Supplementary Information

Zinc-stabilized colloidal polyelectrolyte complexes of chitosan/hyaluronan, a tool for the inhibition of HIV-1 infection

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Methods

1. Preparation and characterization of chitosan

Prior to use, chitosan was purified as follows: dissolution in acetic acid aqueous solution, filtration through Millipore membranes of decreasing porosity (from 3 to 0.45 µm), precipitation with an ammonium solution, rinsing with deionized water until neutrality, and lyophilization.

Purified high M_w of chitosan was N-acetylated in homogeneous media at different DAs with acetic anhydride. The reaction was performed in a hydro-alcoholic mixture according to the procedure previously described by Vachoud *et al.*[1]. After re-acetylation, chitosans were neutralized, rinsed with deionized water, and then lyophilized.

In addition, hydrolysis of the polysaccharide to produce low-molar-mass chitosans was carried out, with a control of the reaction kinetics.[2] Briefly, the hydrolysis process of chitosan was performed as follows: chitosan samples of various DAs were dissolved at 0.5% (w/v) in an acetic acid/ammonium acetate buffer (0.2M/0.15M). A 10 $g \cdot L^{-1}$ of sodium nitrite solution was added to the chitosan solution to attain a nitrite/glucosamine units molar ratio of 0.1. According to the hydrolysis kinetic curves we previously established for each degree of acetylation [3], the reactions were performed under high mechanical stirring for 2 h to obtain M_w close to 1.50×10^5 $g \cdot mol^{-1}$ for chitosan with DA 48%. After hydrolysis reaction, chitosan was precipitated with ammonium hydroxide solution and purified by several washing with deionized water until neutrality, and finally lyophilized.

For the characterization, the weight-average molar mass (M_w) and the polydispersity index

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(I_p) were measured by an aqueous size exclusion chromatography (SEC) system consisting of a Agilent 1260 system equipped with a differential refractometer Wyatt Optilab T-rEX (λ = 658 nm) and interfaced with a multi-angle laser light scattering (MALLS) detector (Wyatt EOS). The separation was carried out on two gel columns (Tosoh TSK PW G2500 and TSK PW G6000). Elution was performed at 22 °C maintaining the flow rate at 0.5 mL·min^{-1}, and the degassed 0.2 M acetic acid/0.15 M ammonium acetate buffer with a pH 4.5 was used as the eluent. The samples were prepared at a concentration of 1 mg·mL^{-1} and filtered through a 0.45 µm pore-size membrane prior to injection.

2. Depolymerization and characterization of hyaluronan

Low molar mass HYAs were obtained by ultrasound assisted depolymerization described by Miyazaki, T. *et al.*[4], R.C. Polexe[5] and Wu & Delair[3]. The sonication was performed with an ultrasound Sonics Vibra Cell generator (Fisher Scientific Bioblock, France). In brief, HYA solution (1%, w/v) was put in a glass reactor of diameter Φ=3.5cm and a maximum liquid height H_{max}=7 cm. It was homogenized by magnetic stirring, and the reactor temperature was kept constant at 25°C during the experiment thanks to a water circulation in the double walled reactor.

For the characterization of hyaluronan (HYA), the SEC columns were PL aquagel-OH mixed M and PL aquagel-OH mixed H (300 × 7.5 mm, bead diameter: 8 µm). An aqueous buffer (0.066M Na_2HPO_4/0.066M KH_2PO_4 (65:35, v:v)) at pH 7.1 was used as the eluent. The dn/dc was fixed at 0.15.

3. Synthesis of fluorescein isothiocyanate-labelled chitosan

To synthesize fluorescein isothiocyanate(FITC)-labelled chitosan(F-CS), 50 mL of 1%wt chitosan was firstly dissolved in a stoichiometric amount of acetic acid solution, with respect to the free amines for the degree of acetylation. 50 mL of dehydrated methanol was slowly added with continuous stirring. FITC, dissolved in methanol at 2.0 mg·mL^{-1}, was then added into chitosan solution, to give a final FITC to D-glucosamine residue ratio of 1:50. After 3 h of reaction in the dark at ambient temperature, the FITC-labelled chitosan was precipitated in an ammonium hydroxide aqueous solution. The precipitate was collected after centrifugation at 11,000 rpm for 15 min, and purified by repeated wash cycles with water until no fluorescence was detected in the supernatant(exc = 490 nm, emi = 520 nm). The labelled chitosan was redissolved in acetic acid solution and dialyzed in the dark against 5L of water for 3 days, with replaced fresh water every 12 h. Finally, the labelled chitosan was lyophilized.

To determine the labelling efficiency (% w/w FITC to FITC-chitosan), the fluorescence intensity

of a solution of FITC-chitosan dissolved in acetic acid solution and diluted with phosphate buffer, pH 8, until a final concentration of 0.5µg·mL^{-1} was measured. The fluorometer was calibrated with standard solutions of 0.01 to 0.10 µg·mL^{-1} of FITC prepared by diluting 100 µg·mL^{-1} methanolic solutions of FITC with phosphate buffer, pH 8.0.

4. Physicochemical Characterization of the Complex Dispersions

Dynamic light scattering measurements of PECs dispersions were carried out using a Malvern Nanosizer SZ equipped with a 5mW He/Ne laser beam operating at λ = 633 nm (at 173° scattering angle). The self-correlation function was expanded in a power series (Cumulants method). The polydispersity value provided by the software is a dimensionless value defined by $\mu_2/(\Gamma)^2$, where μ_2 is the second cumulant of the correlation function and (Γ) is the average decay rate. All measurements were performed at least in triplicate with 10 measurements each at 25 °C.

Zeta potentials were derived from electrophoretic mobility measurements using Smoluchowski's equation. Electrophoretic mobilities (μE) of the particles were determined at 25°C with the Malvern Nanosizer SZ. μE was expressed as the average of 10 measurements with a relative error of 5%.

5. Transmission Electron Microscopy

The morphology of chitosan-HYA particles was examined by transmission electron microscopy (TEM, Philips CM120, UK). A droplet of 0.1% (w/v) washed nanoparticles dispersion was deposited onto a carbon film coated on copper grid. Excess solution was carefully blotted off using filter paper and air-dried at room temperature for imaging. The accelerating voltage used for the observation was 80 kV.

6. Evaluation of the drug encapsulation efficiency

For the determination of drug encapsulation efficiency (*EE*), TF loaded nanoparticles were separated from the suspension by centrifugation at 20,000g for 60 min. The free amount of TF in the supernatants was determined by measuring the absorbance of the supernatant at 259nm after subtraction of the blank value, using a calibration curve established in the same conditions. Each sample was measured in triplicate. *EE* was calculated by the following equations:

$$EE(\%) = ([TF]_{input} - [TF]_{residual})/([TF]_{input}) \times 100$$

Where $[TF]_{input}$ is the total concentration of TF which was put into the nanoparticles, $[TF]_{residual}$ is the concentration titrated from the supernatant, taking into account the background signal from a blank experiment representing particles dispersion without drug.

7. Antibody bioactivities test by ELISA assays

The recognition properties of either anti-OVA or anti-α4β7 antibodies on the CS-HYA particle surface were assayed by an enzyme-linked immunosorbent assay (ELISA).

Briefly, ninety-six-well plates were coated with 100 µL of a 1 µg·mL^{-1} antigen solution in PBS buffer (ovalbumin antigen and recombinant human integrin α4β7, from respectively Sigma Aldrich and R&D System, France) for 2 h at 37°C. The plates were then post-coated with 200 µL of 10% skimmed dry milk in PBS for 1 h at 37°C, and washed three times with 0.05% (v/v) Tween 20 in PBS (T-PBS). Serial dilutions of IgA/chitosan-HYA nanoparticles which were stored for respectively 1, 3 and 7 days, were diluted in 1% bovine serum albumin (BSA)-PBS and 100 µL of particles dispersions were added to the plates in duplicates and incubated for 1 h at 37°C. After three washes with T-PBS buffer, peroxidase-conjugated goat anti-human IgA-HRP (Invivogen) at a concentration of 0.2 µg·mL^{-1} in 1% BSA-PBS was added and the microplate was incubated for 1 h at 37°C. After washing, 100 µL of tetramethyl-benzidine(TMB) (BD Pharmingen, France) which was prepared according to the manufacturer's instructions, were added into the plates and put the plates in the dark for 10 min. The reaction was then quenched with 100 µL of 1N of sulphuric acid. The absorbance at 450 nm was measured with a Model 680 Microplate Reader.

Results and Discussion

1. Particle size distribution of chitosan-hyaluronan nanocomplexes

Particle size distribution of the chitosan-HYA complexes with polymer concentration of 0.1%(w/v), is shown in Fig. S1. The average size of the chitosan-HYA complexes was 295 nm, and the polydispersity index was 0.165, exhibiting relatively narrow particle size distribution.

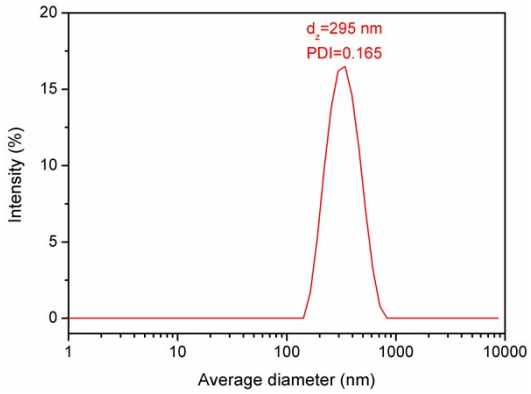

Fig.S1 Particle size distribution of the chitosan-HYA complexes nanoPECs (0.1%, w/v) measured by DLS. Condition: chitosan (DA=48%, M_w =1.23×10^{5}g·mol^{-1}), HYA (M_w=3.90×10^{4}g·mol^{-1}), $R_{n+/n-}$=2.

2. Colloidal stability of TF loaded chitosan-HYA nanocomplexes

The colloidal stability of 0.1% (w/v) TF loaded chitosan-HYA nanoPECs (theoretical final TF concentration of 10 μg·mL^{-1}) in water medium was monitored over time at room temperature. As can be seen in Fig.S2, TF/chitosan-HYA nanocomplexes maintained their stabilities in water for at least three months.

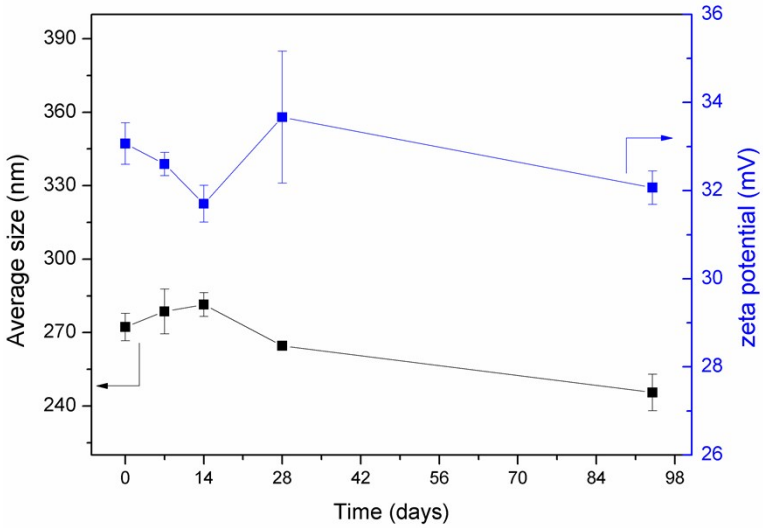

Fig. S2 Colloidal stability of TF loaded chitosan-HYA nanocomplexes

3. Characterization of FITC-labelled chitosan nanoparticles

In our attempt to evaluate the interaction of chitosan nanoparticles with biological structures (by confocal microscopy), the first step of the work was the development of a fluorescent derivative of chitosan that provides a stable fluorescent signal without causing great alteration in the characteristics of the nanoparticles. The association of fluorescein to the chitosan was identified by fluorescence spectrometer. Indeed, the excitation and emission wavelength values observed for the conjugate were 491 and 519 nm respectively, which was in accordance with the relative values of FITC. The effective grafting of the polymer with FITC was also assessed by ^{1}H-NMR spectroscopy (Brucker Avance III 400 MHz, Germany). The successful labelling was shown by ^{1}H-NMR spectra with the peak of the fluoresceinaromatic groups evident at about 7.00 ppm (not shown).

After calculation, the labelling efficiency was found to be approximately one FITC molecule per 60 d-glucosamine residues of chitosan. Typical reaction yield was 87.5%. Once identified the formation of the conjugate, the next step was to verify if the attachment of fluorescein to some amino groups of chitosan would affect both the mean sizes and zeta characteristics of the resulting nanoparticles. The results showed that the average nanoparticles sizes and zeta potential of FITC-CS nanoparticles (size 298 nm, zeta potential 33.6 mV) were similar to that of unmodified chitosan nanoparticles (size 295 nm, zeta potential 36.4 mV). Consequently, these results suggest that the

fluorescent labelling of chitosan should not affect significantly the biological behavior of the nanoparticles.

Table S1 Calculation of effective TF concentration in different nanoparticles concentrations formulation

Nanoparticles formulation	Theoretical final Conc. of TF(μg·mL^{-1})	Effective TF loaded, (μg·mL^{-1}) (particles)				
		Particlesconcentration(μg·mL^{-1})				
		1000	250	125	100	50
CS-HYA nanoparticles	5	4.70	1.17	0.588	0.470	0.235
	10	4.90	1.22	0.612	0.490	0.245
	20	5.80	1.45	0.725	0.580	0.290

References

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