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Elaboration and characterization of thiolated chitosan-coated acrylic nanoparticles

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Abstract

The aim of the present work was to investigate the use of thiolated chitosan in the development of polysaccharide-coated nanoparticles in order to confer specific functionality to the system. After chemical modification of commercial and hydrolysed chitosan (400,000 and 9400 g/mol respectively), thiolated chitosans were used to elaborate particles in the nano-range. They were characterized in terms of size and surface charge measurement. Both analysis showed nanoparticles of mean hydrodynamic diameter around 200 nm and positive zeta potential values, indicating the presence of the cationic polysaccharide at the nanoparticle surface. Moreover, the Ellman's reaction was used to demonstrate the presence of thiol groups at the particle surface. The observation of nanoparticles by scanning electronic microscopy (SEM) showed spherical nanoparticles for all formulations. This new system, combining both the advantages of thiolated polymers and colloidal particles can be proposed as an original drug carrier system for mucosal delivery of biotechnology products.

Keywords: Thiolated chitosan; 2-Iminiothiolane (TBA); Nanoparticles; Poly(alkylcyanoacrylate); Emulsion radical polymerisation

1. Introduction

Surface modified colloidal carriers such as nanoparticles, were demonstrated to be a promising and useful tool in the development of drug carrier systems intended to administer biotechnology engineered products. They offer protection of molecules against in vivo degradation, stability and can control the drug release, especially after oral administration (Janes et al., 2001; Mansouri et al., 2004).

From a technological point of view, most of the approaches used in the development of surface modified nanoparticles have been based on the synthesis of amphiphilic copolymers (Peracchia et al., 1997; Uchegbu et al., 1998; Brigger et al., 2001; Calvo et al., 2001; Rodrigues et al., 2003; Archambault and Brash, 2004; Lemarchand et al., 2004). Chauvierre et al. (2003a,b,c) developed a method for the elaboration of nanoparticles composed of a poly(alkylcyanoacrylate) core coated with polysaccharides, insuring the stability of the colloid. The proposed technique included the formation of a free radical at the end of the polysaccharide chain oxidized by reaction with cerium (IV) ions in aqueous acidic medium. The polymerization of alkyl cyanoacrylate (ACA) monomers is then initiated by these radicals, leading to linear block copolymers that undergo spontaneous auto-association to form nanoparticles with a hydrophobic core coated by the hydrophilic polysaccharide. This radical polymerisation method has been tested using different polysacharides (dextrans of different molecular weight, dextran sulfate, chitosan) (Chauvierre et al., 2003a,b,c; Bertholon-Rajot et al., 2005) providing in all cases very stable suspensions of nanoparticles. The presence of different chemical groups in the polysaccharide structure, on which it is possible to graft different ligands, offers the opportunity to develop delivery systems able to interact more specifically with biological tissues (Lemarchand et al., 2004). In this sense, Bernkop-Schnürch and co-workers have developed several interesting derivatives of chitosan including thiol groups in the

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native chitosan structure (Bernkop-Schnürch and Kast, 2001). Thiolated chitosans were demonstrated to improve the intrinsic mucoadhesive properties of the polysaccharide due to the formation of bonds with cysteine-rich domains of mucus glycoproteins (Bernkop-Schnürch et al., 2000; Bernkop-Schnürch and Steininger, 2000; Bernkop-Schnürch and Kast, 2001; Kast and Bernkop-Schnürch, 2001; Marschutz and Bernkop-Schnürch, 2002; Roldo et al., 2004). Interestingly, these products act also as permeation enhancing promoters according to a glutathion regeneration mechanism (Bernkop-Schnürch et al., 2003b), which differs from the electrostatic interaction with the tight junction-associated proteins mechanism described for plain chitosan. They also have a potential antiprotease activity because they can bind divalent cations, which are typically cofactors of many proteases (Bernkop-Schnürch and Kast, 2001). The most promising thiolated chitosan in terms of stability was the derivative obtained using 2-iminothiolane as ligand (Chitosan-TBA). This component also showed the highest bioadhesiveness, permeation enhancement and antiprotease activity (Bernkop-Schnürch et al., 2003a; Roldo et al., 2004). The aim of the present work was to elaborate and characterize nanoparticles coated with chitosan-TBA, which should provide new drug delivery systems combining both the advantages of the coated nanoparticles and the interesting properties of chitosan-TBA.

2. Materials and methods

2.1. Materials

Isobutylcyanoacrylate (IBCA) was kindly provided as a gift by Loctite (Dublin, Ireland). Chitosan (Mw 400,000 g/mol) and L-cystein HCl were purchased from Fluka (Saint-Quentin Fallavier, France). The Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) was obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France). 2-Iminothiolane HCl (Traut's reagent) was obtained from Pierce (Oud Beijerland, NL). All other chemicals were reagent grade and used as received.

2.2. Methods

2.2.1. Preparation of chitosan-TBA

2.2.1.1. Depolymerization of chitosan. Chitosan was depolymerised following the method developed by Huang et al. (2004). Briefly, 100 ml of a 2% (w/v) commercial chitosan (400,000 g/mol) solution in acetic acid solution (6%, v/v) was depolymerised at room temperature under stirring with 10 ml of $NaNO_3$ (8% in MilliQ® water). After 1 h of reaction, chitosan was precipitated by raising the pH to 9.0 with NaOH (4N). The white-yellow solid was filtrated, extensively washed with acetone and redissolved in a minimum volume of acetic acid 0.1N (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 (Christ Alpha 1–4 freeze-dryer. Bioblock Scientific, Illkirch, France) and the yellowish lyophilizate was then stored at 4 °C until use.

2.2.1.2. Modification of chitosan with 2-iminothiolane. The inclusion of thiol groups in the hydrolysed (low molecular weight) and non-hydrolysed chitosan (high molecular weight) was carried out following the method developed by Bernkop-Schnürch et al. (2003a). 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 (1N). Then the Traut's reagent (2-iminotiolane) 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 products were freeze-dried (Christ Alpha 1–4 freeze-dryer. Bioblock Scientific, Illkirch, France) and stored at −20 °C until use. The resulting polymers were: high molecular weight chitosan-4-thiol-butylamidine (HMW Chito-TBA) and low molecular weight chitosan-4-thiol-butylamidine (LMW Chito-TBA).

2.2.2. Chitosan characterization

2.2.2.1. Nuclear magnetic resonance. ^{1}H NMR spectra were recorded with a Bruker MSL-400 spectrometer (Bruker Instrument Inc. Wissembourg, France) at 25 °C. Samples were dissolved in D_2O, which contained a small amount of DCl. From the NMR spectrum of different chitosan, a peak could be assigned unambiguously, representing the methyl protons ($\delta(CH_3) = 2.1$ ppm). The cyclic structure of glucose residues can be also observed: ($\delta = 3.5$–4.0 ppm for H-3 to H-6 and $\delta = 3.2$ for H-2). The degree of deacetylation (Ddeac) was calculated from these data using the following equation:

$$\text{Ddeac}\,(\%) = \{1 - (2I_{CH_3}/I_{\text{H-2;H-3;H-4;H-5;H-6}})\} \times 100 \quad (1)$$

where I_{CH_3} is the intensity of the methyl proton and $I_{\text{H-2; H-3; H-4; H-5; H-6}}$ is the intensity of all protons from the cyclic glucose structure except the proton on the anomeric carbon, according to Hirai et al. (1991).

2.2.2.2. Measurement of molecular weight. The molecular weight of hydrolysed chitosan was determined from capillary viscosity measurements. Briefly, the reduced viscosity of solutions of hydrolysed chitosan of various concentrations (0.1–2.5 g/l) in acetic acid 0.1 M, NaCl 0.2 M was measured in a Ubbelohde tube (53710/1 Schott Gerate) at 25 °C (Bath CT1450 Schott Gerate and cooling system CK100 Schott Gerate) using a viscometer AVS400 (Schott Gerate). The intrinsic viscosity (η) was then deduced from the reduced viscosity measured for each solution of chitosan by extrapolation at zero concentration. The molecular weight was determined by using the Mark–Houwink Sakurada equation: $(\eta) = K \times M^a$, with $K = 1.81 \times 10^{-3}$ and $a = 0.93$ (Khan et al., 2000; Majeti and Ravi, 2000).

2.2.2.3. Determination of the thiol group content. The degree of modification of thiolated chitosan was analysed by the Ell-

man's reaction. This method is well described in the literature (Bernkop-Schnürch et al., 2003a). Briefly, a solution of 2 mg/ml of polymer was prepared in MilliQ® water. Then 250 μl aliquots were added to 250 μl of 0.5 M phosphate buffer pH 8.0 and to 500 μl of Ellman's reagent (0.3 mg/ml of 5,5-dithiobis(2-nitrobenzoic acid) in 0.5 M phosphate buffer pH 8.0). The reaction was allowed to proceed for 2 h at room temperature and the absorbance was immediately measured at a wavelength of 420 nm (Espectrophotometer UV/VIS lambda 11 Perkin-Elmer. Norwalk, USA). Control samples were elaborated with non-modified chitosan. The amount of thiol moieties was calculated from the corresponding standard curve elaborated between 0.1 and 0.01 μmol/ml of L-cysteine HCl ($y = 5.733x - 0.0113$; $r^2 = 0.9996$) ($n = 3$). This colorimetric reaction measures only reduced thiol groups present in the polymer. A modification of the general protocol consistent with the previous treatment of the polymers with a reducing agent ($NaBH_4$, 4% (w/v) in MilliQ® water) allowed the determination of the total amount of thiol groups present in the polymer (Guggi and Bernkop-Schnürch, 2005).

2.2.3. Preparation of nanoparticles

The preparation of nanoparticles was achieved by radical emulsion polymerization according to previous works (Chauvierre et al., 2003a,b,c). 0.069 g of either HMW or LMW Chito-TBA were dissolved in 4 ml of 0.2 M nitric acid in a glass tube at 40 °C, under gentle stirring and argon bubbling. After 10 min, 1 ml of a solution of 8×10^{-2} M cerium IV ammonium nitrate in 0.2 M nitric acid, and 0.25 ml of IBCA was 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 gentle stirring for 40 min. After cooling to room temperature, NaOH (1N) was added to raise the pH to 4.5. The polymerisation medium was purified by dialysis (Spectra/Por membranes, 100,000 g/mol molecular weight cut off (MWCO), Biovalley, Marne la Vallée, France) against 1 l of acetic acid solution (16 μmol/l) in MilliQ® water twice for 90 min and once overnight.

2.2.4. Nanoparticle characterization

2.2.4.1. Determination of size. The hydrodynamic mean diameter and the size distribution of the nanoparticles were determined at 20 °C by quasi-elastic light scattering using a Nanosizer® N4 PLUS (Beckman-Couter, Villepinte, France). The scattered angle was fixed at 90°. Samples were diluted in acetic acid solution (16 μmol/l) in MilliQ® water to obtained a scattering intensity ranging from 5×10^4 to 5×10^6 counts per seconds as recommended by the apparatus supplier. The results gave the mean hydrodynamic diameter, the standard deviation of the size distribution. The polydispersity index was the average of six determinations.

2.2.4.2. Determination of the ζ potential. The electrostatic surface charge of the polymer particles was deduced from their electrophoretic mobility evaluated using a Zetasizer 4 (Malvern Instruments Ltd., Orsay, France). Dilution of the suspensions (1/200 (v/v)) was performed in NaCl 1 mM.

2.2.4.3. Scanning electron microscopy (SEM). Scanning electron microscopy was performed using a LEO 1530 (LEO Electron Microscopy Inc., Thrnwood, NY) operating at 3 kV with a filament current of about 0.5 mA. Nanoparticle suspensions were diluted in MilliQ® water from 1/1 to 1/10000. Liquid samples were deposed on vitreous carbon conductive double-side tape (Euromedex, France) and dried at room temperature. They were coated with a platinum laker of about 2 nm using a Cressington sputter-coated 208HR with a rotatory-planetary-tilt stage, equipped with a MTM-20 thickness controller.

3. Results and discussion

3.1. Chitosan modification and characterization

The depolymerisation method used to produce low molecular weight chitosan was previously applied by several authors. Mao and co-workers (Mao et al., 2004) demonstrated that the molecular weight of depolymerised chitosan decreased linearly with the ratio chitosan/$NaNO_2$. In this work, this method was used to obtain a LMW chitosan with a molecular weight of 9400 g/mol as determined by capillary viscosity measurements. The production yield was 70%.

The technique of ^{1}H NMR has been used by many authors to determine the degree of deacetylation of chitosan (Varum et al., 1991; Zong et al., 2000; Rinaudo et al., 2001; Chen et al., 2003; Ding et al., 2003; Sun et al., 2003; Liu et al., 2004). After depolymerisation, no structural change of chitosan was observed as indicated by the fact that both the initial and depolymerised chitosan products showed the same degree of deacetylation, 88 and 87%, respectively. This is especially important in the present work, because the thiol group should be included on the free amino groups of the polysaccharide chains. Thus, integrity of chitosan after depolymerisation should be preserved.

Table 1 summarises the characteristics of the chitosan after modification with TBA. The presence of thiol groups in the modified polymers promoted the formation of inter- and intra-chain disulfide bonds. Those bonds change in some extent the characteristics of the polymers and also diminish the percentage of free thiol groups, able to develop both bioadhesive and enhancer absorption behaviour. In this regard, both thiolated polymers showed free thiol group in the same order of magnitude, so a similar biological behaviour might be expected.

3.2. Elaboration of nanoparticles and characterization

Particles of poly(isobutylcyanoacrylate) (PIBCA) with the commercial chitosan (HMW chitosan) were elaborated following the method developed by Chauvierre et al. (2003a,b,c). However, the subsequent particle characterization demonstrated that the particle size was in the micrometer range (~60 μm), in agreement with data obtained by Chauvierre et al. (2003a).

Nanoparticles could not be prepared with HMW Chito-TBA, because this compound was only partially soluble in the polymerization medium. The presence of thiol groups susceptible of being oxidized could explain the formation of a cross-linked polymer of reduced solubility when comparing with the unmodi-

Table 1
Thiolated chitosan characterization

Thiolated chitosan	Production yield (%)	Free thiol groups		Total thiol groups		% of free thiol groups
		% TBA (w/w) of polymer	μmol SH/g of polymer	% TBA (w/w) of polymer	μmol SH/g of polymer	
HMW Chito-TBA	63	2.1 (0.2)	213	5.9 (0.2)	587	36
LMW Chito-TBA	54	3.3 (0.0)	473	5.9 (0.1)	1153	41

The free and total thiol groups content was measured by Ellman's reaction using a calibration curve elaborated between 0.01 and 0.1 μmol/ml of cystein ($y = 5.5375x - 0.0255$, $r^2 = 0.9978$) ($n = 3$).

Table 2
Characterization of formulations elaborated with LMW chitosan and LMW chitosan-TBA

Nanoparticle formulation	Mean particle size (nm)	Standard deviation (nm)[a]	Polydispersity index	*Z* potential (mV)	μmol SH/g of nanoparticles
PIBCA-LMW Chitosan	184	42	<0.1	+37.8 ± 0.3	–
PIBCA-LMW Chito-TBA	229	73	<0.1	+32.0 ± 0.2	342

The free and total thiol groups content was measured by Ellman's reaction using a calibration curve elaborated between 0.01 and 0.1 μmol/ml of cystein ($y = 5.5375x - 0.0255$, $r^2 = 0.9978$) ($n = 3$).

[a] Width of the Gaussian distribution.

fied chitosan. Although the disulfide bond formations should not be enhanced at acidic pH (Guggi et al., 2004), there were already enough such bonds to make impossible the total solubilisation of HMW Chito-TBA at pH 1.

With this method of polymerization it has been reported that the size of the nanoparticles was directly related to the molecular weight of the polysaccharide (Chauvierre et al., 2003c; Bertholon-Rajot et al., 2005; Labarre et al., 2005). Thus, it was assumed that reducing the molecular weight of the commercially available chitosan would allow the synthesis of nanoparticles by this method of polymerisation. Furthermore, having smaller molecules of chitosan would be favourable to improve their solubility in nitric acid after chemical modifications. As reported in Table 2, nanoparticles were actually obtained when using both LMW Chitosan and LMW Chito-TBA.

The hydrodynamic diameter of the nanoparticles obtained with the thiolated chitosan was slightly larger than that of

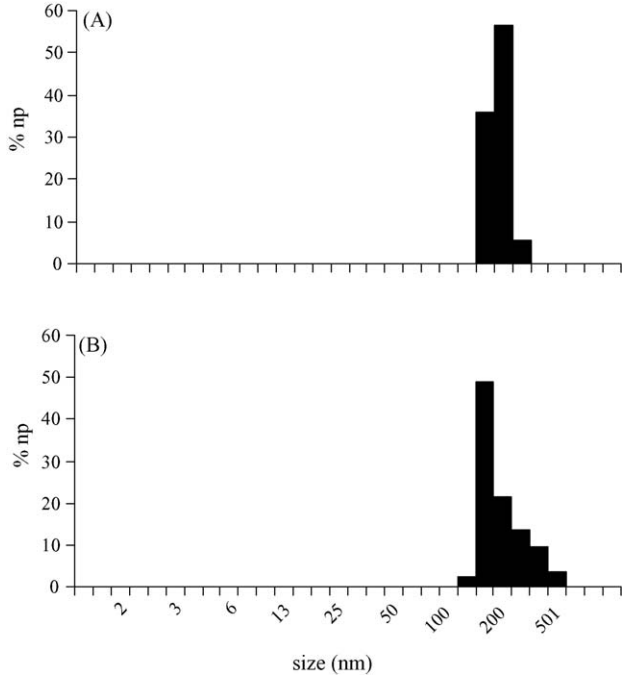

Fig. 1. Hydrodynamic diameter distribution for nanoparticles elaborated with LMW Chitosan (A) and LMW Chito-TBA (B).

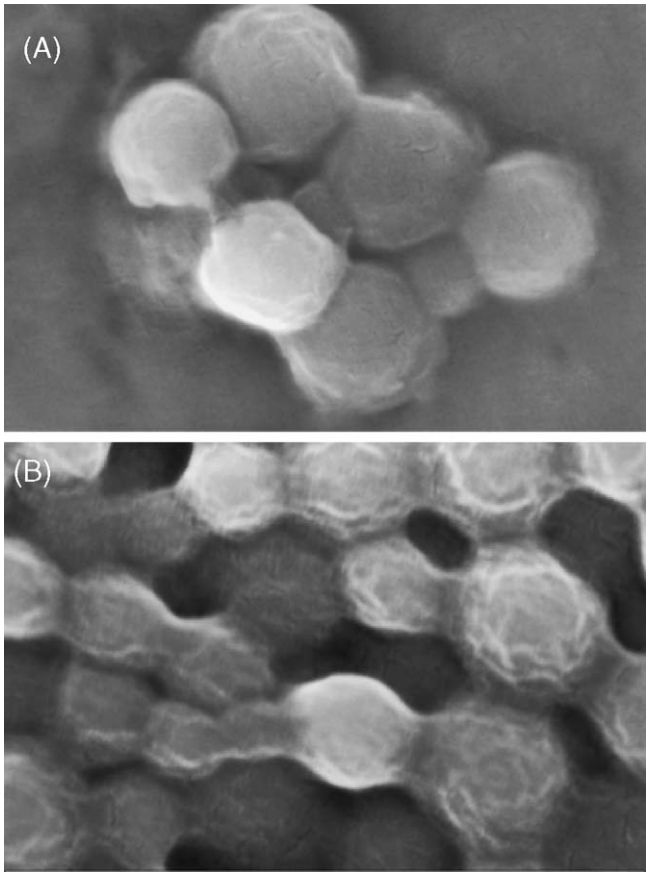

Fig. 2. Scanning electron microscopy photographs (1:150,000) obtained from different formulations: (A) PIBCA-LMW-chitosan and (B) PIBCA-LMW-Chito-TBA.

nanoparticles obtained with non-modified chitosan. The presence of thiol groups should promote the formation of inter- and intra-chain disulfide bonds (Guggi et al., 2004) that might diminish the repulsion interactions between polysaccharide chains and also reduce their mobility. This different spatial arrangement of chains at the nanoparticle surface could affect the hydrodynamic diameter of the nanoparticles. A narrower size distribution was also found for PIBCA-LMW Chitosan nanoparticles (Fig. 1).

The zeta potential of the nanoparticles was positive (Table 2) indicating that the cationic polysaccharide was located at the surface of the nanoparticles. The polysaccharide coating completely masked the negative surface charge values characteristic of non-coated PIBCA nanoparticles (Peracchia et al., 1997). The presence of thiol groups at the surface of nanoparticles elaborated with LMW Chitosan-TBA might be related with the slight decrease in surface charge values observed for this formulation. In Fig. 2 SEM microphotographs showed populations of spherical nanoparticles for both formulations. The particle size values observed in the microphotographs were in the same range than those obtained when measuring the hydrodynamic diameter in aqueous in suspensions. Regular surfaces could be observed for both formulations and no important differences were denoted between the different formulations analysed.

4. Conclusions

In conclusion, the method of free radical emulsion polymerisation is a suitable method for the elaboration of poly(alkylcyanoacrylate) nanoparticles coated by modified polysaccharides. The presence of specific chemical groups (thiol groups) on the particles surface has been demonstrated.

The new system created combines both the advantages of thiolated polymers and colloidal systems so it should be a interesting drug carrier for mucosal delivery of biotechnology products because of potential bioadhesion as well as permeation enhancement properties.

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