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Bioadhesive nanoparticles of fungal chitosan for oral DNA delivery

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ABSTRACT

Chitosan is an ideal candidate for oral DNA delivery due to its mucoadhesive properties. Chitosan (CS) produced under GMP conditions from fungal source was used to encapsulate a plasmid DNA coding for a reporter gene. Nanoparticles made by complex coacervation of CS and DNA had a size around 200 nm, a positive zeta potential, a high association of DNA and protected the plasmid against nuclease degradation. Their transfection ability was assessed in differentiated intestinal Caco-2 cells. An N/P ratio of 4 and a DNA concentration of 8 μg/ml were the optimal conditions leading to a transfection efficiency similar to the one reached with polyethyleneimine (PEI)–DNA complexes without cytotoxicity. M cells in monolayers influenced DNA uptake up to 8 μg of DNA/ml when complexed with CS. Fungal trimethylchitosan was also tested but the complexes interactions were too strong to induce transfection *in vitro*. Confocal microscopy studies showed that CS/DNA and PEI/DNA nanoparticles were found at the apical surface of cell monolayers and DNA was co-localized within the nucleus. Quantification seemed to show that more DNA was associated with the cells when incubated with CS nanoparticles and that the presence of M cells slightly influenced DNA uptake when complexed with CS. In conclusion, we developed a new nanocarrier made of fungal CS promising for oral gene delivery and oral DNA vaccination.

1. Introduction

Gene therapy is a field with real clinical promise for the future involving the insertion of a functioning gene into cells to correct a cellular dysfunction or to provide a new cellular function (Bhavsar and Amiji, 2007). Gene delivery is a promising strategy as the encoded protein can be expressed in the host in its natural form (without denaturation or modification), and caused prolonged expression of the protein. Moreover, upscaled production of DNA in bacteria is possible. Finally DNA is stable at room temperature and has a long shelf-life (Shedlock and Weiner, 2000). However, DNA may present some potential concerns that should be considered early in the development of DNA strategies, like transfer to the host flora, germline integration, adverse effects of encoded peptides and induction of autoimmune responses (Glenting and Wessels, 2005). There are two general approaches for introducing genes into a cell: viral and non-viral. Viral vectors are extremely efficient at transferring genes but can cause some safety concerns (Cotrim and Baum, 2008). Non-viral vectors are considered to be much safer than viral vectors, but at present, their efficiency *in vivo* is low. Currently, several non-viral vectors have been developed among which are cationic polymers. They have an unlimited DNA packaging capacity, well defined physicochemical properties and high degree of molecular diversity (Mao et al., 2010).

Nowadays, plasmid DNA is mainly delivered by intramuscular or intradermal injections. The oral route represents an interesting alternative as it is patient-friendly, painless and safer, which could make large-population immunization more feasible (Galindo-Rodriguez et al., 2005). Nevertheless, DNA molecules are large, hydrophilic and negatively charged, so they do not effectively cross biological membranes (Vasir and Labhasetwar, 2006). Moreover, DNA is very labile in biological environment and is rapidly degraded by nucleases. Then, naked plasmid DNA has a low transfection efficiency (Mao et al., 2010) and, due to dilution and degradation in the gastrointestinal tract, high DNA concentrations would be required to be effective (Nandedkar, 2009).

So the development of efficient gene carriers that protect and deliver plasmid DNA to the target tissue and that allow cellular internalization, endosomal escape and nucleus uptake is a prerequisite for a successful DNA vaccine or gene therapy (Vasir and Labhasetwar, 2006; Bhavsar and Amiji, 2007). Ideal polymeric carrier should form a stable complex with nucleic acids to maintain stability in biological solutions and deliver the complexes to the targeted cells or tissues. It should also be non-reactive, biocompatible, non-pyrogenic and available in pharmaceutical grade purity (Bhavsar and Amiji, 2007; Luten et al., 2008). Among the existing cationic polymers, chitosan is an ideal candidate for oral DNA delivery due to its low toxicity, good biocompatibility and high positive charge density conferring it mucoadhesive properties. Chitosan bioadhesivity is of primary interest for oral delivery since the presence of mucus over the intestinal epithelium layer would increase the residence time of mucoadhesive particles at the cell

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surface (Lai et al., 2009). At last, chitosan can act as a proton sponge, favouring endosomal escape (Ishii et al., 2001). Chitosan from animal origin (crustaceans) have been mainly used as nanocarrier (van der Lubben et al., 2001; Agnihotri et al., 2004). However, batch to batch variability is high, resulting in variable physicochemical properties. Crustacean origin could also induce allergic reactions. Hence, an alternative chitosan produced, i.e. from edible *Agaricus bisporus* mushrooms could be more suitable for pharmaceutical applications, especially when it is available with batch to batch reproducibility and excellent control over the molecular characteristics such as molecular weight and degree of acetylation. Such chitosans from non-animal origin and of pharmaceutical grade are now available with a very narrow range of molecular weight and degree of acetylation at Kitozyme (BE).

Most human vaccines currently available are licensed for non-mucosal administration via subcutaneous or intramuscular routes. But most pathogens gain access to their hosts via the mucosal surfaces, often hijacking the mucosal immune system (Hathaway and Kraehenbuhl, 2000). It would be then interesting to induce a mucosal immune response right at the site of pathogen entry besides the classic systemic protection. Intestinal Peyer's patches are privileged targets for mucosal vaccination due to their crucial role in intestinal mucosal immunity (Hathaway and Kraehenbuhl, 2000; Brayden et al., 2005). While recombinant protein or inactivated virus cannot generate a cytolytic T lymphocytes (CTL) response which requires antigen association with the MHCII molecules (Liu, 2003), DNA can induce both humoral and cellular responses via direct transfection and cross-priming mechanisms (Srivastava and Liu, 2003). In addition, DNA vaccine is unable to revert to a virulent form (Shedlock and Weiner, 2000). DNA produced in bacteria can also play a role of adjuvant because the CpG motifs of bacterial DNA can activate the mammalian innate immune system (Liu, 2003). Besides vaccination, oral gene therapy is promising for diseases such as inflammatory bowel disease (Nandedkar, 2009) and combined immunodeficiency syndromes (Cavazzana-Calvo et al., 2005).

In this study, non-animal-derived chitosan produced from mushrooms under GMP conditions has been used and characterized as DNA carrier. In addition, trimethylchitosan, chemically modified by derivatization of fungal chitosan, to improve water solubility at physiological pH, has been tested. The aim of this study was to evaluate the potential of fungal chitosan as a non-viral carrier for oral DNA delivery. The influence of N/P ratio, DNA concentration and presence of M cells on transfection efficiency has been evaluated. Based on all these observations, a comparison has also been made between chitosan and trimethylchitosan.

2. Material and methods

2.1. Polymers

Ultra-pure chitosan (CS) (KiOmedine-CsU®) with Mv (viscosimetric average molecular weight) of 42 kDa and degree of acetylation of 11.3% as well as trimethylchitosan (TMC) with 33% of degree of substitution were a gift from Kitozyme (BE). The TMC was prepared from a native chitosan with Mv of 108 kDa and DA of 22.3%. Polyethyleneimine (PEI) was obtained from Sigma–Aldrich (BE).

2.2. Plasmid

The luciferase reporter plasmid used for transfection studies was a pGL3LUC (Promega Benelux, NL) containing the cytomegalovirus (CMV) promoter. Plasmids were prepared using EndoFree Plasmid Giga Kit Gigaprep kit (Qiagen, BE) according to the manufacturer's protocol. The quality of resulting plasmid was assessed by measuring the ratio of light absorption (260 nm/280 nm) and by electrophoresis (0.7% agarose gel). Light absorption at 260 nm was used to determine DNA concentration.

2.3. Nanoparticle preparation

CS/DNA and TMC/DNA nanoparticles were prepared by ionic complexation between cationic CS or TMC and anionic DNA. A 2 mg/ml solution of CS or TMC was prepared in acid acetic 1% (v/v) and the pH of the solution was adjusted to 5 with NaOH 10 M and filtered through 0.2 μm. This solution was diluted in acetic acid solution at pH 5 to obtain the different concentrations used to formulate the nanoparticles. DNA was diluted in water (Chew et al., 2003; Zheng et al., 2007; Li et al., 2009a). The nanoparticles were prepared by adding 500 μl of CS or TMC solution to 500 μl of DNA solution, vortexing gently for 20 s. Nanoparticles were incubated at room temperature for 1 h before use.

The N/P ratio is the ratio between the number of polymer positive charges and DNA negative charges. It was calculated taking into account the degree of acetylation for the CS and TMC (related to the quantity of deacetylated groups available for the protonation), the degree of substitution for TMC and the amount of phosphate groups of DNA (1 μg of DNA: 3 nmol of phosphates). PEI/DNA polyplexes were prepared in 0.9% (w/v) sodium chloride with a N/P ratio of 9 according to the supplier's instructions and used as positive control due to its high transfection capacity (Boussif et al., 1995).

2.4. Physicochemical characterization of the nanoparticles

Particle size distribution was determined by means of dynamic light scattering (DLS) using a NanoSizer ZS (Malvern Instruments, UK). The zeta potential of the particles was measured with the NanoSizer ZS by laser Doppler velocimetry. The DNA content of the particles was determined by quantifying the DNA in nanoparticle supernatant with a Quant-it™ Pico Green® ds DNA Assay Kit (Invitrogen, Molecular Probes, BE). The percentage degree of association can be expressed as the amount of DNA encapsulated compared to the amount of DNA used to form the nanoparticles.

$$\text{Degree of association}\,(\%) = \frac{\text{Total amount of DNA} - \text{Free DNA}}{\text{Total amount of DNA}}$$

2.5. Gel electrophoresis

An agarose (Sigma–Aldrich, BE) gel electrophoresis was used to detect non-encapsulated DNA and to check DNA integrity after encapsulation. To assess the ability of nanoparticles to protect DNA from DNase, 0.5 μg of DNA encapsulated in nanoparticles were incubated with 0.5 U DNase (RQ1 RNase free DNase, Promega) for 10 min. Then, DNase was inactivated by incubation with a stop solution according to supplier instructions. To retrieve encapsulated DNA, nanoparticles were incubated with lysozyme from chicken egg white (1 mg/ml) (Sigma–Aldrich) and chitosanase from streptomyces species (160 U/ml) (Sigma–Aldrich) for 60 min. Then, DNA integrity was determined by gel electrophoresis (0.8% agarose gel, migration at 70 V constant voltages for 90 min). Native plasmid DNA, treated and not with DNAse and Supercoiled DNA Marker (New England Biolabs, MA, USA) were used as controls.

2.6. In vitro culture of Caco-2 cells

Human colon carcinoma Caco-2 cell line (clone 1) was obtained from Dr. Maria Rescigno, University of Milano-Bicocca, Milan, Italy (Rescigno et al., 2001). Unless specified all culture media were bought from Gibco™ Invitrogen Corporation (BE). Cells were

maintained in supplemented Dulbecco's Modified Eagle Medium (DMEM) high glucose and L-glutamine, with 10% (v/v) foetal bovine serum (FBS) (Hyclone, Perbio Sciences, BE) and 1% (v/v) non-essential amino acids at 37 °C under a 10% CO_2 water saturated atmosphere. Human Burkitt's lymphoma Raji-B-cell line (American Type Culture Collection, VA, USA), was maintained in RPMI 1640, supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine and 1% (v/v) non-essential amino acids at 37 °C under a 5% CO_2 water saturated atmosphere. Caco-2 cells were seeded on 24-well plates (Costar, Elscolab, BE) at a concentration of 4×10^4 cells per well. Cells were maintained for 21 days and the medium was refreshed every 2 days.

The *in vitro* model of the human follicle-associated epithelium (including M-like cells) was obtained according to the protocol published by des Rieux et al. (2007). Briefly, 5×10^5 Caco-2 cells were seeded on Matrigel™ (Becton Dickinson, Franklin Lakes, USA) coated 12-well Transwell inserts (3.0 μm Transwell® inserts, Costar, Elscolab, BE) and cultivated for 3 days in supplemented DMEM + 1% PEST (Penicillin 10,000 U/ml/Streptomycin 10,000 μg/ml). Inserts were inverted and a piece of silicon rubber (Labo-Modern, Queveaucamps, BE) was placed around the basolateral side. The inserts were then transferred into a pre-filled Petri dish with supplemented DMEM + 1% PEST. Inverted inserts were maintained for 10 days and the basolateral medium was refreshed every 2 days. 2.5×10^5 Raji-B cells, resuspended in supplemented DMEM + 1% PEST, were added to the basolateral compartment of the inserts. The co-cultures were maintained for 5 days. Mono-cultures were cultured under the same conditions, but without the addition of Raji-B cells (Caco-2 cells alone).

2.7. Transfection of differentiated Caco-2 cells on wells or porous inserts

For transfection experiment of differentiated cells in multi-well plates, the cells were washed with HBSS and incubated for 4 h with nanoparticles, diluted in HBSS of pH 6 (unless specified) at desired concentrations. Then, the samples were removed, replaced with fresh culture medium and incubated for 44 h.

For transfection experiments of mono- and co-cultures, inserts were reversed to their original orientation in 12-well plates and washed with HBSS of pH 7.4. After 20 min of equilibration at 37 °C, the transepithelial electrical resistance (TEER) was measured using an Endohm™ tissue resistance chamber (Endohm-12, World Precision Instruments, FL) connected to a Millicell®-RES (Millipore, MA) ohmmeter. Nanoparticles and naked DNA were diluted in HBSS of pH 6 and added to cell apical side; HBSS of pH 7.4 was added to basolateral compartment. Samples were incubated for 4 h at 37 °C with cells and apical and basolateral solutions were replaced by fresh culture medium and incubated for 44 h.

Then, LDH (lactate dehydrogenase kit, Roche, NL) was dosed in apical compartments to evaluate formulation cytotoxicity. Apical media were collected and centrifuged (250 g, 5 min). LDH was quantified according to manufacturer's instructions. For luciferase expression quantification, cells were lysed (lysis buffer from luciferase assay system) and centrifuged. The supernatants were collected and luciferase activity was measured (Luciferase assay system, Promega) and expressed in relative light units (RLU) per mg of total proteins measured by MicroBCA™ Protein assay kit (ThermoScientific, Pierce, USA).

2.8. Confocal microscopy

Differentiated Caco-2 cells, grown on Lab-tek™ (Nunc, DK) or inserts, were incubated for 4 h with formulations (8 or 16 μg/ml of DNA, N/P 4) prepared with yoyo-1-labelled DNA (according to Invitrogen instructions), were washed and fixed with 4% formaldehyde. Cells were permeabilized with 0.2% Triton X-100 in PBS, cytoskeleton (F-actin) labelled with Rhodamine-phalloïdin (1/50, v/v, Invitrogen) and nuclei with Topro-3 (1/100, v/v, Invitrogen). Scanning laser confocal microscopy (Zeiss LSM510 with META detector) allowed the localization of DNA in cells. Images were edited with Adobe Photoshop (CS4 Extended v.11) and quantified with Axio Vision (RGB quantification script, Zeiss, BE). The analyzed surface of each image was $(202 \times 202)\ \mu m^2$ over 28 μm (z stack) ($n = 2$).

2.9. Statistics

Results were analyzed using Mann–Whitney or Kruskal–Wallis test for multiple comparisons followed by post-hoc testing to identify specific conditions (significance $p < 0.05$). Results are expressed as mean ± SEM.

3. Results

3.1. Physicochemical characterization of the nanoparticles

Formulations were characterized in terms of size, zeta potential and degree of association with DNA (Table 1). All nanoparticles made with CS had a mean size around 200 nm with a small polydispersity index. Except for the ratio N/P 1, the zeta potentials of the nanoparticles were positive (30–40 mV). Also, DNA was fully complexed with CS except for the ratio N/P 1 for which the degree of association was slightly lower (93%). TMC nanoparticles (N/P 4) had a higher size (around 500 nm), a positive zeta potential (40 mV) and a complete association with DNA. PEI/DNA complexes (N/P 9) (Boussif et al., 1995) had a smaller size around 100 nm, a slightly lower positive zeta potential and a complete association with DNA.

3.2. Integrity and protection against nuclease degradation of the associated DNA

Integrity of the associated plasmid DNA and protection against nuclease degradation by nanoparticles were determined by agarose gel electrophoresis. Gel electrophoresis indicated that all the CS/DNA formulation efficiently complexed DNA, while preserving its integrity (Fig. 1a). Complexation with CS protected DNA from degradation by DNase (lane 5) while naked DNA was completely degraded (lanes 2 and 3). The integrity of the associated DNA was confirmed after degradation of the nanoparticles with chitosanase and lysozyme (lane 6). Complexation of the DNA with TMC formed nanoparticles (lane 8) which protected the DNA against degradation (lane 9) but were not degraded by incubation with chitosanase and lysozyme (lanes 10 and 11). TMC/DNA complexes incubated with chitosanase and lysozyme for 1, 2 and 4 h to degrade the polymer did not release the plasmid (Fig. 1b).

3.3. Cell transfection in vitro

Transfections were performed on differentiated Caco-2 cell monolayers to mimic the intestinal epithelium. Studies were first performed in 24-wells plates on differentiated cells cultivated during 21 days. Then, to assess the influence of nanoparticles transport across an *in vitro* model of human enterocytes and to study the effect of M cells on DNA transfection, Caco-2 cells were grown on porous inserts as mono-cultures (Caco-2 cells monolayers) or co-cultures with Raji cells (*in vitro* model of the human follicle-associated epithelium).

3.3.1. Transfection of Caco-2 differentiated in multi-well plates

The optimal DNA concentration, eliciting the highest luciferase transgene expression without being cytotoxic, was determined by

Table 1
Physicochemical characteristics of the DNA-loaded nanoparticles ($n = 5$).

Formulation	Average size (nm)	PDI	Zeta potential (mV)	Degree of association (%)
CS N/P1	187 ± 3	0.224 ± 0.031	−29 ± 5	93 ± 0.07
CS N/P2	182 ± 9	0.217 ± 0.007	+26 ± 5	100
CS N/P4	160 ± 1	0.167 ± 0.011	+28 ± 4	100
CS N/P6	221 ± 7	0.349 ± 0.039	+40 ± 3	100
CS N/P8	210 ± 2	0.281 ± 0.021	+26 ± 5	100
CS N/P10	239 ± 12	0.345 ± 0.019	+39 ± 4	100
TMC N/P4	520 ± 41	0.248 ± 0.017	+39 ± 6	100
PEI N/P 9	106 ± 1	0.128 ± 0.007	+15 ± 3	100

incubating differentiated Caco-2 cells with increasing DNA concentrations ranging between 1 and 24 μg/ml (N/P ratio = 4, CS concentration of 2–48 μg/ml). Luciferase expression reached a maximum for 8, 16 and 24 μg/ml of DNA (Fig. 2a). LDH release was below 5% for 1, 4 and 8 μg/ml of DNA and increased up to 10% for 16 and 24 μg/ml. Accordingly, the following experiments were performed at 8 μg/ml of DNA.

Then, the optimal N/P ratio was determined by incubating differentiated Caco-2 cells with formulations whose N/P ratio was ranging from 1 to 20 (8 μg/ml of DNA, 4–80 μg/ml of CS). The luciferase expression induced by nanoparticles with N/P 4, 6, 8, 10 and 15 and by PEI/DNA complexes was significantly higher than the expression induced by naked DNA (Fig. 2b). It reached a plateau at N/P 4 and was enhanced by a factor of 1000 as compared to naked DNA. LDH release increased when cells were incubated with formulations presenting a N/P ratio higher than 6 (from 1% to 8% of LDH release). Accordingly, the following experiments were performed with N/P 4.

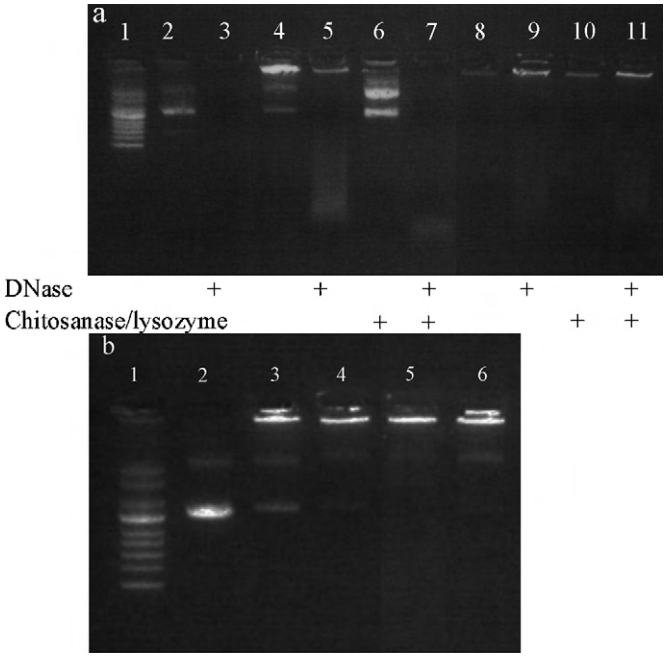

Fig. 1. Integrity and protection against nuclease degradation of the of associated plasmid DNA. Agarose gel electrophoresis (0.8%) of naked or encapsulated plasmid DNA (N/P ratio 4) was performed on the following samples. a – 1: DNA ladder, 2: DNA plasmid, 3: DNA plasmid + DBase, 4: CS/DNA NP, 5: CS/DNA NP + DNase, 6: CS/DNA NP + chitosanase/lysozyme, 7: CS/DNA NP + chitosanase/lysozyme + DNase, 8: TMC/DNA NP, 9: TMC/DNA NP + DNase, 10: TMC/DNA NP + chitosanase/lysozyme, 11: TMC/DNA NP + chitosanase/lysozyme + DNase. b – 1: DNA ladder, 2: DNA plasmid, 3: TMC/DNA NP, 4–6: TMC/DNA NP + chitosanase/lysozyme 1, 2 and 4 h, respectively.

3.3.2. *Influence of CS structure and pH on luciferase transgene expression*

Transfection studies were performed on differentiated Caco-2 cells (on multi-well plates) at different pH (6 and 7.4), and comparing TMC with CS to determine the influence of pH and CS modification (trimethylation of amino groups) on luciferase transgene expression. Luciferase expression of cells incubated with CS-based formulations was significantly lower at pH 7.4 than at pH

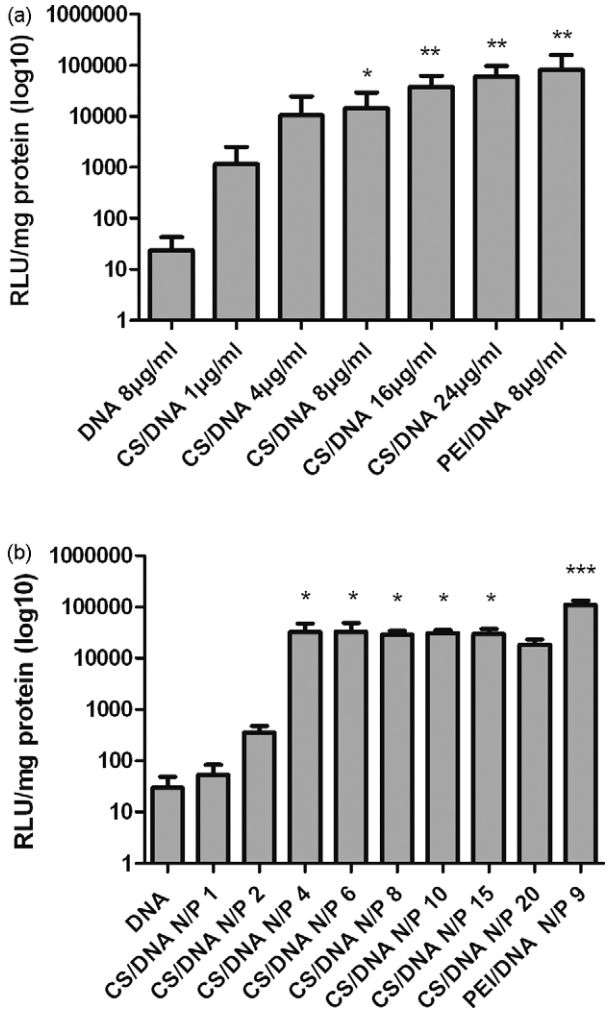

Fig. 2. Influence of DNA concentration and N/P ratio on luciferase transgene expression by Caco-2 cells differentiated in multi-well plates. DNA was complexed with CS (N/P = 4) and formulations were diluted in HBSS pH 6 at DNA desired concentrations. Transfection was performed on differentiated Caco-2 cells. Naked DNA and PEI/DNA were used as negative and positive controls, respectively. (a) Effect of DNA concentration (1 to 24 μg/ml) at N/P = 4. (b) Effect of various N/P (1–20) at 8 μg/ml DNA. * designates samples with luciferase expression that was significantly higher than naked DNA *($p < 0.05$), **($p < 0.01$) and ***($p < 0.0001$) ($n = 8$).

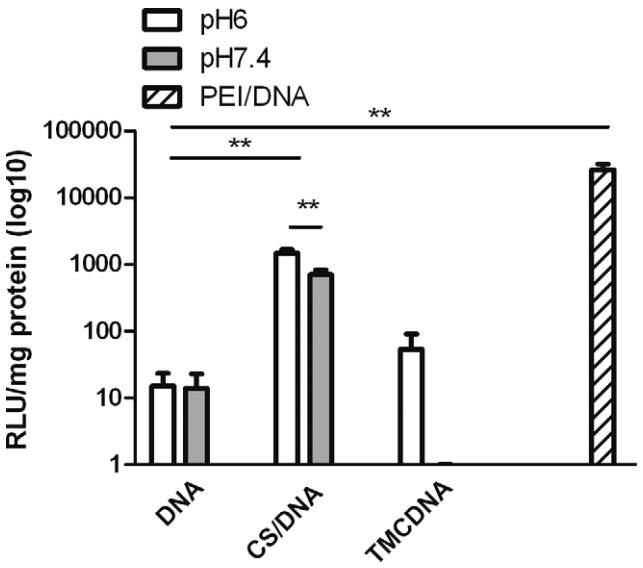

Fig. 3. Influence of pH and chitosan chemistry on luciferase transgene expression. DNA was complexed with CS or TMC at N/P of 4 and formulations were diluted in HBSS pH 6 or pH 7.4 to reach 8 μg/ml of DNA. Naked DNA and PEI/DNA were used as negative and positive controls, respectively. Transfection was performed on multi-well plate differentiated Caco-2 cells. * designates samples with luciferase transgene expression that is significantly higher than naked DNA, $^{**}p < 0.01$ ($n = 16$).

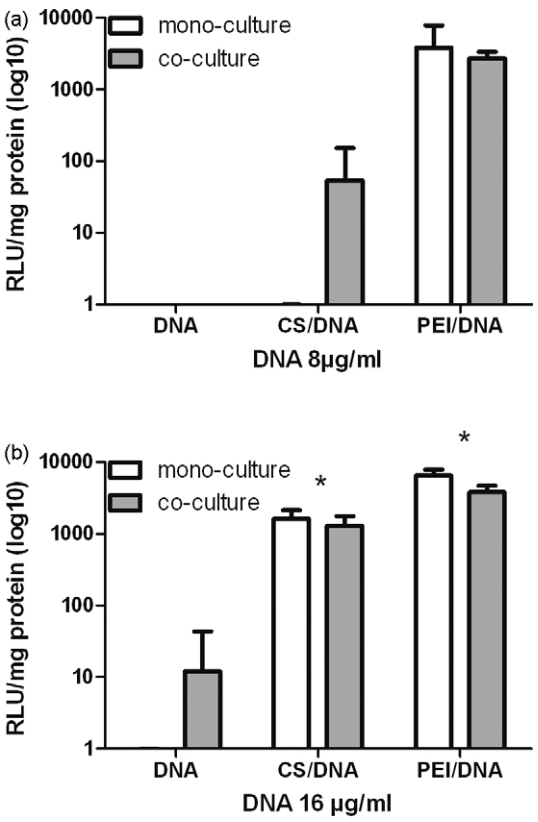

Fig. 4. Influence of M-like cells on luciferase transgene expression. DNA was complexed with CS and formulations were diluted in HBSS pH 6 to reach obtain 8 μg/ml (a) or 16 of DNA μg/ml (b) Transfection was performed on mono- and co-cultures. Naked DNA and PEI/DNA were used as negative and positive controls, respectively. * designates samples with luciferase expression that is significantly higher than naked DNA, $p < 0.05$ ($n = 6$).

6 (Fig. 3). TMC was used to increase nanoparticle stability at pH 7.4 (higher solubility) and hence transfection. However, no expression was detected at pH 7.4 when cells were incubated with TMC nanoparticles. And at pH 6, TMC nanoparticles induced a lower expression compared to CS nanoparticles (Fig. 3). LDH release was less than 5% when cells were incubated with formulations of CS and TMC and around 10% with PEI complexes

3.3.3. *Transfection of mono- and co-cultures on inserts*

Growing Caco-2 cells on porous inserts allowed the transport of nanoparticles from the apical to basolateral side or from basolateral to apical side (des Rieux et al., 2005). Moreover, preparing co-culture of Caco-2 cells with Raji induced 15–30% of M cells mimicing the follicle-associated epithelium (des Rieux et al., 2007). The influence of M cells on transgene expression after transfection by CS/DNA nanoparticles was also measured. Cell transfection was performed in parallel on mono- and co-cultures at two DNA concentrations (8 and 16 μg/ml) with a N/P ratio of 4.

At 8 μg/ml of DNA, luciferase expression was significantly higher when mono- and co-cultures were transfected with CS/DNA nanoparticles or PEI polyplexes compared to naked DNA (Fig. 4a). Luciferase expression by cells transfected with PEI complexes was higher than cells transfected by CS/DNA nanoparticles (in mono- and co-cultures). In presence of M cells, the transgene expression obtained after incubation with CS/DNA nanoparticles was 50-times higher in co-cultures compared to mono-cultures (Fig. 4a). No difference was observed between mono- and co-cultures when cells were transfected with PEI polyplexes.

When DNA concentration increased (16 μg/ml), luciferase transgene expression increased both in mono- and co-cultures, with no significant difference between them (Fig. 4b). Luciferase expression in cells transfected by CS and PEI nanoparticles was similar and significantly higher than with naked DNA ($p < 0.05$).

For both concentrations, incubation with nanoparticles did not alter the tight junction integrity as TEER were between 200 and 300 Ω/cm^2 for the mono-cultures and between 100 and 200 Ω/cm^2 for the co-cultures before and after the experiments.

3.4. *Localization of nanoparticles within cell monolayers*

Fluorescent labeling of both cells and DNA allowed the study of the influence of formulations and cell culture models on DNA localization within the cells. Localization of naked DNA, CS/DNA nanoparticles and PEI polyplexes was performed in Lab Tek® cultured cells (similar to multi-well plate differentiated Caco-2 cells) (Fig. 5) and in insert cultured mono- and co-cultures (Fig. 6).

In Caco-2 cells grown on Lab Tek®, only a little naked DNA was visible on or within cell monolayers (Fig. 5a). When incubated with PEI/DNA and CS/DNA nanoparticles, fluorescent DNA was localized at the cell apical side and in cell nucleus (Fig. 5b and c). Quantification of DNA signal correlated with luciferase expression results, since less DNA was found in cells when incubated with CS/DNA np and naked DNA compared with PEI polyplexes (Fig. 5d).

As observed for Caco-2 cells cultured in Lab Tek®, a small amount of naked DNA was observed in mono- and co-cultures (Fig. 6a and b). After incubation with CS/DNA and PEI/DNA nanoparticles, DNA was found on cell monolayer apical surface as well as co-localized with their nucleus (Fig. 6c–f). Quantification confirmed that more DNA was associated with the cells when incubated with CS and PEI nanoparticles than with naked DNA. Nucleus/DNA co-localization was observed for cells incubated with CS nanoparticles and PEI polyplexes (Fig. 6c–f). More DNA was detected in co-cultures compared to mono-culture when incubated alone or complexed with CS. No difference was observed between mono- and co-cultures for PEI polyplexes (Fig. 6g).

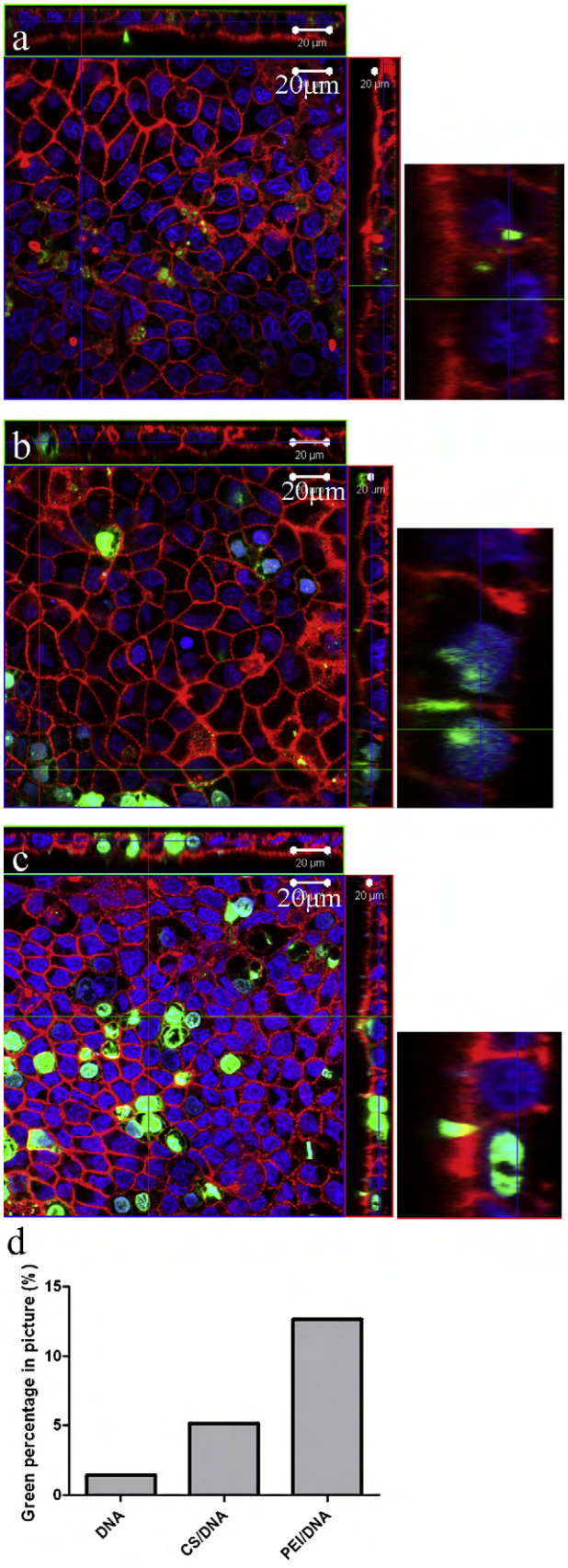

Fig. 5. Influence of formulations on DNA localization in Lab Tek® Caco-2 cultured cells. Orthogonal views of confocal microscopy pictures. Differentiated caco-2 cells were incubated for 4 h with yoyo-1-labelled DNA (Green) (8 μg of DNA/ml, N/P 4): naked DNA (a), CS/DNA nanoparticles (b), PEI polyplexes (c). Cells were fixed in 4% formaldehyde and permeabilized (0.2% Triton X-100 in PBS). Cytoskeleton (F-actin) was labelled with Rhodamine-phalloïdin (Red) and cell nuclei with topro-3 (Blue). d: Quantification of DNA (green fluorescence) expressed as the percentage of green pixels over the total analyzed surface (RGB quantification script, Axiovision) ($n = 2$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

The aim of this study was to validate CS of fungal source as an efficient carrier for oral DNA delivery. CS has already been extensively characterized as non-viral vector for gene delivery applications (Mao et al., 2010) but little is known about CS/DNA nanoparticle potential to transfect human intestinal enterocytes (Kadiyala et al., 2010). In addition, the vast majority of the work achieved in the field has been done using CS from animal origin. KiOmedine-CsU®, a novel CS from non-animal origin and produced according to GMP rules, could overcome the problems of reproducibility of other chitosan sources due to batch to batch variations. We showed that KiOmedine-CsU® successfully complexed DNA, forming 200 nm nanoparticles, which protected DNA from degradation by DNase. CS nanoparticle transfection of human intestinal epithelial cells was characterized using two *in vitro* models: epithelial enterocytes (differentiated Caco-2 cells) and follicle-associated epithelium containing M cells (co-cultures of Caco-2 and Raji cells). Transfection by CS/DNA nanoparticles was found to be dependent of DNA concentration and N/P ratio. Luciferase expression was slightly influenced by presence of M cells as DNA seemed to be more localized in M cells compared to "classic" enterocytes when complexed with CS. CS successfully induced transgene expression of luciferase in differentiated cells, and although it was not as efficient as PEI, CS was less cytotoxic. To complete our study, we compared transfection of nanoparticles made of fungal CS to that obtained with nanoparticles of a higher molecular weight animal-derived CS and largely described in the literature (Ma and Lim, 2003; Huang et al., 2005; Rajeshkumar et al., 2009; Li et al., 2009b; Kadiyala et al., 2010) and found a similar efficiency (data not shown).

4.1. Influence of DNA nanoparticle properties on luciferase transgene expression

An important prerequisite for non-viral gene delivery is the formation of small sized and stable polyplexes with plasmid DNA under mild, organic solvent-free conditions. Particle size is especially important for oral delivery since it is determinant for cellular uptake by intestinal enterocytes. Particularly, M cells required a size under 1 μm and more specifically, between 100 and 200 nm for a successful uptake (Jani et al., 1990; Desai et al., 1996; des Rieux et al., 2005). The size of CS nanoparticles is dependent on the N/P ratio but remains between 150 and 250 nm, making them eligible for oral delivery application.

Intrinsic properties of nanoparticles also influence cellular uptake. For instance, nanoparticles with a low N/P ratio will yield physically unstable complexes and poor transfection, while highly stable complexes with high N/P ratio may show reduced transfection (Mao et al., 2010). The highest transfection efficiencies observed for CS nanoparticles with N/P ratios of 4 and 6 confirmed Mao et al.'s (2010) observations. This could be related to the charges present at the nanoparticle surface (zeta potential), suggesting that surface charges would be more determinant than size for an efficient transfection (Koping-Hoggard et al., 2001; Huang et al., 2005).

In addition to have suitable properties for transfection, nanoparticles have to be stable. The colloidal instability of CS seems to be its major drawback. Indeed, CS is a weak base due to its pK_a value of

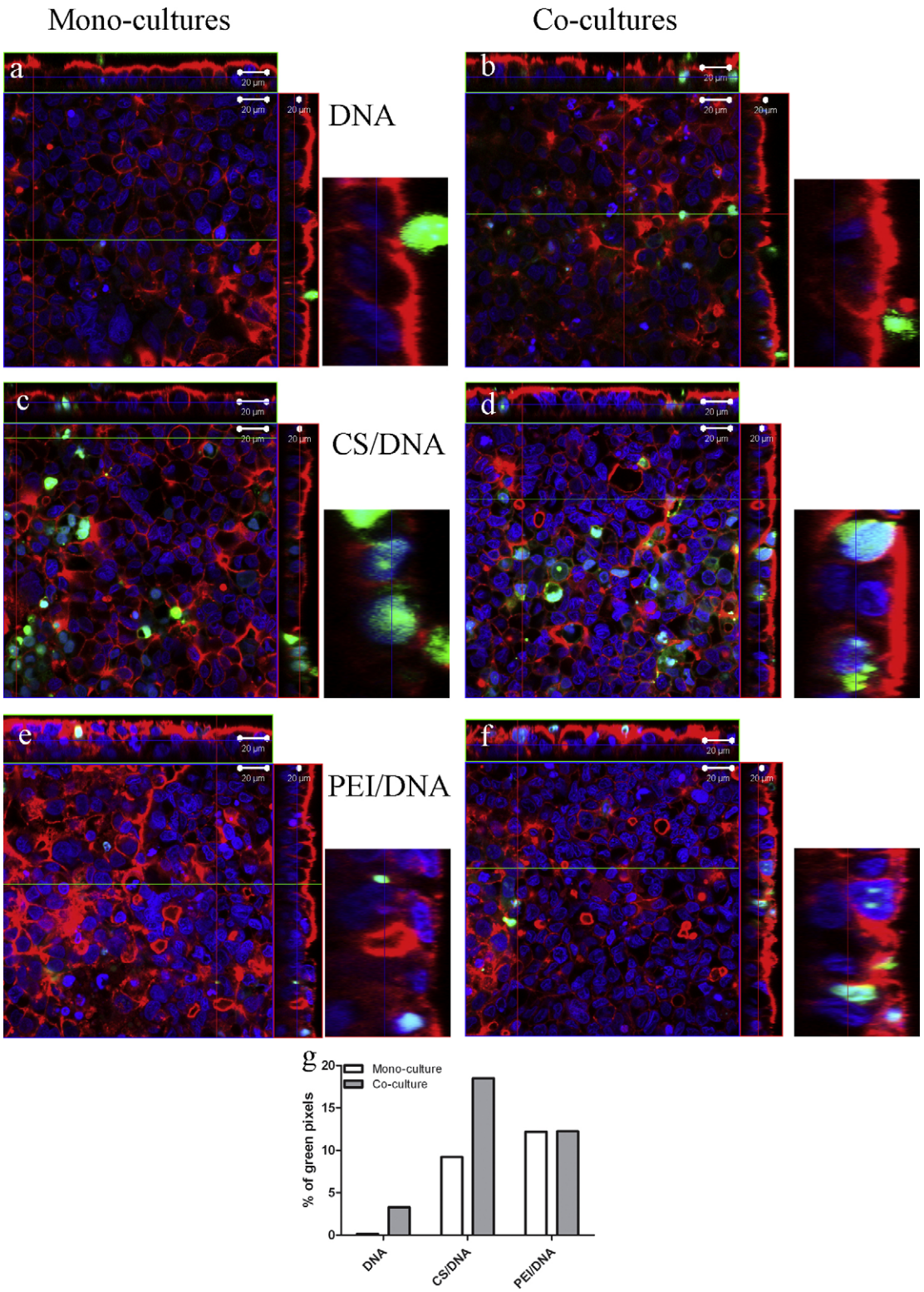

Fig. 6. Influence of formulations on DNA localization in mono- and co-cultures. Orthogonal views of confocal microscopy pictures. Mono- (left column) and co-cultures (right column) were incubated for 4 h with yoyo-1 DNA-labelled formulations (Green) (16 μg/ml of DNA, N/P 4): naked DNA (a, b), CS/DNA nanoparticles (c, d), PEI polyplexes (e, f). Cells were fixed in 4% formaldehyde and permeabilized (0.2% Triton X-100 in PBS). Cytoskeleton (F-actin) was labelled with Rhodamine-phalloïdin (Red) and cell nuclei with topro-3 (Blue). g: Quantification of DNA (green fluorescence) expressed as the percentage of green pixels over the total analyzed surface (RGB quantification script, Axiovision) ($n = 2$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

5.5–6.5 of the D-glucosamine moiety making it insoluble at neutral and alkaline solutions. This could explain the drop in transfection efficiency between pH 6 and pH 7.4 (Fig. 3) due to possible aggregation of nanoparticles at pHs higher than 6.5. Other studies showed the same effect of the pH on transfection efficiency (Opanasopit et al., 2009). Ionic strength and pH of the solution used to prepare, store and apply CS nanoparticles seem to have a high influence on the colloidal stability and thus on transfection efficiency (Chen et

al., 2004; Strand et al., 2005). For instance, an acid buffer could partially neutralize the alkaline condition in small intestine increasing CS nanoparticles transfection efficiency (Chen et al., 2004).

The carrier should form stable nanoparticles and protect the integrity of DNA. However, an intermediate degree of stability is needed to obtain high levels of transfection. Indeed, the carrier should present a good balance between extracellular DNA protection and efficient intracellular DNA release (Mao et al., 2010). Fig. 1 shows a good protection of polyplexes against degradation by DNase. The CS polyplexes were degraded by chitosanase and lysozyme delivering intact DNA while TMC polyplexes could not be degraded. It might be due to the trimethylation of amino groups that increases the charge density and that could induce a stronger affinity between DNA and TMC (Mao et al., 2007). This could explain the low transfection efficiency of TMC (Fig. 3). However, this balance between stability and instability can induce different results *in vitro* and *in vivo*.

The difference observed between mono- and co-culture at 8 μg of DNA/ml complexed with CS could be due to the higher M-cell endocytosis capacity, allowing then more nanoparticles to enter cells. Besides, nanoparticles being bigger than polyplexes (200 nm vs. 100 nm) the transport rate was probably lower and/or the transport mechanism across cell membrane different, leading to reduced transfection efficiency. Differences disappeared when the nanoparticle concentration was increased. When cells were transfected with PEI polyplexes, luciferase expression did not increase with concentration, probably due to PEI cytotoxicity.

4.2. Interaction of nanoparticles with cells

CS is known to interact with cell membranes by electrostatic forces (Ma and Lim, 2003). It has some effects on cell membrane and can perturb it by interactions with the membrane bilayer (Fang et al., 2001).

Nanoparticles of CS have been described to be internalized by adsorptive endocytosis (Ma and Lim, 2003). Nanoparticles can be absorbed by different mechanisms: the clathrin-mediated endocytosis, the macropinocytosis and the uptake via caveolae. The most efficient route of uptake for polyplexes is not yet clearly identified and may depend both on the cell line and the polyplexe properties. But some data suggest that the clathrin-mediated endocytosis is predominant for the internalization of nanoparticles (Ma and Lim, 2003).

Following endocytocis, polyplexes have to escape the endosome and release the DNA. The buffering capacity of some cationic polymers, such as chitosan, seems to have some important effects on the endosomal escape (Ishii et al., 2001). It protects plasmids from lysosomal nucleases by reducing acidification of endosome and thus the activity of lysosomal enzymes (Nelson, 1991). It may also perturb the trafficking of endosomes by osmotic swelling and subsequent endosome disruption (Ishii et al., 2001). Polymers that are partially protonated, such as chitosan could retain a substantial buffering capacity while TMC, with permanent charges, could have a lower buffering capacity. It could explain the lower DNA release into the cells when transfected with TMC nanoparticles. In the endosome vesicle at pH 5, CS still has the ability to buffer the acidification. DNA will then escape the endosome and enter the nucleus. DNA co-localization with the nucleus demonstrated the efficiency of the CS to conduct it into the nucleus.

In conclusion, we developed a new nanocarrier made of fungal CS for DNA delivery. The nanoparticles were found to be suitable for the oral delivery and also promising for the oral DNA vaccination, due to the low amount of DNA required to induce a complete systemic and mucosal immune response with cellular immune activation.

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