Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 340 (2007) 173–181
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Characterization of chitosan thiolation and application to thiol quantification onto nanoparticle surface

I. Bravo-Osuna [a], D. Teutonico [a], S. Arpicco [b], C. Vauthier [a], G. Ponchel [a,*]

[a] *Laboratoire de Physicochimie, Pharmacotechnie et Biopharmacie, UMR CNRS 8612, Université Paris-Sud 11, Faculté de Pharmacie, 5, Rue J.B. Clément, 92296 Chatenay Malabry, France*
[b] *Laboratorio di Chimica Farmaceutica Applicata, Dipartimento di Scienza e Technologia del farmaco, Facolta di Farmacia, Via P. Giuria, 9, 10125 Torino, Italy*

Received 28 July 2006; received in revised form 7 March 2007; accepted 15 March 2007
Available online 19 March 2007

Abstract

The objective of the present work was to establish a simple and appropriated method for the quantification of thiol groups standing on the surface of core–shell nanoparticles elaborated with poly(isobutyl cyanoacrylates) and thiolated chitosan.

A critical analysis of the widely used Ellman's method for the determination of thiol groups in various compounds was made. The reduced solubility of the thiolated polymer at the optimal pH of the Ellman's assay (pH 8–8.5) made difficult the accessibility of the Ellman's reagent to thiol groups in the cross-linked polymer. Furthermore, the lack of stability of the Ellman's reaction with time lead to the conclusion that the Ellman's method was of limited value to evaluate thiol groups in thiolated polymers like thiolated chitosan.

An alternative and very simple thiol quantification method was developed on the bases of the classical iodine titration. The new method allowed the determination of thiol groups in small amount of samples at acidic pH, and the monitoring of the thiol determination kinetic with time. It was successfully applied to the quantification of active thiol groups on the surface of poly(isobutyl cyanoacrylates) nanoparticles coated with thiol chitosan.

Keywords: Surface modified nanoparticles; Thiolated chitosan; Ellman's reagent; DNTB; Iodine titration

1. Introduction

In recent years, interesting works have been focused on the elaboration of thiol-surface modified systems: thiolated polyurethane films for the covalent immobilization of biomolecules (Alferiev and Fishbein, 2002); thiol-modified silica particles for the synthesis of chromatographic stationary phase, or as enzyme support (Nogueira et al., 2005; Hodgkins et al., 2005), etc. In the field of colloidal drug delivery systems, several authors have also successfully elaborated micro and nanoparticles with thiol groups on the surface (Weber et al., 2000; Leitner et al., 2004; Nobs et al., 2004; Bilicic et al., 2005; Pfeifer et al., 2005; Bravo-Osuna et al., 2006). The presence of thiol groups on the nanoparticle surface offers very interesting characteristics for drug mucosal administration, because they can give the system bioadhesive characteristics, permeation enhancing and antiprotease properties (Bernkop-Schnürch et al., 2004). Moreover, they can be an intermediate step in the development of surface modified particles by the covalent linking of specific peptides and proteins or antibodies (Nobs et al., 2004, 2006), being very promising tools in the development of active target drug delivery systems.

In a recent work, we have design thiolated chitosan poly(alkyl cyanoacrylates) (PACA) nanoparticles to propose a new formulation for the administration of peptides and proteins. Indeed, it was believed that the combination of nanotechnology like nanoparticles with bioadhesive and enhancing permeation properties of chitosan reinforced by the antiprotease activity due to the presence of thiol groups could be highly benefit to improve the absorption of peptides and proteins.

Many methods have been developed for the quantification of thiol groups; electromagnetic resonance spectroscopy (Prezelj et al., 2003), high-performance liquid chromatography with electrochemical detection (González-García et al., 2005), cap-

* Corresponding author. Tel.: +33 146835919; fax: +33 146619334.
E-mail address: gilles.ponchel@psud.fr (G. Ponchel).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.03.019

illary electrophoresis (Russell and Rabenstein, 1996; Glatz and Maslanova, 2000), liquid chromatography with mass spectrometry (Guan et al., 2003), enzymatic methods (De Cruz Vieira and Fatibello-Filho, 1999; Zhou et al., 2000; Rover et al., 2001; Liu and Itoh, 2006) and, the traditional analytical approaches based on the thiol derivation procedure, obtaining compounds detectable by UV–visible spectroscopy or fluorimetric (Alferiev and Fishbein, 2002; Root and Mutus, 2003).

Although they are less sensitive and accurate than other methods, the main advantage of the traditional spectroscopic analytical approaches is that they are rapid, simple and generally they do not involve aggressive procedure. Among them, the Ellman's assay, is surely the most commonly technique used (Iznaga Escobar et al., 1996; Russell and Rabenstein, 1996; Gergel and Cederbaum, 1997; Weber et al., 2000; Egwim and Gruber, 2001; Rover et al., 2001; Alferiev and Fishbein, 2002; Riener et al., 2002; Guan et al., 2003; Owusu-Apenten et al., 2003; Ramachandran et al., 2000; Hendricks et al., 2004; Nobs et al., 2004; Ricci et al., 2004; Bilicic et al., 2005; Fabel et al., 2005; Pfeifer et al., 2005; Adsersen et al., 2006).

The Ellman's reaction is based on the reaction of the thiolate anion ($R–S^-$) with 5,5′-dithio-bis(2-nitrobenzoic acid) or $DTNB^{2-}$ (Ellman's reagent) according to the reaction scheme given in Fig. 1a.

The 5-thio-2-nitrobenzoic acid (TNB^{2-}) form exhibits intense light absorption at a wavelength of 410–420 nm (Ellman et al., 1961). The main limit of this method is that the intensity of the light absorption of TNB^{2-} is pH-independent only if the pH of the medium is above 7.3 (Riener et al., 2002). Thus, this assay must be only employed above this pH value, with an optimal value around pH 8–8.5 (Nogueira et al., 2005).

Aiming to explain the biopharmaceutical behaviour of such thiolated colloidal systems, it is important to be able to verify and accurately quantify the presence of thiol groups at the nanoparticles surface, because these groups are highly reactive and may react with biological compounds in vivo. Due to the pH limitation imposed by the use of chitosan, biopolymer insoluble at pH > 6.5 (Mao et al., 2005), it can be suggested that a critical analysis of the Ellman's method must be done. Thus, in the present work the feasibility of this method for the quantification of thiol groups in thiolated chitosan has been analysed. Moreover, a thiol quantification method based on the modification of the classical iodine titration was proposed as a non-aggressive easy and simple alternative for the quantification of thiol groups in acidic conditions. The ultimate goal of this work was to develop an efficient for the determination of thiol groups on the surface of thiolated chitosan poly(butyl cyanoacrylate) nanoparticles under typical working conditions.

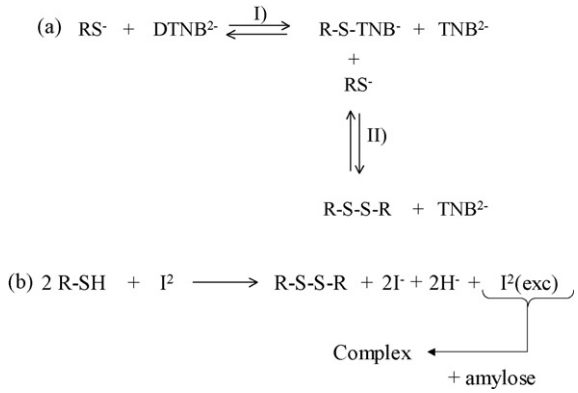

Fig. 1. (a) Scheme of the Ellman's reaction; (b) scheme of iodine titration.

2. Materials and methods

2.1. Materials

Isobutyl cyanoacrylate (IBCA) was kindly provided as a gift by Loctite (Dublin, Ireland). Chitosan M_w 400,000 g/mol and cysteine–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 synthesised in the Department of Organic Chemistry (Biocis UMR CNRS 8076), Faculty of Pharmacy, University Paris-XI (Chatenay-Malabry), France.

All other chemicals were reagent grade and used as received.

2.2. Methods

2.2.1. Chitosan modifications and characterization

Chitosan was selectively 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 $NaNO_2$ solutions in MilliQ® water at different concentrations (7.0%, 2.7% and 1.6%), in order to obtain the desired final molecular weight: 20,000, 50,000 and 100,000 g/mol, respectively. After 1 h of reaction, chitosan was precipitated by raising the pH to 9.0 with NaOH (4 mol/l). The white-yellowish solid was filtrated, extensively washed with acetone and redisolved in a minimum volume of acetic acid 0.1 mol/l (around 20–30 ml). Purification was carried out by subsequent dialyses against MilliQ® water (Spectra/Por® 3 membrane MWCO: 3500). The 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. Products obtained were called: Chito20, Chito50 and Chito100 depending of its theoretical molecular weight.

^{1}H NMR analysis (Bruker MSL-400 Spectrometer, Bruker Instrument Inc., Wissembourg, France) showed no changes in the deacetylation percentage after the depolymerization process, with values around 86–88% in all cases (Bravo-Osuna et al., 2007). The capillary viscosity (viscometer AVS400, Schott Gerate) measurements showed molecular weight values according to the predicted ones.

The inclusion of thiol groups in the different hydrolysed chitosan was carried out following the method developed by Bernkop-Schnürch et al. (2003). One gram of chitosan was solubilized in 100 ml of acetic acid solution (1%, v/v). The pH of the solution was adjusted to 6.5 with NaOH (1 mol/l). Then, the Traut's reagent (2-iminothiolane) was added in a chitosan:2-

iminothiolane weight ratio of 5:2. After an incubation period of 24 h at room temperature under continuous stirring, the resulting thiolated polymer was dialyzed (Spectra/Por® 3 membrane MWCO: 3500) against different aqueous media: 8 h against 5 l of 5 mmol/l HCl, 8 h against 5 l of 5 mmol/l HCl containing 1% NaCl two times, 8 h against 5 l of 5 mmol/l HCl and finally, 8 h against 5 l of 1 mmol/l 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 chitosan-4-thiol-butylamidine, named Chito20-TBA, Chito50-TBA and Chito100-TBA according with the original molecular weight of the respective unmodified polymers.

2.2.2. Nanoparticles preparation

Nanoparticles were elaborated following the radical polymerisation method developed by Chauvierre et al. (2003a,b,c). Briefly, 0.069 g of mixtures of modified and unmodified hydrolysed chitosan (%chitosan/%chitosan-TBA: 0/100, 25/75, 50/50, 75/25, 100/0) were dissolved in 4 ml of 0.2 mol/l nitric acid in MilliQ® water 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} mol/l cerium(IV) ammonium nitrate in 0.2 mol/l nitric acid, and 0.25 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 gentle stirring for 40 min. After cooling to room temperature, NaOH (1 mol/l) was added to raise the pH to 4.5. The nanoparticle suspensions obtained were 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. The so-prepared nanoparticles were characterized. The hydrodynamic mean diameter and size distribution of the nanoparticles were measured by quasi-elastic light scattering using a Nanosizer® N4 PLUS (Beckman-Couter, Villepinte, France). The electric surface charge of the polymer particles was deduced from the electrophoretic mobility of the particles measured by Laser Doppler Electrophoresis (Zetasizer Nanoserie, Malvern Instruments Ltd., Worcestershire, UK) in a NaCl 1 mmol/l solution after suitable dilutions (1/200, v/v) of the different nanoparticles suspensions.

2.2.3. Determination of the thiol group content

2.2.3.1. Ellman's reaction. The degree of modification of thiolated chitosan was firstly analysed by the Ellman's reaction, following the method described in the literature by other authors studying very similar compounds (Bernkop-Schnürch et al., 2003). Briefly, a 2 mg/ml solution of polymer was prepared in MilliQ® water. Then 250 μl aliquots were added to 250 μl of 0.5 mol/l phosphate buffer pH 8.0 and to 500 μl of Ellman's reagent (0.3 mg/ml of DTNB in 0.5 mol/l phosphate buffer pH 8.0) (final polymer concentration of 0.5 mg/ml). The reaction was allowed to proceed for 2 h at room temperature and the absorbance was measured at a wavelength of 420 nm (Spectrophotometer 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 15 and 62 μmol/l of cysteine–HCl.

Several parameters of this method were analysed. Firstly, the effect of chitosan concentration and molecular weight was studied by analysing polymer concentrations from 6×10^{-3} to 0.5 mg/ml for Chito20-TBA, Chito50-TBA and Chito100-TBA. Secondly, the effect of incubation time was also determined so samples were measured between 1 and 24 h of incubation. Finally, possible interferences of oxygen in the thiol oxidation process were avoided by degassing samples by argon bubbling for 20 min previously to the assay (Iznaga Escobar et al., 1996; Hendricks et al., 2004).

2.2.3.2. Iodine titration. The degree of modification of thiolated chitosan was also analysed by iodine titration. Different solutions of thiolated polymers were prepared (0.5–0.05 mg/ml) in 0.5 mol/l CH_3COOH/CH_3COONa buffered solution (pH 2.7). Then 1 ml of starch aqueous solution (1%, w/v) and 0.5 ml of iodine (1 mmol/l) were added to 2 ml of each preparation. A storage iodine solution was weekly prepared at concentration 0.1 mol/l by dissolving 0.63 g of I_2 and 1.93 g of KI in 25 ml of MilliQ® water. The so prepared solution was stored protected from light and diluted just before the assay.

The oxidation reaction was allowed to proceed for 24 h at room temperature and protected from light. Then, samples were centrifuged (10 min, 3500 rpm) and the supernatant was measured at 560 nm (Spectrophotometer UV/VIS Lambda 11 Perkin-Elmer). Control samples were elaborated with non-thiolated hydrolysed chitosan. The amount of thiol moieties was calculated from the corresponding standard curve elaborated in the same conditions with cysteine–HCl solutions (20–70 μmol/l). Iodine promotes the oxidation of free thiol groups. To avoid interferences of oxygen, buffer was degased by bubbling argon for 20 min before the assay. The excess of iodine reacts with starch giving a blue complex whose intensity depends on the remaining iodine in the solution, which has not reacted with thiol groups. It can be easily measured at 560 nm.

Once the method was optimized for the analysis of thiolated chitosan, it was slightly modified in order to quantify thiol groups onto the nanoparticles surface. Two hundred and fifty microliters of nanoparticle suspensions (10–15 mg np/ml of suspension, depending on the formulation), elaborated with different proportions of chitosan/chitosan-TBA (Table 1), were incubated with 250 μl of acetate buffer (pH 2.7) and 500 μl of starch aqueous solution (4%, w/v). As low percentage of thiol groups was expected than in polymers previously analysed, the increase in the starch concentration was necessary to determine the excess of iodine. The so-prepared samples were incubated with 300 μl of iodine solution (1 mmol/l) at room temperature and protected from light. After 24 h of incubation, each sample was ultracentrifugated (15 min 30,000 rpm). The supernatant was measured. The calibration curve was elaborated with cysteine–HCl in the same experimental conditions. It is necessary to underline that previous works discarded interaction between PIBCA and iodine (Peracchia et al., 1997).

Table 1
Mean hydrodynamic diameter (H_D), polydispersity index (PI) and surface charge (ζ) measurements of nanoparticles elaborated

Chitosan (g/mol)	%Chitosan/%chitosan-TBA	H_D (nm)	PI	ζ potencial (mV)
20,000	100/0	181	0.110	+41.2 ± 0.9
	75/25	210	0.161	+37.0 ± 0.7
	50/50	360	0.139	+36.3 ± 0.7
	25/75	463	0.162	+36.0 ± 0.4
	0/100	491	0.200	+26.9 ± 0.8
50,000	100/0	240	0.029	+40.9 ± 0.7
	75/25	249	0.181	+31.5 ± 0.5
	50/50	465	0.125	+37.1 ± 0.7
	25/75	548	0.086	+35.2 ± 0.8
100,000	100/0	509	0.133	+51.2 ± 0.9
	75/25	512	0.091	+53.0 ± 0.3
PIBCA	–	260	0.088	−17.7 ± 0.3

2.2.3.3. Elemental analysis. The total amount of sulphur in thiolated polymers and nanoparticles was determined by elemental analysis using an Analyser LECO SC144 (Service central d'analyse du CNRS, Vernaison, France). Samples of 10 mg were burned at 1350 °C over oxygen flux and the detection of SO_2 was performed by infrared measurements.

3. Results and discussion

3.1. Nanoparticle preparation and characterization

Size and zeta potential of nanoparticles prepared with different proportions of chitosan/chitosan-TBA are presented in Table 1. While for the lowest chitosan molecular weight (Chito20) it was possible to elaborate nanoparticles with different proportions of chitosan/chitosan-TBA up to 100% of chitosan-TBA, the partial insolubilization of Chito50-TBA and Chito100-TBA in the polymerisation medium made impossible the elaboration of nanoparticles with percentage of chitosan-TBA higher than 75% for Chito50-TBA and 25% for Chito100-TBA.

For each series of nanoparticles elaborated with chitosan of the same molecular weight, the hydrodynamic mean diameter of particles increased with the percentage of chitosan-TBA. This can be explained by the presence of intra- and inter-molecular disulphide bonds formation as was suggested for other thiolated products (Bilicic et al., 2005). The mean hydrodynamic diameter of these types of nanoparticles showed a clear increase with the molecular weight of chitosan, which is in agreement with previous works (Chauvierre et al., 2003a; Bertholon-Rajot et al., 2005). Chitosan free amino groups were responsible for the measured positive ζ potential values obtained for all formulations.

3.2. Thiol groups determination

During the reaction of chitosan with the Traut's reagent (2-ininothiolane), part of the SH included underwent spontaneous oxidation in aqueous medium forming inter- and intra-chain disulphide bonds. This phenomenon has been already reported for chitosan-TBA and for many other thiolated compounds (Guggi et al., 2004; Owusu-Apenten and Chee, 2004; Bilicic et al., 2005; Bravo-Osuna et al., 2006). In addition, once elaborated and lyophilised, thiol groups can be easily oxidized in presence of atmospheric oxygen (Egwim and Gruber, 2001), so they must be stored at −20 °C in inert (argon) atmosphere to try to minimize this effect.

The thiol spontaneous oxidation could be avoided by the eventual protection with different chemical groups (Arpicco et al., 1997). However, the presence of a cross-linked structure is not necessarily a disadvantage in the development of drug delivery systems, so they can be very useful in the control of the drug release or in the modulation of the gel viscosity (Bernkop-Schnürch et al., 2004; Greindl and Bernkop-Schnürch, 2006).

The sulphur content of the three chitosan-TBA elaborated were: 6.6, 6.0 and 5.1% (w/w) for Chito20-TBA, Chito50-TBA and Chito100-TBA, respectively, as determined by elemental analysis. These values give the total amount of sulphur present in the sample, but they do not provide any information about the chemical state of the sulphur, whether it exists as a reactive sulfhydryl or whether it has been oxidised (unreactive disulphide) (Nogueira et al., 2005). Determinations of the reactive sulfhydryl groups were performed using the Ellman's reaction and a new analytical method in acid conditions.

3.2.1. Ellman's reaction

The Ellman's reaction was developed in 1959 for the determination of enzymatic activity of cholinesterase (Ellman, 1959; Ellman et al., 1961). Since then, it has been used for the specific determination of thiol groups (Boyne and Ellman, 1972; Adsersen et al., 2006). Several variations have been introduced, such as the inclusion of organic solvents (Fabel et al., 2005; Hodgkins et al., 2005), the inclusion of a "mediator" such as cystamine to accelerate the reaction (Riener et al., 2002) or even the covalent linkage of the Ellman's reagent (DTNB) to proteins, such as casein, to obtain new derivative reagents (Owusu-Apenten and Chee, 2004).

In this work, the simplest classical Ellman's reaction conditions were employed. First, a calibration curve was elaborated with different cysteine–HCl concentrations between 15 and

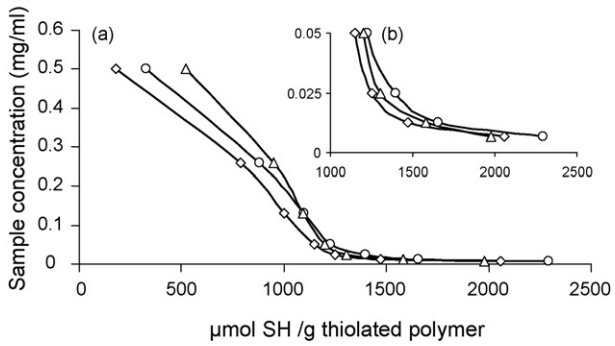

Fig. 2. Thiol determination by Ellman's reaction (µmol/g) for the three thiolated chitosan under study as function of the polymer sample concentration between 6.5×10^{-3} and 0.5 mg/ml (a). Details of the thiol values obtained for low chitosan concentration (between 6.5×10^{-3} and 0.05 mg/ml) have been presented (b). *Keys*: Samples of Chito20-TBA (triangles); Chito50-TBA (circles); Chito100-TBA (diamonds).

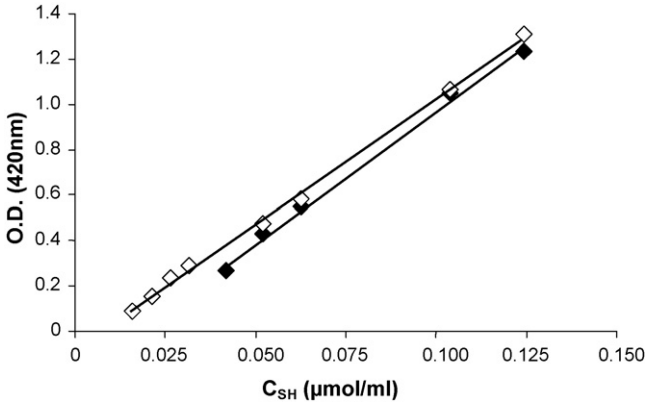

Fig. 3. Calibration curves elaborated with different concentrations of cysteine–HCl by Ellman's reaction, in absence (white diamond) or in presence (black diamond) of Chito100 (1 mg/ml). Without chitosan: $y = 11.05x - 0.07$, $r^2 = 0.998$; with chitosan: $y = 11.68x - 0.18$, $r^2 = 0.996$.

62 µmol/l. Typically, Ellman's assay has been reported to be reliable for the measurement of thiol concentrations higher than 3 µmol/l (Wright and Viola, 1998). Absorbance values at 420 nm between 0.214 and 0.850 were measured and a linear relation between both parameters expressed by Eq. (1) was obtained:

$$\text{O.D.}\,(\lambda = 420) = 10.80(C_{\text{L-Cys}(\mu\text{mol/ml})}) - 0.06 = 10.80(C_{\text{SH}(\text{mmol/ml})}) - 0.06\,(r^2 = 0.997). \quad (1)$$

Then, different concentrations of Chito20-TBA, Chito50-TBA and Chito100-TBA were treated in the same way than cysteine–HCl samples and were analysed after 2 h incubation. Results of thiol determination for different sample concentrations are presented in Fig. 2. A very important concentration-dependence in the thiol determination was obtained. According to those data, the lower the sample concentration was, the higher was the amount of thiol groups determined per gram of thiolated polymer. This clearly highlighted a problem in the thiol measurement since the concentration of thiol groups per gram of polymer must be found independent of the sample concentration used.

Although many authors have demonstrated that the Ellman's assay is very accurate for small soluble thiolated compounds such as cysteine or glutathion, being the reference assay in a high number of studies (Zhou et al., 2000; Rover et al., 2001; Riener et al., 2002), several problems have been detected when trying to use this method for determination of thiol groups in macromolecules. For instance, a number of protein sulfhydryls were seen to give an incomplete reaction with Ellman's reagent, due to steric or electrostatic constraints. (Egwim and Gruber, 2001; Riener et al., 2002).

The analysis of the different parameters concerning the specific characteristic of the thiolated polymer considered in the present study was carried out in order to explain the influence of its concentration in the thiol quantification by the Ellman's assay. Firstly, the charge of the polymer was taken into account. DTNB is negatively charged at pH 8 and can develop electrostatic attraction with cationic polymers, which accelerate the oxidation reaction (Owusu-Apenten et al., 2003). Chitosan has a cationic nature due to the presence of amino groups able to be protoned. However, the pK_a of such amino groups is around 6.5, so at the pH of the Ellman's reaction (pH 8) only a low amount of amino groups might remain positively charged. In those conditions, a low electrostatic attraction between DTNB and chitosan could explain the difficult in developing the Ellman's assay when increasing the polymer content, it seems indeed not to be very favoured.

Secondly, the presence of thiolated chitosan induces the formation of a viscous medium, which could diminish the accessibility of the reagent molecules to the free thiol groups of the samples. To analyse this effect a second calibration curve was prepared using unmodified chitosan solutions as diluents. The high Chito100 concentration used (1 mg/ml) was chosen to clearly identify any interference in the colorimetric reaction. In Fig. 3, the two calibrations curves obtained, with and without chitosan, are shown. It can be observed that the presence of the non-modified polymer does not have any influence in the measurement of cysteine–HCl content.

The third hypothesis explored the effect of the reduced solubility of chitosan and thiolated chitosan at the pH of the assay (pH 8.0) and the presence of a cross-linked system. This can diminish in some extent the accessibility of thiol groups of the polymer. As can be observed in Fig. 2, the fact that the higher molecular weight chitosan-TBA seemed to be more influenced, supports this hypothesis.

Previous works describing the quantification of surface-bound thiol groups with Ellman's reagent rely on the assumption that matrix effects due to polymers were absent and that a quantitative yield of thiol group detection was achieved, like in the reaction with the soluble standard (Nogueira et al., 2005). However, some authors have demonstrated that the Ellman's regent can only interact with very "accessible" thiol groups. Indeed, this characteristic was used by Ramachandran et al. (2000), for example, to monitor the process of proteins unfolding by measuring natively buried cysteine residues. Owusu-Apenten et al. (2003) analysed the rate constant of the thiol oxidation reaction for glutathion, BSA (protein with one SH surface group)

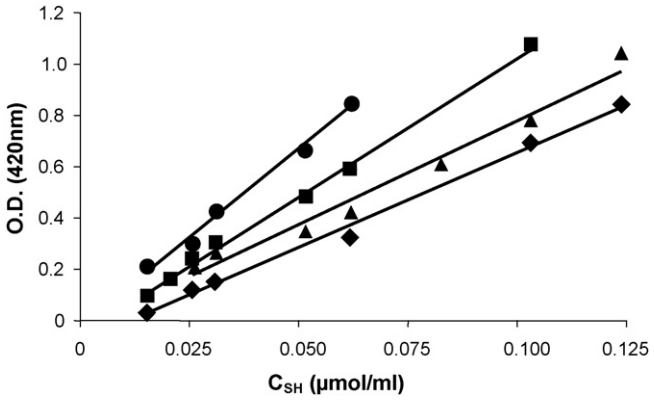

Fig. 4. Calibration curves elaborated with different concentrations of cysteine–HCl by Ellman's reaction, after different incubation times: 1 h (circle) $y = 13.89x - 0.02$, $r^2 = 0.990$; 2 h (square) $y = 10.80x - 0.06$, $r^2 = 0.997$; 4 h (triangle) $y = 8.09x - 0.03$, $r^2 = 0.980$; 24 h (diamond) $y = 7.37x - 0.09$, $r^2 = 0.995$.

and BLG (protein with SH buried into the globular structure) demonstrating an influence of the accessibility of thiol groups on the rate of reaction with DTNB. In addition, Badyal et al. (2001) examined the use of Ellman's reagent for the quantification of the thiol groups immobilized onto different resin supports, obtaining non-reproducible results when using the standard aqueous conditions.

Another factor that may influence the Ellman's reaction is the time between the addition of the reagent and the measurement of the optical density. So, experiments were planned to measure the same chitosan-TBA samples after different incubation times to monitorize the kinetic of the colorimetric reaction. Before that, the assay was carried out with different cysteine–HCl concentrations (calibration curve) to verify the stability of the reaction with time. Cysteine–HCl samples were prepared as has been already explained, protected from light, at room temperature well closed and measured after 1, 2, 4 and 24 h of storage. As can be observed in Fig. 4, an important decrease in TNB concentration was observed with time, as can be deduced from the lowering in the calibration curve slopes observed.

Although the complete Ellman's reaction is the one expressed in reaction (1a.I), it is only performed in presence of an excess of RSH (Dietz and Rubinstein, 1972). On the contrary, Several authors have demonstrated that, in an excess of DTNB, as is the case in our experiments, the reaction (1a.I) with small soluble molecules such as cysteine is instantaneous and the concentration of the formed Ellman's derivative ($R–S–TNB^-$) remained constant a minimum for 24 h at room temperature (Russell and Rabenstein, 1996; Guan et al., 2003; Ricci et al., 2004). In these conditions, the decrease of TNB observed with time might not be attributed to an inversion of the Ellman's reaction but to its oxidation in the aqueous medium. Indeed, several authors have already pointed out the existence of a possible reoxidation phenomenon of TNB with time in the presence of water (Thanenhauser et al., 1984; Gergel and Cederbaum, 1997; Okonjo et al., 2006).

In summary, it seemed that the stability of the colorimetric determination of the Ellman's reaction was compromised with time. It was then impossible to develop kinetic studies of the Ellman's reaction of chitosan-TBA. Our hypothesis is that the accessibility of thiol groups in the thiolated chitosan is limited due to the typical presence of a cross-linked network in its structure further decreased at pH 8 due to the limited solubility of the polymer. In these conditions, the reaction with DTNB might be a time-consuming process, that cannot be quantified because of the low stability of the coloured product of the Ellman's reaction in aqueous medium.

According to our data, this reaction should not be used to quantify thiol groups associated with polymers like polysaccharides before the influence of polymer solubility and of the time dependency of the reaction have been evaluated. Although widely used by other authors (Bernkop-Schnürch et al., 2003), it seems that this reaction is not appropriated to determine the thiol content in thiolated chitosan.

Due to the limitations of pH and sometimes to the lack of reproducible results observed with the Ellman's assay performed in aqueous medium, several authors have suggested alternative thiol determination methods. For instance, the Ellman's reagent can be successfully used in organic solvents for the determination of immobilized cysteine derivatives in solid resins (Badyal et al., 2001), or can be substituted by pyridine-based products (Glatz and Maslanova, 2000; Egwim and Gruber, 2001; Riener et al., 2002; Owusu-Apenten et al., 2003). In the following section, we propose an alternative thiol determination method based on a classical oxydo-reduction titration reaction using iodine.

3.2.2. *Modified iodine titration method*

Iodine presents oxidizing properties, able to oxidize SH groups to disulphuric bonds (Samoulinov and Zweier, 1998). It has been widely used for the determination of thiol groups in acidic conditions (Sherman et al., 1996; Sherman and Kuselman, 1999; Baker, 2000), which might be more appropriated for chitosan.

Typically, the titration is performed using an aqueous solution of starch as indicator. The addition of complexing ligands, such as iodine, induces changes in the conformation of amylose. The iodine/amylose complex presents a brilliant-blue colour (Heinemann et al., 2003). It can be used to quantify the excess of iodine in the reaction medium remaining after reaction with free available thiol groups. This is illustrated in Fig. 1b.

This reaction has been already applied to the titration of thiol groups in thiolated chitosan (Kast and Bernkop-Schnürch, 2001). However, the limitation of this technique is the subjective evaluation of the end point of the reaction and also the larger amounts of products generally needed to carry out the quantification. In the present work, we proposed a modified version of this method that involved standarization and adjustment for small amounts of samples. In addition, the use of a spectrophotometer was introduced to make the technique independent of subjective considerations.

After 30 min of mixing, the calibration curve for L-cysteine in acetate buffer (pH 2.7) showed the following Eq. (2):

$$\text{O.D.}\,(\lambda = 560) = -23.45(C_{\text{L-Cys}(\mu\text{mol/ml})}) + 1.40 = -23.45(C_{\text{SH}(\mu\text{mol/ml})}) + 1.40\,(r^2 = 0.998). \quad (2)$$

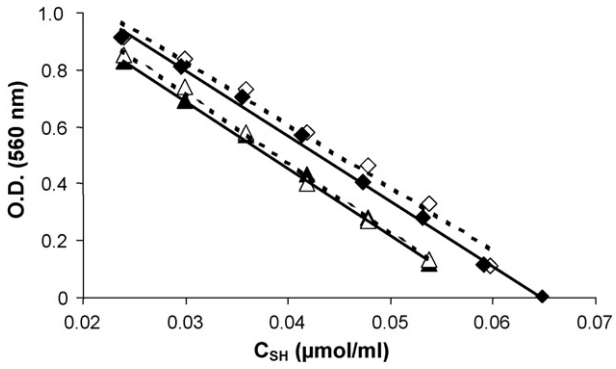

Fig. 5. Calibration curves elaborated with different concentrations of cysteine–HCl by iodine titration, in absence (triangles) or in presence (diamond) of Chito100-TBA (1 mg/ml), measured after 30 min (black) or 24 h (white) of incubation. The following regression curves were obtained: without chitosan (30 min) $y = -23.50 + 1.40$, $r^2 = 0.998$; with chitosan (30 min) $y = -22.92 + 1.50$, $r^2 = 0.996$; without chitosan (24 h) $y = -24.65 + 1.46$, $r^2 = 0.996$; with chitosan (24 h) $y = -22.16 + 1.50$, $r^2 = 0.984$.

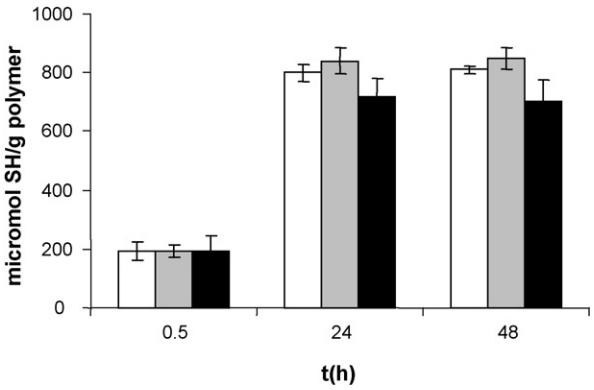

Fig. 6. Evolution of thiol determination with time obtained by the iodine titration method for the three thiolated chitosan analysed. Chito20-TBA (white); Chito50-TBA (grey); Chito100-TBA (black).

Both, the influence of chitosan (Chito100 1 mg/ml) and the influence of incubation time (30 min and 24 h) in the cysteine–HCl thiol determination were analyzed. As it can be observed in Fig. 5, no influence was found in the calibration curve for the two parameters evaluated. This is in agreement with the well-known high stability of the iodine/amylose complex that forms during titration of the excess of iodine (Heinemann et al., 2003).

Samples of thiolated chitosan were prepared at different concentrations and were measured after 30 min of stirring. These samples were then stored well-closed and protected from light, to be measured again after 24 and 48 h. Results presented in Fig. 6 showed that the maximum of the absorbance reached after a time of 24 h. At low time, the reaction was incomplete, in agreement with our previous hypothesis suggesting that the kinetic for the determination of thiol groups in thiolated chitosan is slow. Twenty-four hours were then accepted as optimal reaction time.

Once the method was optimized for the analysis of thiolated chitosan, it was further slightly modified to quantify thiol groups on the nanoparticle surface. The main modification consisted on the introduction of a centrifugation step to remove the nanoparticles from the system, prior to the measurement of the absorbance. The calibration curve was elaborated with cysteine–HCl in the same experimental conditions. It is necessary to underline that previous works discarded interaction between PIBCA and iodine (Peracchia et al., 1997).

Results of sulphur determination are reported in Table 2. As can be observed, the presence of total amount of sulphur in nanoparticles increased with the percentage of chitosan-TBA used in their preparation. The elemental analysis of sulphur content involved the thermal degradation of the nanoparticle, so these results are encounted all sulphur present in the whole nanoparticle. However, taking into account the core–shell structure demonstrated for those nanoparticles, it is expected that most of sulphur might be located in the hydrophilic shell of the nanoparticles, i.e. the nanoparticle surface. Accordingly, the amount of "active" or "reactive" (reduced) thiol groups on the nanoparticle surface increased with the increase of the total amount of sulphur in the nanoparticles.

Calculations were made to evaluate the percentage of total sulphur presented in the "reactive" (reduced) form. Interestingly, this percentage decreased with the amount of total sulphur in the formulation. The presence of high percentages of chitosan-TBA

Table 2
Total sulphur determination in nanoparticles (np) measured by elemental analysis and reduced thiol groups determination onto nanoparticles surfaces obtained by the modified iodine titration method

Chitosan (g/mol)	%Chitosan/%chitosan-TBA	Total S in np (%w/w)	µmol SH/cm^2 np	% of total S on the np surface
20,000	100/0	–	–	–
	75/25	0.19	114×10^{-6}	50
	50/50	0.52	127×10^{-6}	11
	25/75	0.77	170×10^{-6}	8
	0/100	0.99	209×10^{-6}	7
50,000	100/0	–	–	–
	75/25	0.24	53×10^{-6}	14
	50/50	0.54	107×10^{-6}	7
	25/75	0.75	206×10^{-6}	7
100,000	100/0	–	–	–
	75/25	0.18	104×10^{-6}	27

in formulations might promote the formation disulphide bonds, leading to a higher cross-linked structure.

The method proposed for the measurement of thiol groups on the chitosan-coated nanoparticles offered several interesting advantages. It allows the quantification in acidic medium, with mild conditions that do not drastically change the organization and nature of copolymers on the nanoparticle surface. In addition, no problems related to instability of reagents were found. The method developed in this work is simple and can be performed with small amount of samples. The only limitation, which was also applied for the Ellman's reaction, is that the measurement of thiol groups is based on the formation of inter- and intra-chain disulphide bonds during the reaction with iodine, thus there be surely subestimation of thiol groups in some extent. However, the data obtained could be related with the tendency to develop disulphide bonds in vivo.

4. Conclusions

Although Ellman's reaction is a well-known method for the determination of thiol groups in small soluble molecules, it has been many works trying to solve problems related to its use in the quantification of thiol groups in macromolecules. In this work, we demonstrated that this reaction is of limited value to determine thiol groups in thiolated chitosan because of the restricted accessibility of thiol groups in the samples, due to the presence of a cross-linked structure and to the scarce solubility of this chitosan derivative at pH 8. To improve the performance of this method, increase of the incubation time of the reagent with the polymer may be requested but this is compromised because of the poor stability of the Ellman's reaction in aqueous media.

Thus, an alternative method was proposed. It is based on a classical iodine titration modified to be applied on small samples and to have rational detection of the colorimetric reaction. The method developed allowed the measurement of the total "reactive" thiol groups in the sample. The acidic pH used in the method is more favourable to ensure the total solubility of chitosan-TBA, allowing the measurements under typical working conditions. Finally, the method proposed allowed the objective evaluation of the resulting colorimetric reaction by the use of spectroscopy. This non-aggressive analytical method was then successfully used for the determination of thiol groups on the surface of chitosan-TBA coated PIBCA nanoparticles.

The modified iodine titration method proposed in this work can be suggested as alternative method for the thiol quantification of products with limited solubility at basic pH.

Acknowledgements

Authors want to thank Dr. K. Broadley from Loctite (Dublin, Ireland) for his kindness in providing the isobutyl cyanoacrylate monomer. Authors want to thank the Department of Organic Chemistry (Biocis UMR CNRS 8076), Faculty of Pharmacy, University Paris-XI (Chatenay-Malabry, France) for their help in the synthesis of 2-iminothiolane and the "Service central d'analyse du CNRS" (Vernaison, France) for the elemental analysis of thiolated polymers. Authors are especially grateful to Gioconda Millotti for her help and personal implication in the development of the experimental work.

References

Adsersen, A., Gauguing, B., Gudiksen, L., Jager, A.K., 2006. Screening of plants used in Danish folk medicine to treat memory dysfunction for acetylcholinesterase inhibitory activity. J. Ethnopharmacol. 104, 418–422.

Alferiev, I.S., Fishbein, I., 2002. Activated polyurethane modified with latent thiol groups. Biomaterials 23, 4753–4758.

Arpicco, S., Dosio, F., Brusa, P., Crosasso, P., Cattel, L., 1997. New coupling reagents for the preparation of disulfide cross-linked conjugates with increased stability. Bioconjugate Chem. 8, 327–337.

Badyal, J.P., Cameron, A.M., Cameron, N.R., et al., 2001. A simple method for the quantitative analysis of resin bound thiol groups. Tetrahedron Lett. 42, 8531–8533.

Baker, W.L., 2000. Ascorbic acid reaction with disulphide compounds: effects and applications. Talanta 52, 425–433.

Bernkop-Schnürch, A., Kast, C.E., Guggi, D., 2003. Permeation enhancing polymers in oral delivery of hydrophilic macromolecules: thiomer/GSH systems. J. Control. Release 93, 95–103.

Bernkop-Schnürch, A., Krauland, A.H., Leitner, V.M., Palmberger, T., 2004. Thiomers: potential excipients for non-invasive peptide delivery systems. Eur. J. Pharm. Biopharm. 58, 253–263.

Bertholon-Rajot, I., Labarre, D., Vauthier, C., 2005. Influence of the initiator system, cerium–polysaccharide, on the surface properties of poly(isobutylcyanoacrylate) nanoparticles. Polymer 46, 1407–1415.

Bilicic, M.B., Filipovic-Grcic, J., Martinac, A., et al., 2005. Synthesis and characterization of thiomers of polyaspartamide type. Int. J. Pharm. 291, 211–219.

Boyne, A.F., Ellman, G.L., 1972. A methodology for analysis of tissue sulfhydryl components. Anal. Biochem. 46, 639–653.

Bravo-Osuna, I., Schmitz, T., Bernkop-Schnürch, A., Vauthier, C., Ponchel, G., 2006. Elaboration and characterization of thiolated chitosan-coated acrylic nanoparticles. Int. J. Pharm. 316, 170–175.

Bravo-Osuna, I., Ponchel, G., Vauthier, C., 2007. Tuning of shell and core characteristics of chitosan-decorated acrylic nanoparticles. Eur. J. Pharm. Sci. 30, 143–154.

Chauvierre, C., Labarre, D., Couvreur, P., Vauthier, C., 2003a. Radical polymerization of alkylcyanoacrylates initiated by the redox system dextran-cerium (IV) under acidic aqueous conditions. Macromolecules 36, 6018–6027.

Chauvierre, C., Labarre, D., Couvreur, P., Vauthier, C., 2003b. Plug-in spectrometry with optical fibres as a novel analytical tool for nanoparticles technology: application to the investigation of the emulsion polymerisation of the alkylcyanoacrylate. J. Nano. Res. 5, 365–371.

Chauvierre, C., Labarre, D., Couvreur, P., Vauthier, C., 2003c. Novel polysaccharide-decorated poly(isobutyl cianoacrylate) nanoparticles. Pharm. Res. 20, 1786–1793.

De Cruz Vieira, I., Fatibello-Filho, O., 1999. L-Cysteine determination using a polyphenol oxidase-based inhigbition flow injection procedure. Anal. Chem. Acta 399, 287–293.

Dietz, A.A., Rubinstein, H.H., 1972. Laboratory note. Standarization of the Ellman reaction. Clin. Biochem. 5, 136–138.

Egwim, I.O.C., Gruber, H.J., 2001. Spectrophotometric measurement of mercaptans with 4,4′-dithiodipyridine. Anal. Biochem. 288, 188–194.

Ellman, G.L., 1959. Tissue sulphydryl groups. Arch. Biomed. Biophys. 82, 70–77.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Fabel, S., Niessner, R., Séller, M.G., 2005. Effect-directed análisis by hight-performance liquid chromatography with gas-segmented enzyme inhibitor. J. Chromatogr A 1099, 103–110.

Gergel, D., Cederbaum, I., 1997. Interaction of nitric oxide with 2-thio-5-nitrobenzoic acid: Implications for the determination of free sulphydryl groups by Ellman's reagent. Arch. Biochem. Biophys. 347, 282–288.

Glatz, Z., Maslanova, H., 2000. Specific thiol determination by micellar electrokinetic chromatography and on-column detection reaction with 2,2′-dipyridyyldisulfide. J. Chromatogr. A 895, 179–187.

González-García, O., Ariño, C., Diaz-Cruz, J.M., Esteban, M., 2005. Comparison of voltametric detection assisted by multivariate curve resolution with amperometric detection in liquid chromatographic analysis of cysteine-containing compounds. J. Chromatogr. A 1062, 95–101.

Greindl, M., Bernkop-Schnürch, A., 2006. Development of a novel method for the preparation of thiolated polyacrylic acid nanoparticles. Pharm. Res. 23, 2183–2189.

Guan, X., Hoffman, B., Dwivedi, C., Matthees, D.P., 2003. A simultaneous liquid chromatography/mass spectrometric assay of glutathione, cysteine, homocysteine and their disulfides in biological samples. J. Pharm. Biomed. Anal. 31, 251–261.

Guggi, D., Marschütz, M.K., Bernkop-Schnürch, A., 2004. Matrix tablets based on thiolated poly(acrylic acid): pH-dependent variation in disintegration and mucoadhesion. Int. J. Pharm. 274, 97–105.

Heinemann, C., Escher, F., Conde-Petit, B., 2003. Structural features of starch-lactone inclusion complex in aqueous potato starch dispersions: the role of amylose and amylopectin. Carbohydrate Polym. 51, 159–168.

Hendricks, C.L., Ross, J.R., Pichersky, E., Noel, J.P., Zhou, Z.S., 2004. As enzyme-coupled colorimetric assay for *S*-adenosylmethionine-dependent methyltransferase. Anal. Biochem. 326, 100–105.

Hodgkins, R.P., Garcia-Bennett, A.E., Wright, P.A., 2005. Structure and morphopogy of propylthiol-functionalised mesoporous silicas templated by non-ionic triblock copolymers. Micropor. Mesopor. Mater. 79, 241–252.

Huang, M., Khor, E., Lim, L.Y., 2004. Uptake and cytotoxicity of chitosan molecules and nanoparticles: effects of molecular weight and degree of deacetylation. Pharm Res. 21, 344–353.

Iznaga Escobar, N., Morales, A., Núñez, G., 1996. Micromethod for quantification of SH groups generated after reduction of monoclonal antibodies. Nucl. Med. Biol. 23, 641–644.

Kast, C.E., Bernkop-Schnürch, A., 2001. Thiolated polymers-thiomers: development and in vitro evaluation of chitosan-thioglycolic acid conjugates. Biomaterials 22, 2345–2352.

Leitner, V.M., Guggi, D., Krauland, A.H., Bernkop-Schnurch, A., 2004. Nasal delivery of human growth hormone: in vitro and in vivo evaluation of a thiomer/glutathione microparticulate delivery system. J. Control. Release 100, 87–95.

Liu, J., Itoh, J.I., 2006. Kinetic determination of cysteine on flow injection system by utilizing catalytic complexation reaction of Cu(II) with 5,10,15,20-tetrakis (4-*N*-trimethylammino-phentyl) porphyrin. Talanta 70, 791–796.

Mao, S., Shuai, X., Unger, F., Wittmar, M., Xie, X., Kissel, T., 2005. Synthesis, characterization and cytotoxicity of poly(ethylene glycol)-graft-trimethyl chitosan block copolymers. Biomaterials 26, 6343–6356.

Nobs, L., Buchegger, F., Gurny, R., Allemann, E., 2004. Poly(lactic acid) nanoparticles labelled with biologically active Neutravidin™ for active targeting. Eur. J. Pharm. Biopharm. 58, 483–490.

Nobs, L., Buchegger, F., Gurny, R., Allemann, E., 2006. Biodegradable nanoparticles for direct or two-step tumor immunotargeting. Bioconjugate Chem. 17, 139–145.

Nogueira, R., Lammerhofel, M., Maier, N.M., Lindner, M.W., 2005. Spectrophotometric determination of sulphydryl concentration on the surface of thiol-modified chromatographic silica particles using 2,2′-dipyridyl disulfide reagent. Anal. Chem. Acta 533, 179–183.

Okonjo, K.O., Fodeke, A.A., Kehinde, A.T., 2006. Reversible reaction of 5-5′-dithiobis(2-nitrobenzoate) with the CysF9[93]β sulfhydryl groups of the hemoglobins of the domestic cat: variation of the equilibrium and reverse rate constant with pH. Biophys. Chem. 121, 65–73.

Owusu-Apenten, R.K., Chee, C., 2004. Sulphydryl group activation for commercial β-lactoglobulin measured using K-casein 2-thio, 5′nitrobenzoic acid. Int. Dairy J. 14, 195–200.

Owusu-Apenten, R.K., Chee, C., Hwee, O.P., 2003. Evaluation of a sulphydryl-disulphide exchange index (SEI) for whey proteins-β-lactoglobulin and bovine serum albumin. Food Chem. 83, 541–545.

Peracchia, M.T., Vauthier, C., Passirani, C., Couvreur, P., Labarre, D., 1997. Complement consumption by poly(ethylene glycol) in different conformations chemically coupled to poly(isobutyl 2-cyanoacrylate) nanoparticles. Life Sci. 61, 749–761.

Pfeifer, B.A., Burdick, J.A., Langer, R., 2005. Formulation and surface modification of poly(ester-anhydride) micro- and nanoparticles. Biomaterials 26, 117–124.

Prezelj, A., Strancar, J., Gubensek, F., Pecar, S., Degand, G., 2003. Quantification of binding of some thiol-reactive clenbuterol analogues to bovine serum albumin by electron paramagnetic resonance spectroscopy. Anal. Biochem. 315, 202–207.

Ramachandran, S., Rami, B.R., Udgaonkar, J.B., 2000. Measurements of cysteine reactivity during protein unfolding suggest the presence of competing pathways. J. Mol. Biol. 297, 733–745.

Ricci, F., Arduini, F., Amine, A., Moscone, D., Palleschi, G., 2004. Characterisation of Prussian blue modified screen-printed electrodes for thiol detection. J. Electroanal. Chem. 563, 229–237.

Riener, C.K., Kada, G., Gruber, H.J., 2002. Quick measurement of protein sulfhydryls with ellman's reagent and with 4,4′-dithiodipyridine. Anal. Bioanal. Chem. 373, 266–276.

Root, P., Mutus, P., 2003. *O*-Aminobenzoil-*S*-nitrosohomocysteine, a fluorogenic probe for cell-surface thiol determinations via microtiter plate assay. Anal. Biochem. 320, 299–302.

Rover, L., Kubota, L.T., Hoehr, N.F., 2001. Development of an amperometric biosensor based on glutathione peroxidase immobilized in a carbodiimide matrix for the analysis of reduced glutathione from serum. Clin. Chim. Acta 308, 55–67.

Russell, J., Rabenstein, D.L., 1996. Speciation and quantitation of underivatized and Ellman's derivatized biological thiols and disulfides by capillary electrophoresis. Anal. Biochem. 242, 136–144.

Samoulinov, A., Zweier, J.L., 1998. Development of chemiluminescence-based methods for the specific quantification of nitrosylated thiols. Anal. Biochem. 258, 322–330.

Sherman, F., Kuselman, I., 1999. Water determination in drugs containing thiols. Int. J. Pharm. 190, 193–196.

Sherman, F., Kuselman, I., Shenhar, A., 1996. Determination of water samples and ene-diols or thiols in samples inaccessible for direct K. Fischer titration. Talanta 43, 1035–1042.

Thanenhauser, T.W., Konishi, Y., Scheraga, H.A., 1984. Sensitive quantitative analysis of disulphide bonds in polypeptides and proteins. Anal. Biochem. 138, 181–188.

Weber, C., Riss, S., Langer, K., 2000. Preparation of surface modified protein nanoparticles by introduction of sulfhydryl groups. Int. J. Pharm. 211, 67–78.

Wright, S.K., Viola, R.E., 1998. Evaluation of methods for the quantitation of cysteines in proteins. Anal. Biochem. 265, 8–14.

Zhou, Y., Guo, X.C., Yi, T., Yoshimoto, T., Pei, D., 2000. Two continuous spectrophotometric assays for methionine aminopeptidase. Anal. Biochem. 280, 159–165.