4) for 4 h. All the experiments were performed at 37°C. Released insulin was quantified by HPLC using the European pharmacopeia monography. Data are presented as mean ± SEM (n ≥ 3).

**Figure 4.** Cell viability. Cell viability was measured by using the MTS assay; 5 different types of NPs, +PVA NPs, -PVA NPs, SDS NPs, chitosan NPs, and SDS-chitosan NPs, were evaluated by using 2 cell culture models, Caco-2 cells (A) and Caco-2/RevHT29 MTX co-culture model (B). Cells without incubation were used as negative control and set as 100% of viability. Data are presented as mean ± SEM (n ≥ 3). All datasets were compared to the negative control. The level of significance was set at *p < 0.05 vs. negative control.

**Figure 5.** The percentage of transepithelial electric resistance (TEER) of the cell monolayer compared to the initial level. TEER was measured with an ohmmeter, 5 types of NPs were evaluated on 2 cell culture models, Caco-2 cells (A) and Caco-2/RevHT29 MTX co-culture model (B). EGTA was used as a positive control of gap junction opening. Data are presented as mean ± (n ≥ 3). All datasets were compared to the negative control. The level of significance was set at **p < 0.01 vs. negative control.

**Figure 6.** Uptake of insulin loaded PLGA particles in Caco-2 cells. Associated fluorescence measurements were performed by flow cytometry; 10 000 events were
recorded per well and data are expressed as the mean fluorescence intensity. Data are presented as mean ± SEM (n = 6). All datasets were compared to the negative control and +PVA NPs. The level of significance was set at * p < 0.05 vs. negative control, ** p < 0.01 vs. negative control, and $ p < 0.05$ vs. +PVA particles.

**Figure 7. Blood glucose levels in diabetic rats after oral administration of insulin loaded PLGA NPs:** + PVA particles and SDS particles at an insulin dose of 100 or 250 UI/kg (n ≥ 6) and intraperitoneal insulin (5UI/kg) (n=3). NPs without insulin served as a blank control. All datasets were compared to NPs without insulin. The level of significance was set at *** p < 0.005 vs. NPs without insulin and ** p < 0.01 vs. NPs without insulin.
Graphical abstract