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RESEARCH PAPER

The Counterbalanced Effect of Size and Surface Properties of Chitosan-Coated poly(isobutylcyanoacrylate) Nanoparticles on Mucoadhesion Due to Pluronic F68 Addition

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ABSTRACT

Purpose To evaluate of the effect of size and surface characteristics of poly(isobutylcyanoacrylate) nanoparticles coated with pluronic F68 and thiolated chitosan on mucoadhesion.

Methods Nanoparticles were obtained by radical emulsion polymerization in presence of different amounts of F68 (0–4%w/v). Mucoadhesion was *ex vivo* evaluated by applying nanoparticle suspension on rat intestinal mucosa and quantifying the amount of attached nanoparticles after incubation.

Results F68 unimers added in the polymerization medium allowed decreasing nanoparticle size from 251 to 83 nm, but resulted in nanoparticle surface modification. The amount of thiolated chitosan onto nanoparticle surface was decreased resulting in lower thiol groups and zeta potential. Consequently, the decrease of nanoparticle hydrodynamic diameter resulted in eight-fold-increase of the number of nanoparticles attached to the mucosa but a significant decrease of the weight of attached nanoparticles was observed. This unexpected result was due to a decrease of the amount of chitosan and thiolated chitosan available to interact with mucus upon addition of F68 in the polymerization medium.

Conclusions Addition of F68 should not be recommended to improve the amount of mucoadherent nanoparticles. Further studies could allow understanding if the low amount of small size nanoparticles could be able to improve oral bioavailability.

KEY WORDS chitosan · mucoadhesion · nanoparticles · pluronic F68 · poly(isobutylcyanoacrylate)

INTRODUCTION

Nowadays, it is well known that the low residence time in the gastrointestinal tract of drugs (such as paclitaxel (1) or insulin (2) among others) represents an important obstacle for their oral administration. So far, pharmaceutical investigations have produced several oral drug-delivery systems such as nanoparticles, which retain drugs in the gastrointestinal tract (3). Particularly, when mucoadhesive nanoparticles are orally administered in the form of a suspension, they can be immobilized at the intestinal surface by a mucoadhesion mechanism (4–6). Mucoadhesion slows the particle transit time through the gastrointestinal tract thereby enhancing drug absorption.

In this context, nanoparticles composed of poly(isobutylcyanoacrylate) (PIBCA) coated with thiolated chitosan for the oral delivery of drugs have been recently designed (7–11). They are core-shell nanoparticles formed by the auto-assembling of amphiphilic polymers composed of hydrophobic PIBCA and hydrophilic thiolated chitosan. The improvement of mucoadhesion is due to the covalent attachment of thiols on glycoproteins of the cystein residues

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of the mucus gel (12). However, in spite of the promising approach of mucoadhesion to increase the bioavailability of drugs delivered via mucosal tissues, important fundamental limitations of this approach still exist. Indeed, the elastic and viscous mucus layer has evolved to protect the body by rapidly trapping and removing nanoparticles. It can be suggested that mucus layer is divided into adherent mucus layer and luminal mucus layer. Adherent mucus layer which is close to the intestinal epithelial cells is cleared much slowly than the luminal mucus layer (for review see 13,14).

Due to the high mucus turnover in the luminal mucus layer, mucoadhesive nanoparticles attached to this external layer of the mucus are not expected to adhere for more than few hours. In order to overcome rapid mucus clearance and reach the underlying epithelia, nanoparticles must quickly traverse at least the outermost layers of the mucus barrier which are cleared most rapidly. As previously exposed in the review of Lai *et al.* (13), rapid mucus clearance mechanisms could be avoided by the design of mucus-penetrating nanoparticles providing thus sustained drug delivery for localized therapies in mucosal tissues (13).

The factors controlling intestinal absorption of nanoparticles are now better known. The nanoparticle size and shell composition have been determined as critical factors influencing their uptake (15,16 for review see 13). Particularly, many previous research works demonstrated that the diffusion of small-size nanoparticles through the mucus gel layer allowed enhancing drug bioavailability by escaping the mucus turnover (review see 13).

Interestingly, the size of nanoparticles composed of PIBCA coated with chitosan can be controlled by adding a surfactant to the polymerization medium. The addition of pluronic F68 (also known under the generic name of poloxamer P188 and the trade name of Lutrol F68) composed of $(\text{ethylene oxide})_{80}(\text{propylene oxide})_{27}(\text{ethylene oxide})_{80}$ block copolymers allowed to dramatically decrease the size of the obtained nanoparticles. This was reported with PIBCA nanoparticles without using polysaccharide (17) or by using non-thiolated chitosan (18). However, despite the evidence of the decrease of PIBCA nanoparticle size by addition of pluronic F68 in the polymerization medium, there is no research work about the effect of nanoparticle size decrease on mucoadhesion and possible nanoparticle surface modifications due to pluronic F68 addition. Thus, many questions remained to be explained: Is pluronic F68 adsorbed on chitosan-coated PIBCA nanoparticles? Or is it covalently linked to PIBCA? Are micelles required for decreasing nanoparticle size? Is it in the form of micelles or in the form of unimers in the polymerization medium? Dialysis is the method generally used to purify nanoparticle suspension. Is it an efficient purification method regarding to pluronic F68? What is the effect of pluronic F68 addition in the polymerization medium on the size of nanoparticles, their surface properties and their mucoadhesion?

In this context, the main objectives of the present work are to answer these questions and gain further insights on the real effect of pluronic F68 on mucoadhesion and surface modifications of nanoparticles.

MATERIALS AND METHODS

Materials

Isobutylcyanoacrylate (IBCA) was kindly provided as a gift by Henkel Biomedical (Dublin, Ireland). PolyFluor 570 (methacryloxyethyl thiocarbamoyl rhodamine B) was purchased from Biovalley Polyscience Inc® (Warrington, USA). Solvents (acetonitril, acetic acid and acetone) were provided from Carlo Erba®. Pluronic F68 (pharmaceutical grade) was a gift from BASF. Chitosan molecular weight (M_w) 400,000 g/mol, L-cystein HCl, ammonium cerium (IV) nitrate, sodium nitrite ($NaNO_2$), deuterium chloride solution (DCl) and starch from potato were purchased from Sigma (Saint-Quentin Fallavier, France). Nitric acid (63% v/v) was provided from Acros Chemicals, France. Sodium hydroxide, sodium chloride (NaCl), sodium dodecyl sulphate (SDS), $BaCl_2$, KI, and I_2 were purchased from Prolabo® (France). Finally 2-iminothiolane HCl (Traut's reagent) was synthesized in the Department of Organic Chemistry (Biocis UMR CNRS 8076), School of Pharmacy, University of Paris-Sud XI (Châtenay-Malabry) France. All other chemicals were of analytical grade. Phosphate buffer (pH 6.5) was prepared according to the US pharmacopeia.

Methods

Depolymerization and Thiolation of Chitosan

Chitosan was depolymerized following the method developed by Huang *et al.* (19). Briefly, 100 mL of a 2% (w/v) commercial chitosan (400,000 g/mol) solution in acetic acid (6%, v/v) was depolymerized at room temperature under stirring with 10 mL of $NaNO_2$ (8 g/L in MilliQ® water). After 1 h of reaction, chitosan was precipitated by raising the pH to 9.0 with NaOH (4 N). The white-yellow solid was filtrated, extensively washed with acetone and re-dissolved in a minimum volume of acetic acid 0.1 N (around 20–30 mL). Purification was carried out by subsequent dialyses against MilliQ® water (2× 1 L for 90 min and 1× 1 L over night) (Spectra/Por® 3 membrane MWCO: 3500). Dialysed product was freeze-dried (SMH15, Usifroid

Procédés Rieutord, Maurepas, France) and the yellowish lyophilizate was then stored at 4°C until use.

The depolymerized chitosan batch was prepared with an average M_w of 20,000 g/mol (Chito20), as evaluated by capillary viscosimetry (viscosimeter AVS400, Schott Geräte). Deacetylation percentage of chitosan was 60% as determined by ^{1}H-NMR analysis (Bruker MSL-400 spectrometer, Bruker Instrument Inc. Wissembourg, France) according to the method of Hirai *et al.* (20).

Thiolation of hydrolyzed chitosan was carried out following the method developed by Bernkop-Schnürch *et al.* (12) which was well described in previous works (12,21). One gram of chitosan was solubilised in 100 mL of acetic acid solution (1% v/v). The pH of the solution was adjusted to 6.5 with NaOH (1 N). Then, the Traut's reagent (2-iminothiolane) was added in a chitosan:iminothiolane weight ratio of 5:2. After an incubation period of 24 h at room temperature under continuous stirring, the resulting thiolated polymer was dialysed (Spectra/Por® 3 membrane MWCO: 3500) against different aqueous media: 8 h against 5 L of 5 mM HCl, two times 8 h against 5 L of 5 mM HCl containing 1% NaCl, 8 h against 5 L of 5 mM HCl and finally, 8 h against 5 L of 1 mM HCl (40 h in total). Dialysed product was freeze-dried (Christ Alpha 1–4 freeze-dryer. Bioblock Scientific, Illkirch, France) and stored at −20°C until use. The corresponding thiolated polymer was chitosan-4-thiol-butylamidine, and was named Chito20-TBA according to the original M_w of the unmodified polymer.

Nanoparticle Preparation

Radical emulsion polymerization was carried out according to previous works (22) and adapted to chitosan (7). Briefly, 0.069 g of mixtures of chitosan and thiolated chitosan (Chito20/Chito20-TBA 75/25% w/w) were dissolved in 4 mL of 0.2 mol/L nitric acid containing different percentages of pluronic F68 (0, 1, 2, 3 and 4% w/v) in a glass tube at 40°C, under vigorous stirring and argon bubbling. After 10 min, 1 mL of a solution of 8×10^{-2} M ammonium cerium (IV) nitrate in 0.2 mol/L nitric acid, and 0.250 mL of IBCA were added under vigorous magnetic stirring. Argon bubbling was kept for additional 10 min and stopped. The reaction was allowed to continue at 40°C under vigorous stirring for 50 min.

Control rhodamine labelled PIBCA nanoparticles were elaborated by anionic emulsion polymerization at room temperature without adding chitosan in the polymerization medium (control A). Pluronic F68 solution (1% w/v) was prepared in HCl 0.01 N. After 10 mn of vigorous stirring and argon bubbling, 0.250 mL of IBCA were added. Then 1 mL of PolyFluor 570 (2 mg/mL in acetonitrile) were added after 5 mn. Argon bubbling was kept for additional 10 min and stopped. The reaction was allowed to continue under vigorous stirring for 6 h.

Rhodamine-labelled nanoparticles were elaborated similarly with adding 1 mL of PolyFluor 570 acetonitrile solution (2 mg/mL) to the polymerization medium immediately after the addition of ammonium cerium (IV) nitrate. After 2 min, 0.250 mL of IBCA were added to the medium and allowed to react 110 min protected from light.

Control rhodamine-labelled PIBCA nanoparticles coated with chitosan (without thiolated chitosan) were elaborated similarly without thiolated chitosan (Chito20/Chito20-TBA 100/0% w/w) (control B).

Tthe purification of nanoparticles was achieved by dialysis using a Spectra/Por membrane with a molecular weight cut-off of 100,000 g/mol (Biovalley, Marne la Vallée, France) twice for 30 min, twice for 60 min and once overnight against 1 L of acetic acid 16 µmol/L. Rhodamine labelled nanoparticle dialysis should be achieved protected from light.

Nanoparticle Characterization

Particle Size Distribution. The hydrodynamic diameter of the nanoparticles was determined at 25°C by quasi-elastic light scattering using a Zetasizer Nanoseries Nano-ZS (Malvern Instruments, France). The scattered angle was fixed at 90° and 30 µL of each sample was diluted in 1 mL of MilliQ® water.

Zeta Potential Determination. The zeta potential of nanoparticles was measured using Zetasizer Nanoseries (Malvern Instruments, France). Dilution of the suspensions (1:33 (v/v)) was performed by adding 60 µL of each sample in 2 mL of NaCl (1 mmol/L).

Thiol Content Determination. The quantification of reduced thiol groups on the nanoparticle surface was determined using the iodine titration method (23). In brief, 0.250 mL of nanoparticle suspension was mixed with 0.250 mL of acetate buffer solution at 1 mol/L (pH 2.7). Then, 1 mL of starch solution (1% w/v) and 0.1–0.5 mL of iodine (1 mmol/L) were added at each preparation. The reaction was allowed to proceed for 24 h at room temperature and protected from light. Then, samples were centrifuged (10 min, 3,500 rpm) and the supernatants were measured at 560 nm (Spectrophotometer UV/VIS lambda 11 Perkin Elmer. Norwalk, USA). Control samples were prepared from nanoparticles elaborated with non-thiolated chitosan. The amount of thiol moieties was calculated from the corresponding standard curve elaborated under the same conditions with L-cystein HCl solutions (0.04–0.124 mmol/L).

Iodine promotes the oxidation of the free thiol groups. To avoid interferences with oxygen, all samples were degassed with argon after the addition of iodine. The excess of iodine was able to react with starch giving a quantitative blue complex easily measured by spectrometry (λ=560 nm). This indirect dosage method allowed the determination of the amount of reduced thiol groups present in the sample.

Determination of Pluronic F68 Concentration. The concentration of pluronic F68 was determined by a colorimetric method, adapted from Leo *et al.* (24), based on the formation of a colored insoluble complex between two hydroxyl groups of pluronic F68, Ba^{2+} and iodine molecule. In brief, 1 mL of nanoparticles suspension (diluted 1:20 in MilliQ® water) was mixed with 0.25 mL of $BaCl_2$ solution (5% w/v in HCl 0.1 M), and 0.25 mL of I_2/KI solution (0.05 M/0.15 M). The eppendorf was kept in a shaker for 15 min at 25°C, protected from light. The insoluble colored complex was formed. The complex was separated by centrifugation at 11 000 rpm for 15 min (6×10^3 g). The supernatant was analyzed by using a spectrophotometer at 540 nm (Shimadzu, Roucaire Instruments).

The amount of pluronic F68 contained in the samples was calculated from the corresponding standard curve elaborated under the same conditions with an aqueous solution of pluronic F68 (concentrations were ranging from 0.02 to 1 mg/mL).

The determination of pluronic F68 concentration concerned specific steps of the manufacturing process (e.g. dialysis efficiency and adsorption on ultracentrifuge tube). In the aim to investigate the efficacy of dialysis method for the purification of pluronic F68, the concentration of solutions of pluronic F68 (1, 2, 3 and 4% w/v) was determined before (denoted F68-A) and after dialysis (denoted F68-B). In the aim to investigate the possible adsorption of pluronic F68 onto Microcon® centrifugal filters, the concentration of solutions of pluronic F68 (1, 2, 3 and 4% w/v) was determined before and after ultrafiltration. The concentration of pluronic F68 eliminated by dialysis (denoted F68-C) was calculated from the difference between F68-A and F68-B.

Furthermore, pluronic F68 concentration on nanoparticle shell (F68-D) was evaluated after dialysis step. Pluronic F68 contained on nanoparticles was separated from pluronic F68 in solution that has not been removed by dialysis (F68-E) by using ultrafiltration over Microcon® centrifugal units YM-30 (30,000 MWCO) (15 min, 6×10^3 g) (8).

Micro-DSC

The measurements of micro-DSC were carried out with a calorimeter Micro-DSC III Setaram in the aim to determine the critical micellization temperature (CMT) of pluronic F68 solution at different concentrations (1, 2, 3 and 4% w/v). Pluronic F68 solution at a concentration of 20% w/w was used as a control. The cells used to deposit the sample and the reference (distilled water) were the type batch (1 mL). Two empty cells with the caps were weighed after complete drying and the joints were chosen to obtain the same mass (±0.2 mg). The sample and the reference should be introduced to the cells at room temperature and weighed for the identical mass (±0.3 mg). After their insertions into the oven at room temperature, the temperature of the oven was reduced until 5°C with 1°C/min. After 1 h at 5°C to equilibrate the thermal flow, the scan of temperature was performed at 0.1°C/min until 70°C. After this, the oven should stay 1 h at 70°C to balance the thermal flow before the beginning of fusion with −0.1°C/min to 5°C.

The analysis of the enthalpograms led to the determination of the CMT according to the previously described and well known method (25).

Ex Vivo Evaluation of Nanoparticle Mucoadhesion

Ex vivo mucoadhesion studies on rat intestinal mucosa were carried out according to a previous method developed by Durrer *et al.* (26,27).

Preparation of the Mucosa. Male Wistar rats (200–250 g) (Charles River, Paris) were used for the *ex vivo* mucoadhesion evaluation of nanoparticles. Animals were sacrified with an overdose of anaesthesia pentobarbital. Fresh small intestine (jejunum) of sacrificed rats was excised, rinsed with Ringer solution (pH 6.8) and cut into segments of 4 cm length. Each segment was opened lengthwise along the mesentery and spread in an aluminium plate. A second aluminium plate with a 2 cm^2 slit in the centre was then fixed on the so-prepared mucosa sample. Due to the important role of peyer's patches and M-cells in nanoparticle uptake (28), all mucosal samples were free from peyer's patches and M-cells. The agreement for animal experiments is A92-019-01.

Nanoparticle Mucoadhesion Experiment. Briefly, 0.5 mL of fluorescent labelled nanoparticles (20 mg/mL) in MilliQ® water was led in contact with a delimited intestinal mucosa (the surface was equal to 2 cm^2) at room temperature and protected from light. After a contact time of 120 min, nanoparticle suspensions were sucked off. Samples were rinsed three times with 1 mL of Ringer solution (pH 6.8) for 1 min each, to eliminate non-attached nanoparticles. Subsequently, the mucus layer, including the attached nanoparticles, was scraped off the membrane and dispersed in 5 mL solution composed of NaOH1%/SDS2%. Samples were treated for 2 h by ultrasonication and left overnight at room temperature

under magnetic stirring until mucus and nanoparticles were completely dissolved.

Nanoparticle Quantification. The fluorescent signal of attached nanoparticles was measured in a fluorimeter (Perkin Elmer spectrometer L550B, Norwalk, USA) with excitation and emission wavelengths of 561 and 577 nm, respectively. Data were compared with the corresponding calibration curves elaborated in the same conditions. Results were expressed as the amount of attached nanoparticles per apparent surface (g/m^2). Each sample was tested in three different rats.

In a further step, the number of attached nanoparticles was calculated as follows:

$$\mathcal{N} = \frac{m_{attached}\, 6}{\rho S d} \quad (1)$$

where $\mathcal{N}$ is the number of attached nanoparticles, $m_{attached}$ the mass of attached nanoparticles (g), ρ the nanoparticles density (1.2 g/cm^3), d the nanoparticle diameter (cm) and S the nanoparticle surface: $S=4\pi(d/2)^2$. Furthermore, assuming that the surface occupied by a spherical particle on the biological surface must be considered as the surface of the square whose length is the particle sphere diameter, it was possible to calculate the number of layers that theoretically cover the 2 cm^2 of biological external surface used in this study (Υ):

$$\Upsilon = \frac{\mathcal{N} d^2}{2} \quad (2)$$

Statistical Analysis. The results obtained were statistically analysed by using Mann–Whitney's *t*-test with a 95% confidence level. (n=4).

RESULTS

Physico-Chemical Characterization of Nanoparticles

As reported from previous works on PIBCA nanoparticles, the mean hydrodynamic diameter of non-labelled nanoparticles decreased by increasing pluronic F68 in the polymerization medium from 0 to 4% w/v. This was observed when preparations were performed in the absence of polysaccharide in the polymerization medium (17) and in the presence of a define concentration of chitosan (18) (Table I).

Results obtained in the polymerization conditions considering the presence of thiolated chitosan, the increase of pluronic F68 concentration from 0 to 4% w/v decreased nanoparticle size from 206 to 68 nm. In parallel, the amount of free thiol groups was decreased from 1.789 to 0.031 mol/mg respectively and the zeta potential of the nanoparticles was reduced from +38 to +12 mV. Free thiol groups and zeta potential were the actual parameters that were considered to evaluate the effect of the presence of pluronic F68 in the polymerization medium on the nanoparticle surface characteristics.

Rhodamine-labelled nanoparticles were prepared by the covalent anchoring of rhodamine labelled monomer to the nanoparticle acrylic core during the polymerization process. As we can observe from Table I, the hydrodynamic diameters, zeta potential and thiol content of labelled and non-labelled nanoparticles were not significantly different except for control PIBCA nanoparticles elaborated without chitosan.

Micro-DSC Characterization of Pluronic F68

Pluronic F68 co-polymer is able to auto-associate in the form of micelles at concentrations higher than the critical micelle concentration or at temperatures above the critical

Table I Physicochemical Characterization of PIBCA/(Chito20/Chito20-TBA) (75/25)% Nanoparticles Elaborated by Radical Emulsion Polymerization as Function of the Initial Percentage of Pluronic F68 Present in the Polymerization Medium

	Sample code	D ± SD (nm)	ζ potential (mV)	Concentration of –SH[a] (mol/mg)
Non-labelled nanoparticles	Np 0%	206 ± 2	+38	1.79
	Np 1%	134 ± 2	+31	0.58
	Np 2%	101 ± 1	+23	0.21
	Np 3%	93 ± 2	+14	0.06
	Np 4%	68 ± 1	+12	0.03
	Np Control[b]	103 ± 3	−7	0.00
Rhodamin labelled nanoparticles	Np Rh 0%	221 ± 3	+38	1.90
	Np Rh 1%	134 ± 2	+30	0.60
	Np Rh 2%	106 ± 1	+20	0.19
	Np Rh 3%	99 ± 3	+15	0.05
	Np Rh4%	83 ± 3	+12	0.03
	Np Control Rh[b]	262 ± 2	−10	0.00

[a]iodo titration, [b]PIBCA nanoparticles not coated with chitosan stabilized with pluronic F68

micelle temperature. As reported with other pluronics such as F127 and L64 (29–31), the CMC of pluronic F68 was found to be strongly dependent on temperature (32–34).

Upon temperature increase, micellization of pluronic F68 solution at 20% w/w used as control appears on thermogram as broad and asymmetric exothermic peak spreading over approximately 22°C (Fig. 1a). Two temperatures could be defined from the thermogram T_{onset} and T_{endset}, the temperature of the beginning and the end of the micellization process respectively. T_{onset} corresponding to the critical micelle temperature of pluronic F68 solution at a concentration of 20% w/w was equal to 52°C.

Samples with concentrations ranging from 1 to 4% w/v showed no micellization even at high temperatures (Fig. 1b). This means that at the polymerization temperature of 40°C, pluronic F68 was in the form of unimers whatever its concentration.

The MicroDSC experiments did not allow to confirm the hypothesis of the covalent linkage of pluronic F68 to PIBCA but allowed to conclude that whatever its concentration in the polymerization medium (from 1 to 4% w/v), pluronic F68 is in the form of unimers and not in the form of micelles. This result was important as it excluded a micellization mechanism for reducing PIBCA nanoparticle size.

Determination of Pluronic F68 Concentration before and after Dialysis and at the Surface of Nanoparticles

In the aim to investigate the effectiveness of dialysis method to remove pluronic F68, the concentrations of pluronic F68 solutions (1, 2, 3 and 4% w/v) were determined before and after dialysis by using cellulose membranes of 100 kDa molecular weight cut off. As we can see from Table II, the dialysis method was efficient for the purification of pluronic F68 because all concentrations after dialysis were too low and can not be detected by the analytical method used.

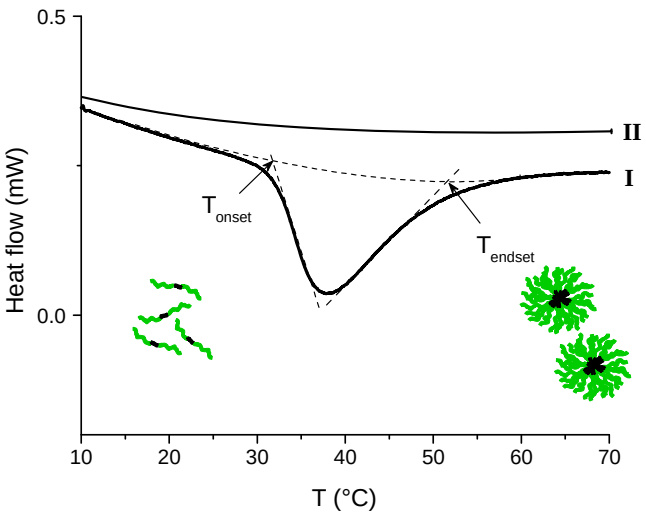

Fig. 1 Micro DSC trace of the pluronic F68 aqueous solution at concentration of 20% w/w (**I**) and concentrations of 1, 2, 3 and 4% w/v (**II**). Exothermic peak of micellization and definition of T_{onset} and T_{endset}.

Table II Effect of Dialysis and Ultrafiltration of F68 Concentration. The Concentration of Pluronic F68 was Determined by Colorimetric Method Described in "Determination of Pluronic F68 Concentration"

Theoretical initial concentration of F68 solution (% w/v)	Experimental F68 concentration before dialysis (% w/v)	F68 concentration after dialysis (% w/v)	F68 concentration in the ultrafiltrate (% w/v)
0	0.00	–	–
1	1.05	–	1.05
2	1.89	–	1.89
3	3.05	–	3.05
4	3.93	–	3.93

(–) Non-detectable concentration of pluronic F68

In the aim to investigate the possible adsorption of pluronic F68 onto Microcon® centrifugal filters used to separate nanoparticles from the aqueous medium, the concentrations of solutions of pluronic F68 (1, 2, 3 and 4% w/v) were determined before and after ultrafiltration. As we can see from Table II, no adsorption of pluronic F68 was observed on Microcon® centrifugal filters. Total pluronic F68 was found in the ultrafiltrate.

The pluronic F68 concentrations into nanoparticle suspensions were determined before and after dialysis (F68-A and F68-B respectively). Each nanoparticle suspension was then separated by using ultrafiltration through Microcon® centrifugal filters. The concentrations of pluronic F68 into nanoparticle suspension after ultrafiltration (F68-D) and in the ultrafiltrate (F68-E) were then determined.

As we can see from the results in Table III, the concentrations of pluronic F68 into nanoparticle suspensions after dialysis were lower than the initial concentration of pluronic F68 indicating that a part of pluronic F68 was removed by dialysis (F68-C).

Because all the concentrations of pluronic F68 in the ultrafiltrates (F68-E) were below resolution limit, the pluronic F68 found in the nanoparticle suspension after dialysis (F68-B) can be attributed to pluronic F68 covalently linked to PIBCA core that cannot be removed by dialysis.

Mucoadhesion Results

Mucoadhesion results of controls A and B presented in Fig. 2 confirmed that the presence of chitosan on PIBCA nanoparticle surface (control B) clearly enhanced mucoadhesion behaviour in comparison with PIBCA (control A) after 120 min of contact time with rat intestinal jejunum. The mucoadhesion can be two-fold-enhanced by the

Table III Determination of the Concentration of Pluronic F68 Before Dialysis (F68-A) and After Dialysis (F68-B). The Concentration of Pluronic F68 Eliminated by Dialysis was Calculated from F68-A and F68-B*. The Concentration of Pluronic F68 Associated to Nanoparticles (F68-D) and in the Ultrafiltrate were then Evaluated. All the Concentrations of Pluronic F68 were Determined by Colorimetric Method Described in "Determination of Pluronic F68 Concentration" Lower Scheme Represents a Microcon® Centrifugal Filter

Sample code	F68-A: Total F68 concentration into nanoparticle suspension before dialysis (% w/v)	F68-B: Total F68 concentration into nanoparticle suspension after dialysis (% w/v)	F68-C: Concentration of F68 eliminated by dialysis (% w/v)*	F68-D: F68 concentration still associated with Nps after ultrafiltration (% w/v)	F68-E: F68 concentration in the ultrafiltrate (% w/v)
Np 0%	0.00	0.00	0.00	0.00	0.00
Np 1%	1.04	0.55	0.49	0.55	0.00
Np 2%	1.89	1.08	0.81	0.85	0.02
Np 3%	3.04	1.18	1.86	1.15	0.02
Np 4%	3.93	1.49	2.44	1.43	0.06

*(F68-C) = (F68-A)-(F68-B)

introduction of thiol groups at the surface of nanoparticles composed of PIBCA/(Chito20/Chito20-TBA) (75/25)% (Fig. 2). These results were in agreement with previous data (35).

The effect of pluronic F68 addition on mucoadhesion was then evaluated from two points of views. On one hand we considered the effect of pluronic F68 added to the polymerization medium on nanoparticle mucoadhesion, and on the other hand, we considered the effect of pluronic F68 added to the nanoparticle suspension on their mucoadhesion. This second investigation allowed understanding the effect of pluronic F68 on mucoadhesion independently on the size or the surface modifications of nanoparticles. Indeed, a previous work by Hillery and Florence, the adsorption of pluronic F68 surfactant on polystyrene nanoparticles appeared to completely inhibit particle uptake in the small intestine (36).

As we can see from Fig. 2, the decrease of nanoparticle size resulted in a decrease of the weight of attached nanoparticles on mucosa (g/m²) (Fig. 2I) and the calculated number of nanoparticle layers that cover the intestinal mucosa (Table IV). However, the number of nanoparticles attached to the mucosa increased dramatically (Fig. 2II).

Finally, the effect of the addition of pluronic F68 into final nanoparticle suspension (Np Rh 0%) on mucoadhesion was investigated. The concentrations of pluronic F68 added to nanoparticle suspension were similar to the ones found into nanoparticles (Np Rh 1%, Np Rh 2%, Np Rh 3%, Np Rh 4%) after dialysis (0.55, 0.85, 1.15 and 1.43% w/v). As we can see from the results in Fig. 3, whatever the concentration of pluronic F68 added to nanoparticle suspension, the amount of nanoparticles attached to the mucosa and the calculated number of nanoparticle layers that cover the intestinal mucosa

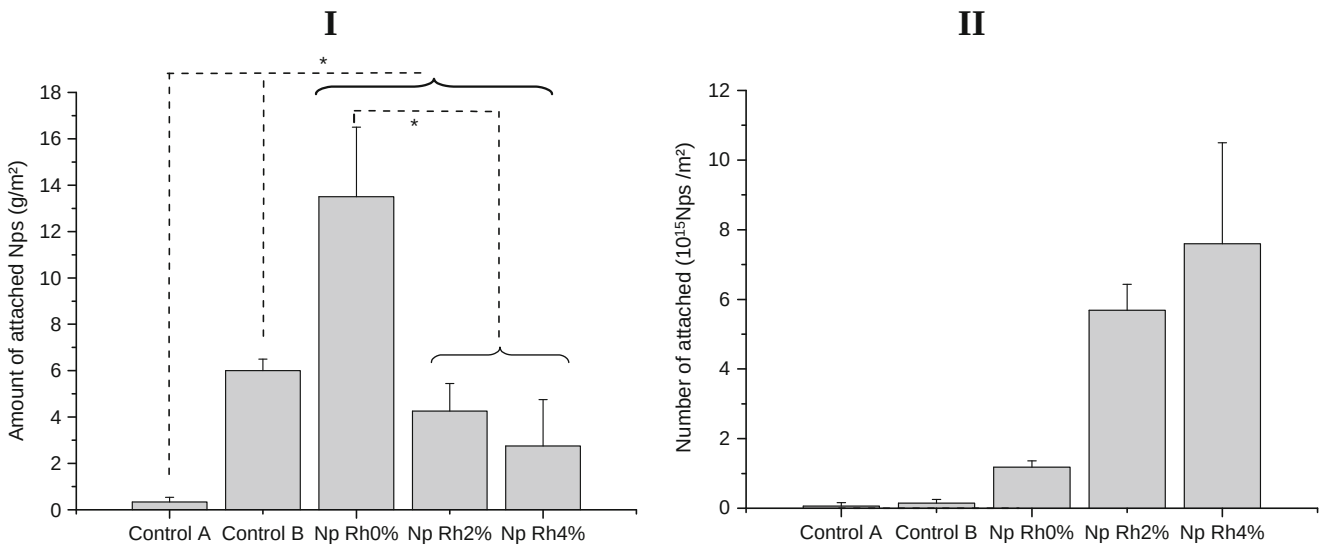

Fig. 2 Effect of nanoparticle composition on mucoadhesion. In the left graph (**I**), mucoadhesion is expressed by g/m². In the right graph (**II**), mucoadhesion is expressed in terms of number of attached nanoparticles on m² of intestinal mucosa. Mucoadhesion experiments were performed with nanoparticles at a concentration of 20 mg/mL after 120 min of contact time. Controls consisted on PIBCA nanoparticles without chitosan (control A) and PIBCA nanoparticles coated with non-thiolated chitosan (PIBCA/Chito20) (control B). (*) represents significant difference ($P < 0.05$).

Table IV Number of Layers that Theoretically Cover 2 cm^2 of Biological External Surface Calculated According to Eq. 2

	Sample code	Number of layers/cm^2
Effect of F68 addition in the polymerization medium	Np Rh 0%	22
	Np Rh 2%	16
	Np Rh 4%	13
Effect of F68 addition to nanoparticle suspension Rh 0%	Np Rh 0%	22
	Np Rh 0% + 0.85%	21
	Np Rh 0% + 1.43%	25
Control A	Np Control Rh[a]	1

[a] PIBCA nanoparticles not coated with chitosan stabilized with pluronic F68

(Table IV) are not significantly changed. It means that pluronic F68 did not preclude the nanoparticle adhesion to mucosa.

DISCUSSION

A major part of administered poly(alkylcyanoacrylate) nanoparticles do not adhere the mucus layer, but undergoes direct transit through the gastrointestinal tract (37). The consequent short transit time in the gastrointestinal tract can be inadequate for poly(alkylcyanoacrylate) nanoparticles to release a significant fraction of encapsulated drugs. Consequently, the realization of a high local drug concentration over extended periods of time was precluded leading to low bioavailability and poor efficacy. To overcome the short transit time, many researchers have sought to enhance the mucoadhesion of poly(alkylcyanoacrylate) nanoparticles in order to improve their retention at the mucosal surfaces by modifying their surface composition with chitosan (38). The analysis of mucoadhesion results of the controls A and B in Fig. 2 confirmed that the presence of chitosan on PIBCA nanoparticle surface (control B) clearly enhanced mucoadhesion behaviour in comparison with PIBCA (control A) after 120 min of contact time with rat intestinal jejunum (38). In this case, mucoadhesion of chitosan-coated PIBCA nanoparticles was obtained by the building of non-specific weak chemical bonds such as electrostatic attraction, hydrophobic interactions, van der Waals' forces and hydrogen bonds. Ionic interactions are driven by the positive charge of the chitosan due to primary amino groups and negatively charged sialic acid and sulfonic acid of the mucus (39).

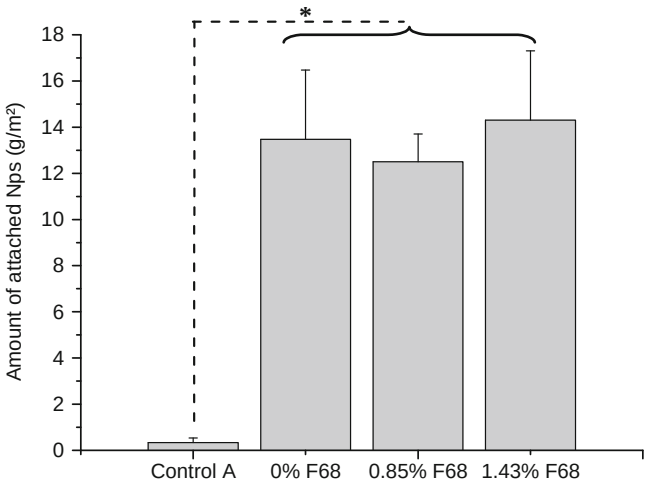

Fig. 3 Effect of addition of pluronic F68 done to the suspension of already formed nanoparticle on their mucoadhesion (g/m^2). Mucoadhesion experiment performed with nanoparticles composed of PIBCA/(Chito20/Chito20-TBA) (75/25)% at a concentration of 20 mg/mL after 120 min of contact time. Control A consisted on PIBCA nanoparticles without chitosan. (*) represents significant difference ($P < 0.05$).

Mucoadhesion can also be improved through specific interactions when a ligand attached to the nanoparticle is used for the recognition and the interaction with a specific site at the mucosal surface. The mucoadhesion can be two-fold-enhanced by the introduction of thiol groups at the surface of nanoparticles composed of PIBCA/(Chito20/Chito20-TBA) (75/25)% (Fig. 2). This result confirmed that the mucoadhesion capacity of nanoparticles coated with thiolated chitosan was increased by forming covalent bonds with the cystein residues of the mucus glycoproteins by Bravo-Osuna *et al.* (35).

In this work, we investigated the effect of pluronic F68 addition in the polymerization medium and after nanoparticle preparation on mucoadhesion. As demonstrated for PIBCA nanoparticles without chitosan (17) and PIBCA nanoparticles coated with non-thiolated chitosan (18), it was possible to significantly decrease PIBCA/(Chito20/Chito20-TBA) nanoparticle size by adding pluronic F68 in the polymerization medium. The polymerization was initiated by the reaction of Cerium ions with chitosan dissolved in the continuous phase of the emulsion formed by the hydrophobic isobutylcyanoacrylate monomer. The amphiphilic polymer formed by PIBCA and thiolated chitosan auto-associates to form positively charged nanoparticles (D_h=206 nm, ζ potential=+38 mV) (Table I) (Fig. 4I). In the presence of both thiolated chitosan and pluronic F68 unimers in the polymerization medium, a competition occurred between the two polymers (Fig. 4II). The confirmation of this hypothesis comes from the surface analysis of the obtained nanoparticles because the increase of pluronic F68 concentration from 0 to 4% w/v decreased the amount of free thiol groups from 1.8 to 0.031 mol/mg and the total positive charge of nanoparticles from +38 to +12 mV (Table I).

Because the excess of un-reacted pluronic F68 was totally removed by dialysis (Table II), the pluronic F68 found in the purified nanoparticle suspension (F68-D) was attributed to pluronic F68 covalently bound to PIBCA polymer and not

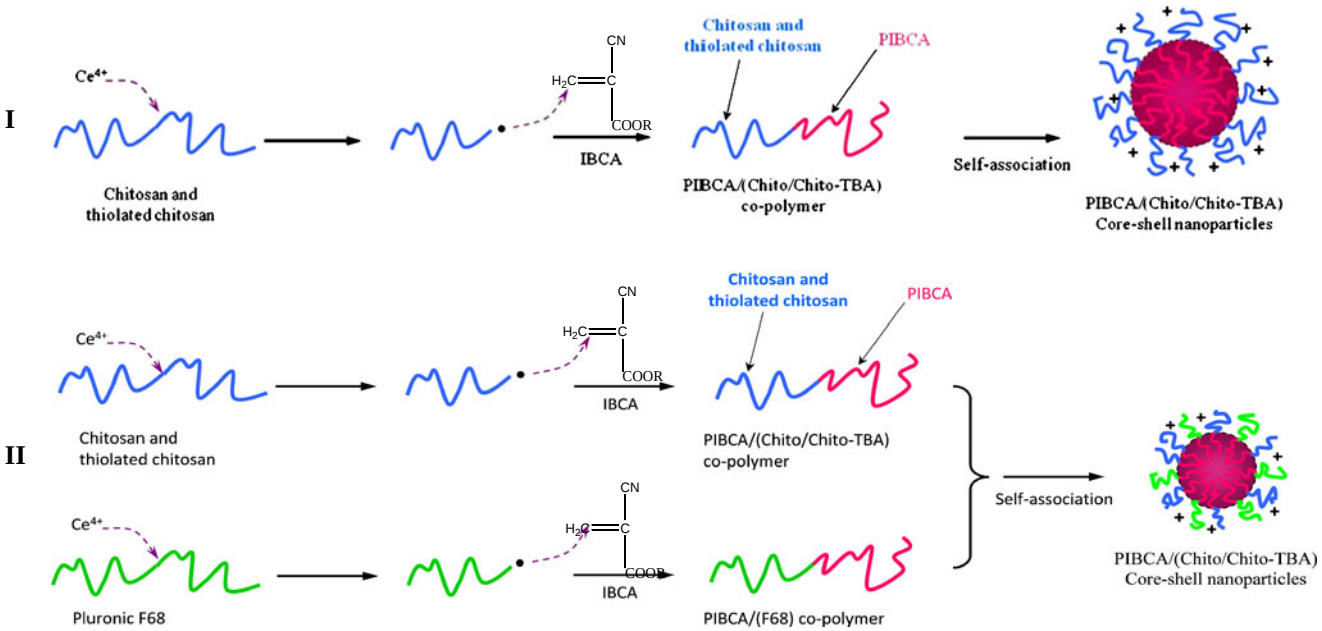

Fig. 4 Hypothetic sketch of PIBCA/(Chito/Chito-TBA) core-shell nanoparticles formation without pluronic F68 (**I**) and in the presence of pluronic F68 (**II**).

to pluronic F68 adsorbed onto nanoparticles. These results demonstrated that the addition of pluronic F68 in the polymerization medium resulted in a decrease of nanoparticle size but also in modification of nanoparticle surface characteristics. These size and surface modifications directly affected the nanoparticle mucoadhesion. As we can see from Fig. 2, the decrease of nanoparticle size resulted in a decrease of the weight of attached nanoparticles on mucosa (g/m²), while their number increased dramatically. The decrease of the amount of attached nanoparticles to the mucosa could be related to the lower zeta potential and thiol amounts at the surface of nanoparticles. Both non-specific and specific interactions between nanoparticle surface and mucus layer were hence decreased. It is noteworthy that the presence of free pluronic F68 did not preclude the nanoparticle adhesion to mucosa. As we can see from the results in Fig. 3, no significant difference was found when pluronic F68 was added.

The combination of all these results led to the conclusion that pluronic F68 added in the polymerization medium resulted in a decrease of nanoparticle size but also in nanoparticle surface modification by the competition between pluronic F68 and thiolated chitosan chains during polymerization. However, the weight of attached nanoparticles (g/m²) was lowered meaning that lower amount of the drug would be available at the mucosal surface if the total drug-loaded nanoparticle weight attached is reduced. Further *ex vivo* and *in vivo* tests could be useful to understand if the low amount of small-size nanoparticles will be able to improve oral bioavailability of the encapsulated drug in comparison with a higher amount of drug likely to be brought by larger-sized nanoparticles.

CONCLUSIONS

We proved that the addition of pluronic F68 in the polymerization medium decreased thiolated chitosan-coated PIBCA nanoparticle size but also the total thiol groups and the positive charge at the nanoparticle surface. Consequently, the addition of pluronic F68 decreased the total weight of nanoparticles attached to rat intestinal mucosa because both specific and non-specific interactions of chitosan and thiolated chitosan with mucus were decreased. Thus the systematic use of pluronic F68 to decrease nanoparticle size should be used carefully because it resulted in a decrease in the weight of attached nanoparticles. Further *ex vivo* and *in vivo* studies should be useful to understand if size decrease could result in an improved diffusion of the particles in the mucus layer, favoring drug absorption.

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REFERENCES

1. Agüeros M, Ruiz-Gatón L, Vauthier C, Bouchemal K, Espuelas S, Ponchel G, *et al.* Combined hydroxypropyl-β-cyclodextrin and poly(anhydride) nanoparticles improves the oral permeability of paclitaxel. Eur J Pharm Biopharm. 2009;38(4):405–13.
2. Sarmento B, Ribeiro A, Veiga F, Ferreira D, Neufeld R. Oral bioavailability of insulin contained in polysaccharide nanoparticles. Biomacromolecules. 2007;8:3054–60.
3. Mc Clean S, Prosser E, Meehan E, O'Malley D, Clarke N, Ramtoola Z, *et al.* Binding and uptake of biodegradable poly-DL-lactide micro- and nanoparticles in intestinal epithelia. Eur J Pharm Sci. 1998;6:153–63.
4. Galindo-Rodriguez SA, Allemann E, Fessi H, Doelker E. Polymeric nanoparticles for oral delivery of drugs and vaccines: a critical evaluation of *in vivo* studies. Crit Rev Ther Drug Carr Syst. 2005;22: 419–64.
5. Ponchel G, Irache J. Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract. Adv Drug Deliv Rev. 1998;34:191–219.
6. Woodley J. Bioadhesion: new possibilities for drug administration? Clin Pharmacokinet. 2001;40:77–84.
7. Bravo-Osuna I, Schmitz T, Bernkop-Schnürch A, Vauthier C, Ponchel G. Elaboration and characterization of thiolated chitosan-coated acrylic nanoparticles. Int J Pharm. 2006;316: 170–5.
8. Bouchemal K, Ponchel G, Mazzaferro S, Campos-Requena V-H, Gueutin C, Palmieri G-F, *et al.* A new approach to determine loading efficiency of Leu-enkephalin in poly(isobutylcyanoacrylate) nanoparticles coated with thiolated chitosan. J Drug Del Sci Tech. 2008;18(6):392–7.
9. Mazzaferro S, Bouchemal K, Gallard J-F, Iorga BI, Cheron M, Gueutin C, *et al.* Bivalent sequantial binding of docetaxel to methyl-β-cyclodextrin. Int J Pharm. 2011; doi:10.1016/j.ijp.2011.06.034.
10. Sajeesh S, Bouchemal K, Sharma CP, Vauthier C. Surface functionalized polymethacrylate acid based hydrogel microparticles for oral drug delivery. Eur J Pharm Biopharm. 2010;74(2):209–18.
11. Sajeesh S, Bouchemal K, Vauthier C, Sharma CP. Cyclodextrin complexed insulin encapsulated hydrogel microparticles: an oral delivery system for insulin. J Control Release. 2010;147:377–84.
12. Bernkop-Schnürch A, Kast CE, Guggi D. Permeation enhancing polymers in oral delivery of hydrophilic macromolecules: thiomer/GSH systems. J Control Release. 2003;93:95–103.
13. Lai SK, Wang YY, Hanes J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tiussues. Adv Drug Deliv Rev. 2009;61:158–71.
14. Cone RA. Barrier properties of mucus. Adv Drug Deliv Rev. 2009;61:75–85.
15. Lai S, O'hanlon E, Harrold S, Man ST, Wang YY, Cone R, *et al.* Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus. PNAS. 2007;104(5):1482–7.
16. Tang BC, Dawson M, Lai SK, Wang YY, Suk JS, Yang M, *et al.* Biodegradable polymer nanoparticles that rapidly penetrate the human mucus barrier. PNAS. 2009;106(46):19268–73.
17. Seijo B, Fattal E, Roblot-Treupel L, Couvreur P. design of nanoparticles of less than 50 nm diameter: preparation, characterization and drug loading. Int J Pharm. 1990;62:1–7.
18. De Martimprey H, Bertrand J-R, Fusco A, Santoro M, Couvreur P, Vauthier C, *et al.* siRNA nanoformulation against the ret/PTC1 junction oncogene is efficient in an *in vivo* model of papillary thyroid carcinoma. Nucleic Acids Res. 2008;36:e2.
19. Huang M, Khor E, Lim LY. Uptake and cytotoxicity of chitosan molecules and nanoparticles: effects of molecular weight and degree of deacetylation. Pharm Res. 2004;21:344–53.
20. Hirai A, Odani H, Nakajima A. Determination of degree of deacetylation of chitosan by ^{1}H NMR spectroscopy. Polym Bull. 1991;26:87–94.
21. Bravo-Osuna I, Ponchel G, Vauthier C. Tuning of shell and core characteristics of chitosan-decorated acrylic nanoparticles. Eur J Pharm Sci. 2007;30:143–54.
22. Chauvierre C, Labarre D, Couvreur P, Vauthier C. Radical polymerization of alkylcyanoacrylates initiated by the redox system dextran-cerium (IV) under acidic aqueous conditions. Macromolecules. 2003;36:6018–27.
23. Bravo-Osuna I, Teutonico D, Arpicco S, Vauthier C, Ponchel G. Characterization of chitosan thiolation and application to thiol quantifictaion onto nanoparticle surface. Int J Pharm. 2007;340:173–81.
24. Leo C, Contado F, Bortolotti B, Pavan A, Scatturin G, Tosi S, *et al.* Nanoparticle formulation may affect the stabilization of an anti-ischemic prodrug. Int J Pharm. 2006;307(1):103–13.
25. PhamTrong LC, Djabourov M, Ponton A. Mechanisms of micellization and rheology of PEO-PPO-PEO triblock copolymers with various architectures. J Colloid Interf Sci. 2008;328:278–87.
26. Durrer C, Irache JM, Puisieux F, Duchene D, Ponchel G. Mucoadhesion of latexes. II. Adsorption isotherms and desorption studies. Pharm Res. 1994;11:680–3.
27. Durrer C, Irache JM, Puisieux F, Duchene D, Ponchel G. Mucoadhesion of latexes. I. Analytical methods and kinetic studies. Pharm Res. 1994;11:674–9.
28. Pinto-Alphandary H, Balland O, Couvreur P. A new method to isolate poly(alkylcyanoacrylate) nanoparticle preparations. J Drug Target. 1995;3:167–9.
29. Bouchemal K, Agnely F, Koffi A, Ponchel G. A concise analysis of the effect of temperature and propanediol-1,2 on Pluronic F-127 micellization using isothermal titration microcalorimetry. J Colloid Interf Sci. 2009;338:169–76.
30. Roques C, Bouchemal K, Ponchel G, Fromes Y. Parameters affecting organization and transfection efficiency of amphiphilic copolymer/DNA carriers. J Control Release. 2009;138(1):71–7.
31. Aka-Any-Grah A, Bouchemal K, Koffi A, Agnely F, Zhang M, Djabourov M, *et al.* Formulation of mucoadhesive vaginal hydrogels insensitive to dilution with vaginal fluids. Eur J Pharm Biopharm. 2010;76:296–303.
32. Bahadur P, Li P, Almgren M, Brown W. Effect of potassium fluoride on the micellar behavior of pluronic F-68 in aqueous solution. Langmuir. 1992;8:1903–7.
33. Zhou Z, Chu B. Light-scattering study on the association behavior of triblock polymers of ethylene oxide and propylene oxide in aqueous solution. J Colloid Interf Sci. 1988;126(1):171–80.
34. Nakashima K, Anzai T, Fujimoto Y. Fluorescence studies on the properties of a pluronic F68 Micelle. Langmuir. 1994;10: 658–61.
35. Bravo-Osuna I, Vauthier C, Farabollini A, Palmieri G-F, Ponchel G. Mucoadhesion mechanism of chitosan and thiolated chitosan-poly(isobutylcyanoacrylate) core-shell nanoparticles. Biomaterials. 2007;28:2233–43.
36. Hillery AM, Florence AT. The effect of adsorbed poloxamer 188 and 407 surfactants on the intestinal uptake of 60-nm polystyrene particles after oral administration in the rat. Int J Pharm. 1996;132:123–30.
37. Kreuter J, Muller U, Munz K. Quantitative and microautoradiographic study on mouse intestinal distribution of polycyanoacrylate nanoparticles. Int J Pharm. 1989;55:39–45.
38. Bertholon I, Ponchel G, Labarre D, Couvreur P, Vauthier C. Bioadhesive properties of poly(alkylcyanoacrylate) nanoparticles coated with polysaccharide. J Nanosci Nanotechno. 2006;6:1–8.
39. Sogias IA, Williams AC, Khutoryanskiy VV. Why is chitosan mucoadhesive? Biomacromolecules. 2008;9:1837–42.