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Surface modification of poly(lactic acid) nanoparticles by covalent attachment of thiol groups by means of three methods

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Abstract

The aim of the present work was to find a suitable method for the introduction of thiol functions on the surface of poly(DL-lactic acid) (PLA) nanoparticles. Three different approaches were investigated. The modification of the surface involves the activation of PLA carboxylic acid groups followed by the attack of a nucleophile such as cysteine (method #1) or cystamine (method #2 and #3) that provide thiol functions via an amide bond. For the conjugation with cystamine, a second reaction step is required to expose the sulfhydryl function of cystamine that is otherwise protected in a disulfide bond. 1,4-Dithio-DL-threitol (DTT) (method #2) or Tris(2-carboxyethyl)-phosphine hydrochloride (method #3) were evaluated for their ability to reduce this linkage. Method #1 allowed a maximum of 8.5 ± 2.8 mmol of thiol functions per mol of PLA to be attached on the surface of the nanoparticles. Method #2 allowed the introduction of a greater number of thiol functions (up to 190 ± 15 mmol per mol of PLA). However, this latter method has a major drawback: DTT interacts strongly with the nanoparticle matrix during the reduction step. Method #3 has the advantage over method #2 in that it allowed a significant number of thiol functions to be covalently bound to the particles (up to 107.6 ± 0.6 mmol per mol of PLA) without the problem of undesired interaction between DTT and the nanoparticle matrix. The introduction of thiol groups onto the surface of PLA nanoparticles is possible with all three suggested methods. The method #3 provides a straight forward approach for the substitution of carboxylic acid groups with a high number of activated sulfhydryl at the surface of PLA nanoparticles.

Keywords: Nanoparticles; Poly(DL-lactic acid); Sulfhydryl functions; Surface modification

1. Introduction

In recent years there has been growing interest in using poly(α-hydroxy carboxylic acid) nanoparticles as carrier systems for an increasing number of compounds such as anti-infectious agents or anticancer drugs, mainly due to the excellent

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biocompatibility and the controlled biodegradability properties of these polymers. To-date the use of such nanoparticles carrying drugs for parenteral administration is still limited due to the undesirable short circulating time in the blood compartment. After intravenous administration, poly(DL-lactic acid) (PLA) nanoparticles are rapidly taken up by the cells of the mononuclear phagocyte system (MPS) (Krause et al., 1985; Allémann et al., 1993) and consequently, a dramatic decrease in the efficacy of the colloidal carrier is observed. This has led several researchers to investigate the possibility to achieve longer circulating nanoparticles that are coated with hydrophilic and flexible polymers such as poly(ethylene glycol) (Bazile et al., 1995; Zambaux et al., 1999). The reported results represent a major breakthrough in this field reducing significantly the recognition by the MPS and thus increasing their circulation time in the vascular compartment when compared to non-coated nanoparticles (Dunn et al., 1997; Gref et al., 1994; Verrecchia et al., 1995; Leroux et al., 1996; De Jaeghere et al., 1999).

However, such nanoparticles are not able to deliver selectively active molecules to specific tissues or organs, where the therapeutic effect is required. Therefore, the next challenge for nanoparticles is to combine long circulating time in the blood with highly selective target binding capacity. To achieve this goal, antibodies have been adsorbed onto the particle surface, but this approach has significant drawbacks, such as potential competitive displacement of the adsorbed antibodies by blood components (Illum et al., 1983). An alternative approach to physical adsorption is the covalent binding of the antibody to the particle surface (Rolland et al., 1987; Akasaka et al., 1988). For this reason, it is essential to develop methods that enable functional groups to be generated on the nanoparticle surface, which can covalently bind substrates (e.g. antibodies, specific peptides, etc.), and thereby provide an active targeting mechanism. Commonly, antibody or protein conjugation is realised by the help of bifunctional cross-linkers such as sulfo-*m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (sulfo-MBS) (Jiang et al., 1990) that offer two binding sites, one for primary amino groups (antibodies, active drugs, specific peptides) and one for thiol functions. Thus, it is necessary to have accessible thiol groups on the surface of the particles to achieve the coupling of various compounds to the colloidal carrier. Since PLA does not have such functions, there is a need to modify the end groups of the polymer.

The aim of this study was to add sulfhydryl groups by a covalent linkage at the surface of nanoparticles using as a target site free carboxylic acid groups on PLA chain ends. This modification of the surface was accomplished by means of the well known two-step carbodiimide method (Irache et al., 1994; Hussain et al., 1997; Ezpeleta et al., 1999) followed by coupling of L-cysteine hydrochloride or cystamine dihydrochloride.

2. Materials and methods

2.1. Materials

PLA (Mw 57 kDa) was a gift from Alkermes (Cincinnati, Ohio), magnesium chloride hexahydrate, 1,4-Dithio-DL-threitol (DTT), cystamine dihydrochloride and L-cysteine hydrochloride monohydrate were purchased from Fluka (Buchs, Switzerland). Poly(vinyl alcohol) (Mowiol 4–88) was purchased from Hoechst (Frankfurt/M, Germany). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and 2-(*N*-Morpholino)-ethanesulfonic acid (MES) were obtained from Sigma (Buchs, Switzerland) and Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) was supplied by Pierce (Rockford, IL). 5,5′-Dithiobis(2-nitrobenzoic acid) (DNTB, Ellman's reagent) was purchased from Aldrich (Buchs, Switzerland).

2.2. Preparation of plain nanoparticles

Nanoparticles were prepared by a salting-out process according to a previously described method (De Jaeghere et al., 2000). Typically, 10 g of a saturated aqueous solution containing 10% (w/w) poly(vinyl alcohol) and 60% (w/w) magnesium chloride hexahydrate were added dropwise under vigorous stirring to 4 g of an organic phase constituted of 18% (w/w) PLA dissolved in ace-

tone. The miscibility of the two phases was prevented by the high concentration of the salt. This allowed the formation of an oil-in-water emulsion. Then, the emulsion was diluted by adding 10 g of pure water to allow the diffusion of acetone into the aqueous phase, inducing thus, the formation of raw nanoparticles. The resulting nanoparticles were centrifuged five times to remove poly(vinyl alcohol) and the salt. Thereafter, the purified nanoparticles were lyophilised and stored at −20 °C for subsequent chemical modification. The size of nanoparticles was characterised by photon correlation spectroscopy using a Zetasizer 5000 (Malvern Intruments Ltd, UK).

2.3. Introduction of thiol functions with EDAC and cysteine (method #1)

The carboxylic groups on the surface of PLA nanoparticles (60 mg) were activated in 2.4 ml phosphate buffer (pH 4.0, 100 mM) by dropwise addition of an equal volume of EDAC solution (50 mg/ml). The mixture was incubated at room temperature for 30 min to 3 h. The unreacted EDAC was removed by centrifugation and the nanoparticles were resuspended in 2.4 ml phosphate buffer. The same volume of L-cysteine hydrochloride solution (100 mg/ml) was added to the suspension containing the activated nanoparticles suspension and the mixture was shaken gently at room temperature for 3 h (Fig. 1a). The conjugated nanoparticles were centrifuged to remove free L-cysteine and the clear supernatant analysed using Ellman's reagent (description of the assay below). Finally, cysteine-conjugated nanoparticles were lyophilised and stored at −20 °C.

2.4. Introduction of thiol function with EDAC and cystamine dihydrochloride

2.4.1. Procedure with a final cleavage of disulfide bonds by DTT (method #2)

PLA nanoparticles were suspended in phosphate buffer (pH 4.7, 100 mM) and aliquots (1000 μl) of the suspensions (25 mg/ml) were mixed with different volumes (0.0–3400 μl) of a solution of cystamine dihydrochloride (26 mg/ml). To this mixture 400 μl of EDAC solution (110 mg/ml) were added dropwise to activate the carboxylic groups. The samples were gently shaken at room temperature for 24 h. Thereafter, each sample was purified to eliminate EDAC and the unbound cystamine dihydrochloride. The disulfide functions on the surface of the nanoparticles provided by the cystamine were reduced by treating each sample with 1000 μl of a DTT solution (30 mg/ml in phosphate buffer). The mixtures were shaken for 24 h at room temperature and then centrifuged to remove DTT (Fig. 1b). The supernatant of each centrifugation step was used to determine the amount of DTT remaining in the samples by Ellman's reagent. The purified nanoparticles were lyophilised and then frozen for storage.

2.4.2. Procedure with a final cleavage of disulfide bonds by TCEP (method #3)

PLA nanoparticles (100 mg) were suspended in MES buffer (0.1 M MES, 0.9% NaCl, pH 4.7) and the reaction was initiated by adding consecutively 10 ml of a solution of EDAC (24 mg/ml) and an increasing volume (0–6 ml) of cystamine dihydrochloride (59 mg/ml). The final suspension was completed to 25 ml with the same buffer. The mixture was shaken under mild stirring during 24 h at room temperature. Thereafter, EDAC and the unbound cystamine dihydrochloride were removed by 4 successive centrifugations of the mixture and the particles were resuspended in the same activation buffer.

The reduction of disulfide bonds was carried out in 20 ml of MES buffer (pH 4.7) by adding 1 ml of a solution of TCEP (6 mg/ml). After 3 h of incubation, the nanoparticle suspension was centrifuged until no free TCEP was detected in the supernatant using DTNB (Fig. 1b).

2.5. Quantitative determination of thiol functions with Ellman's reagent

The number of thiol groups on the nanoparticle surface was determined using Ellman's reagent (Ellman, 1959). Lyophilised nanoparticles (10 mg) were suspended in 780 μl of phosphate buffer pH 7.2 (1 mM EDTA) and to this suspension 15 μl of DTNB solution (0.4% w/v) were added and the mixture was incubated for 15 min at room

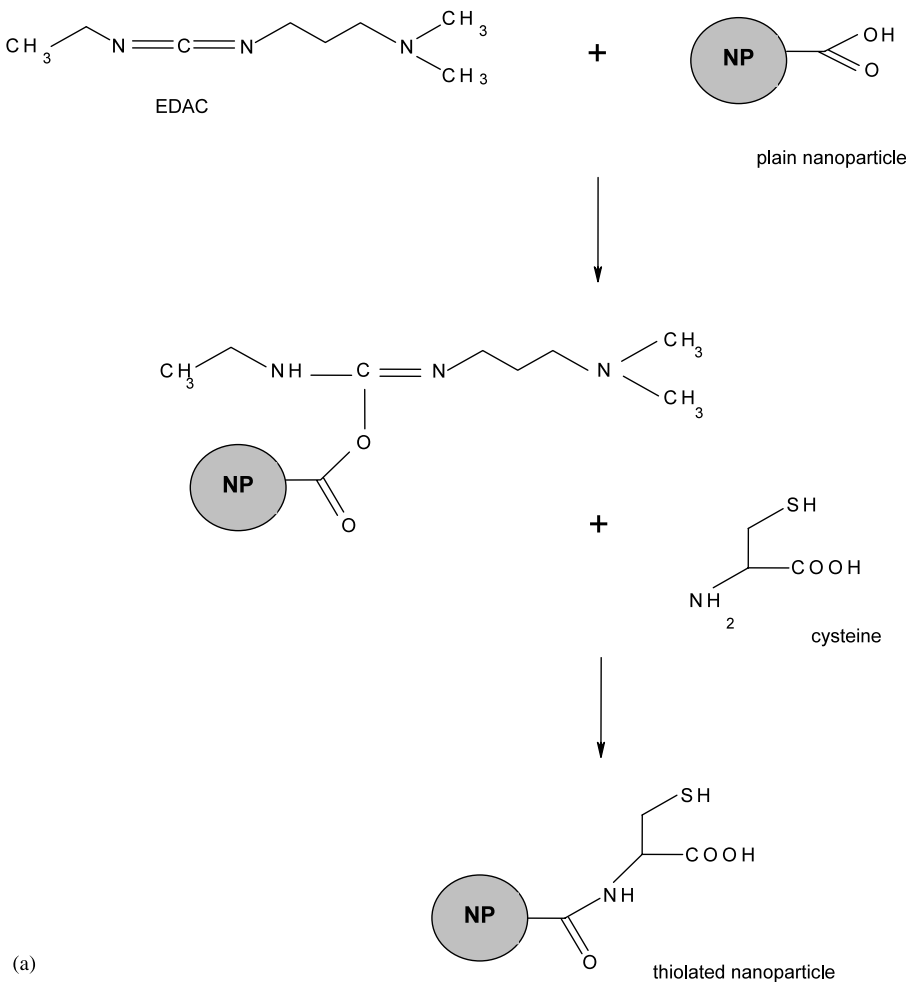

Fig. 1. Schematic presentation of the coupling of thiol groups on nanoparticle (NP) using cysteine (a) or cystamine (b) as substrate.

temperature under mild stirring. Thereafter, the nanoparticle suspension was centrifuged twice at $48\,000 \times g$ for 10 min to separate the supernatant containing 2-nitro-5-thiobenzoic acid (NTB) from the nanoparticles. The NTB concentration was determined spectrophotometrically at 412 nm and the concentration of thiol functions at the nanoparticle surface was then calculated.

The amount of thiol functions obtained on the surface of one nanoparticles was determined using a density of 1.5 g/cm^3 and the mean size of nanoparticles.

$$n = aN(d(4/3)\pi r^3) \quad (1)$$

n, amount of thiol function per nanoparticle; a, mol of SH per g PLA; d, density of nanoparticles; r, mean radius of nanoparticles; N, 6.022×10^{23} (Avogadro Number).

3. Results and discussion

3.1. Thiolation of PLA nanoparticles using cysteine (method #1)

The first method chosen for the preparation of nanoparticles with sulfhydryl groups on their surface is based on a two-step carbodiimide

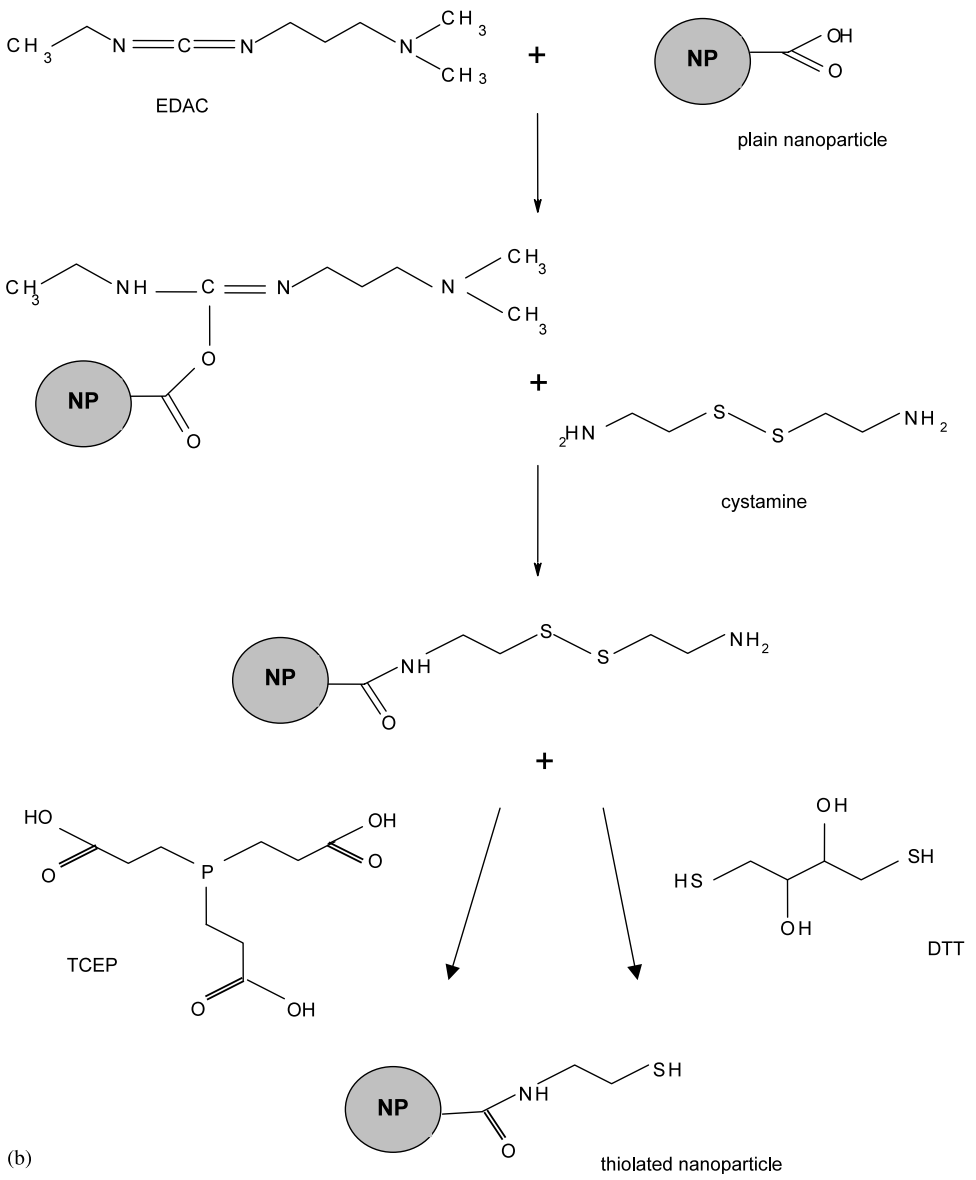

Fig. 1 (*Continued*)

technique (Irache et al., 1994; Hussain et al., 1997; Ezpeleta et al., 1999). Initial activation of PLA carboxylic groups of the nanoparticles by EDAC to form the *O*-isoacylurea is followed by a nucleophilic attack of the amine on the cysteine leading to the formation of nanoparticles with exposed reactive thiol functions on their surface attached by an amide bound. Non-covalently associated cysteine and the urea derivative of EDAC released during the latter reaction were eliminated by centrifugation and supernatants were collected for a subsequent analysis by Ellman's reagent. This approach was recently described by Bernkop-Schnürch et al. for the introduction of sulfhydryl groups on polymers to generate mucoadhesive matrix-tablets (Bernkop-Schnürch, 2000) and by Weber et al. for the preparation of human serum albumin nanoparti-

cles (Weber et al., 2000). We modified this method for PLA nanoparticles by varying different parameters such as incubation time and buffer composition.

The quantification of thiol groups on the nanoparticle surface by Ellman's reagent revealed a small, but detectable number of sulfhydryl functions bound to the particles with the spectrophotometric method. The approximate number of sulfhydryl groups per nanoparticle is shown in Table 1. The small number of thiol functions can be explained by the fact that cysteine, due to its two functional groups (carboxylic acid and sulfhydryl), can on the one hand interact with EDAC by forming an amide bond and thus desactivate the EDAC reagent and on the other hand, after activation of PLA carboxylic acid, cysteine can bind to the surface by its thiol function instead of the amine group and form a thioester bond. In the two cases, a decrease in the total amount of thiol functions on the nanoparticle surface will be observed. This inconvenience has already been described (Carraway and Triplett, 1970; Weber et al., 2000) and to circumvent it the replacement of cysteine by another nucleophile reagent such as cystamine, which has only an amine function, can be envisaged.

Nevertheless, the total amount of sulfhydryl functions obtained with cysteine on the nanoparticles surface appears sufficient for further conjugation since there are almost 1000 groups per nanoparticle.

The number of thiol functions per nanoparticle seems not to depend on the incubation time of EDAC. In fact, there is only a slight difference between the results obtained with the three reaction times. When longer reaction time (up to 24 h) was used there were no thiol groups detectable on the surface of the nanoparticles (data not shown). This could be explained by the fact that a longer incubation time probably leads to the degradation of the *O*-acylisourea complex, formed after the activation of carboxylic functions by EDAC. This intermediate compound is rapidly hydrolysed in aqueous solution leading to the regeneration of the carboxylic acid group if the amine reagent, in our case cysteine, does not react before the hydrolysis (Hoare and Koshland, 1967; Arangoa et al., 2000). Another explanation for the small number of thiol functions could be the formation of a stable *N*-acylureae derivate obtained by the rearrangement of the *O*-acylisourea (Hoare and Koshland, 1967; Arangoa et al., 2000). Consequently, for the EDAC coupling reactions the incubation time chosen was 45 min for all other experiments.

Several trials were also performed in an aqueous solution in the absence of buffer. The pH was only maintained at 4 by the addition of either HCl (0.1 N) or NaOH (0.1 N) solution. Results obtained in unbuffered solution were encouraging, since a slight increase in the number of thiol functions was observed (Table 2). Control experiments were carried out to ensure that cysteine was covalently bound to the surface of the nanoparticles and not physically adsorbed: the reaction was done by adding cysteine to the nanoparticles that were not activated with EDAC. The quantification by Ellman's reagent revealed no trace of thiol functions on the surface of the particles which gives evidence that cysteine is non physically adsorbed to the particles.

The average particle size and the polydispersity index of the thiolated particles were also measured The results shown in Table 2 clearly indicate that in most cases there is only a slight increase in the particle size compared to the plain nanoparticles. Furthermore, no aggregation of the particles was observed.

Stability trials were performed on lyophilised thiolated nanoparticles in order to determine the stability of the sulfhydryl groups at −20 °C over 3 months. At selected time intervals, nanoparticles were assayed for thiol groups using Ellman's

Table 1
Influence of EDAC time reaction on the incorporation of thiol groups on the nanoparticles surface using cysteine as substrate (method #1, in buffered solution)

EDAC incubation time (min)	Thiol groups (mmol per mol) of PLA	Thiol groups per PLA nanoparticle
30	5.5 ± 3.0	715 ± 394
45	7.4 ± 1.5	961 ± 183
180	6.4 ± 2.9	835 ± 371

Mean ± SD; $n = 3$.

Table 2
Quantification of thiol functions on the nanoparticles surface and measure of their average size after lyophilisation

Batch number	Thiol groups (mmol per mol of PLA)	Thiol groups (units per PLA nanoparticle)	Particles average size (nm) before thiolation	Particles average size (nm) after thiolation
1	8.6±5.9	1110±769	233.3 (0.083)[a]	403.9 (0.373)[a]
2	8.2±2.9	1063±363	243.1 (0.039)[a]	265.9 (0.081)[a]
3	8.4±3.0	1094±389	238.9 (0.022)[a]	263.4 (0.362)[a]

Thiolated nanoparticles obtained with method #1 in aqueous solution in the absence of buffer (pH adjusted by addition of either HCl or NaOH). Mean±SD; $n = 3$.
[a] Polydispersity index (0–1).

reagent. Results illustrate that the storage of thiolated nanoparticles was possible (Table 3) and that there is no need to proceed immediately for further conjugation of the nanoparticles after their thiolation.

3.2. Introduction of thiol function with EDAC and cystamine dihydrochloride

3.2.1. Cleavage of disulfide bonds by DTT (method #2)

The second approach to attach thiol functions on the nanoparticles surface is based on the same principle as the one described before (activation of carboxylic acid by EDAC, covalently binding of a compound including amine function) but instead of using cysteine as a substrate, cystamine was chosen which does not contain directly available sulfhydryl groups. Therefore, an additional reaction step is required to reduce the disulfide bonds using a sulfhydryl reductant reagent such as DTT. This method was already described by Weber et al. (Weber et al., 2000) for the introduction of thiol functions on human serum albumin nanoparticles.

Table 3
Influence of the storage time on the amount of thiol functions on the nanoparticle surface using method #1, in buffered solution

Storage period (month)	Thiol groups (mmol per mol) of PLA	Thiol groups per PLA nanoparticle
0	8.4±3.0	1094±389
1	8.5±2.8	1098±360
2	8.3±2.1	1080±275
3	8.1±2.5	1054±327

Mean±SD; $n = 3$.

The main advantage of using cystamine rather than cysteine is the lack of other functional groups such as carboxylic acid and free thiol groups, which may react either with EDAC or with, lately activated carboxylic acid and thus decrease the final number of thiol functions attached to the nanoparticle surface.

In order to evaluate the influence of cystamine dihydrochloride concentration on the number of thiol functions introduced at the surface of nanoparticles, different concentrations of the substrate ranging from 0 to 895 mol per mol of PLA nanoparticles were added in the first step of the reaction. Results demonstrated a good correlation, as shown in Fig. 2, between the amount of cystamine dihydrochloride added and the number of thiol function calculated on the nanoparticle surface (Fig. 2). The increase of sulfhydryl functions at the nanoparticle surface is significant compared to the previous method, with an incorporation of up to 190±15 mmol thiol groups per mol of PLA.

As shown in Fig. 2, the quantification of sulfhydryl groups detects thiol functions introduced via cystamine dihydrochloride as well as a significant quantity of such functions representing DTT. Evidence of this was given by the quantification of blank nanoparticles incubated only in the presence of DTT without having been exposed to EDAC and cystamine. These blank nanoparticles revealed a significant quantity of DTT (95±11 mmol/mol PLA). It is assumed that there is a strong affinity of DTT for PLA nanoparticles. Indeed, even after 20 purification steps (40 000 × g, 15 min) a small amount of DTT was still released from nanoparticles. To prove the exis-

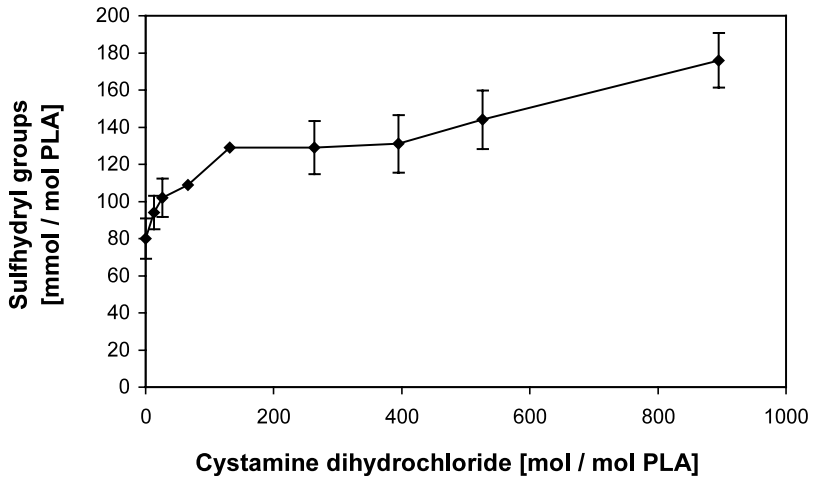

Fig. 2. Influence of cystamine dihydrochloride concentration (0.0–895 mol/mol PLA) on the incorporation of thiol groups at the surface of nanoparticles using DTT as a reducing agent (method #2). Each point represents the mean ± SD; $n = 3$ except 4th and 5th experimental point, $n = 2$.

tence of this strong interaction of DTT for PLA nanoparticles and the fact that more thiol functions were fixed onto the nanoparticles surface by adsorption than by covalent coupling two different trials were carried out. The first consisted in the investigation of the influence of the number of centrifugation steps on the amount of available thiol groups. After the two reactions (cystamine dihydrochloride coupling and disulfide cleavage by DTT), nanoparticles were purified by 6, 12 or 20 centrifugation steps ($40\,000 \times g$, 15 min). After lyophilisation of the three different formulations, quantification of thiol groups by Ellman's reagent was performed and, as shown in Fig. 3, the increase of centrifugation cycles led to a decrease of detectable functions. In fact, when only 6 centrifugation steps were carried out, the concentration of thiol groups reached up to almost 900 mmol/mol PLA; whereas with 12 centrifugation steps a dramatic decrease of sulfhydryl groups was observed. This confirms that a considerable amount of thiol functions are not covalently bound to the nanoparticle surface and that changing the purification conditions influences the final concentration of functional groups. Only a small difference was found upon increasing the number of centrifugation steps from 12 to 20. The assay suggests that the affinity between DTT and PLA nanoparticles is strong enough to potentially use

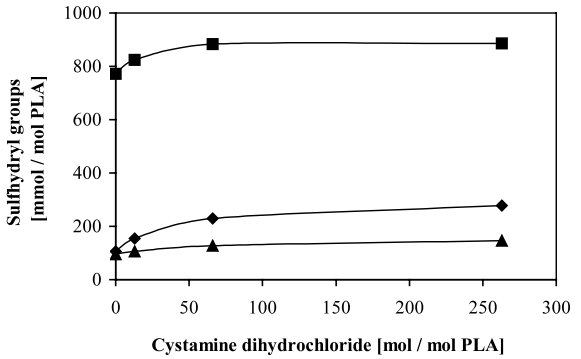

Fig. 3. Influence of the purification on the introduction of thiol functions. Study of the effect of the number of centrifugation steps on the introduction of thiol groups after reduction of disulfide bonds by DTT (method #2). ■, 6 centrifugations; ◆, 12 centrifugations; ▲, 20 centrifugations.

the adsorption of DTT as a method for providing thiol functions without a covalent bond.

With the aim to further study the latter hypothesis a second experiment was performed to evaluate the degree of adsorption of DTT to PLA nanoparticles. Briefly, nanoparticles were incubated with DTT during 6–24 h followed by 5 centrifugation steps to eliminate the free DTT. The amount of available thiol function was quantified by Ellman's reagent and as the results show in Table 4, there is not a significant difference of the

number of thiol function between the three DTT incubation times. This means that the adsorption procedure of DTT on the surface is very rapid and that an incubation time of 6 h is sufficient to have a high number of non-covalently bound thiol groups to the particles. The same quantification was performed on hydrolysed nanoparticles (by adding of NaOH 1 N) to discard the possibility that a fraction of the total amount of DTT may also be incorporated inside the nanoparticles. The results (Table 4) were surprising as the number of thiol functions measured after hydrolysis of the nanoparticles was much higher than the amount measured on intact nanoparticles suggesting thiol functions are mainly located in the nanoparticle matrix and only a small portion is adsorbed on the surface. A prolonged incubation time (24 h) demonstrated a higher entrapment efficacy since the number of total thiol functions was even higher (11.9 mol/mol PLA). While the simple adsorption of DTT on the nanoparticles surface could have been an easy technique for the addition of thiol functions on the surface of the particles, this last study gave evidence that this adsorption technique is not suitable, since a large number of thiol functions are entrapped into the matrix of the particles, this location being undesired for further chemical conjugation; in fact these functions could be released and compete with the small amount of DTT adsorbed on the surface.

The main inconvenience of the reaction with cystamine dihydrochloride and DTT is the significant number of unbound thiol functions taken up by the nanoparticles in the presence of DTT. Decreasing the concentration of DTT to circumvent this problem, avoided the entrapment of DTT but with the loss of the efficacy of the sulfhydryl reductant. In fact, no thiol groups were detectable on the surface of the nanoparticles (data not shown).

Nevertheless, it has been demonstrated that this reaction procedure works since the number of thiol groups increases as a function of cystamine concentration.

3.2.2. *Cleavage of disulfide bonds by TCEP (method #3)*

An interesting alternative to the use of DTT as reducing agent for disulfide bonds is TCEP, a selective, strong and rapid sulfhydryl reductant. Previous studies have compared sulfhydryl reductants and TCEP. It was shown that TCEP was more stable than DTT over a wider pH range (Han and Han, 1994) and was able to cleave disulfide bonds more rapidly and specifically than DTT. In our study the biggest advantage of replacing DTT by TCEP was the absence of interaction between TCEP and PLA nanoparticle. In fact, the control (reaction in the absence of cystamine) revealed no trace of TCEP (Fig. 4) and moreover during the fifth centrifugation step no further detachment of TCEP was detected, which proves that TCEP is not taken up by the nanoparticles. The quantification of the thiol groups on the nanoparticle surface obtained using TCEP proves that this sulfhydryl reductant can favourably replace DTT since the number of covalently bound thiol groups obtained is approximately the same as with DTT but without requiring a large number of centrifugation steps. In addition, the concentration of TCEP can be reduced to 0.3 mg/

Table 4
Influence of the DTT incubation time on the amount of thiol functions taken up by the nanoparticles

DTT incubation time (h)	Intact nanoparticles		Hydrolysed nanoparticles	
	Thiol groups (mol per mol of PLA)	Thiol groups ($\times 10^3$ units per PLA nanoparticle)	Thiol groups (mol per mol of PLA)	Thiol groups ($\times 10^3$ units per PLA nanoparticle)
6	0.9 ± 0.1	112.3 ± 18.6	7.2 ± 0.2	1543 ± 64.4
12	0.7 ± 0.1	95.1 ± 18.6	10.3 ± 0.1	1331 ± 18.6
24	0.7 ± 0.2	95.1 ± 37.2	11.9 ± 0.5	3377 ± 37.2

Mean ± SD; $n = 3$.

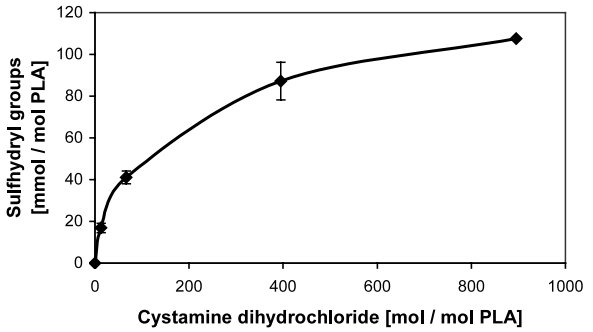

Fig. 4. Influence of cystamine dihydrochloride (0.0–895 mol/mol PLA) concentration on the incorporation of thiol functions at the surface of nanoparticles using TCEP as reducing agent (method #3). Each point represents the mean ± SD; $n = 3$.

ml without loss of efficacy of the sulfhydryl reductant.

The same reaction was also performed with an incubation time of 24 h instead of 3 h. A decrease in the number of thiol groups formed was observed (data not shown). This could be explained by the fact that the thiol functions formed by means of TCEP are oxidized during the reaction leading to reformation of disulfide bonds, if a larger incubation time is chosen. Furthermore, the concentration of TCEP might not be sufficient to protect the formed thiol functions in aqueous solution.

Although it has been stated (Getz et al., 1999) that TCEP was unreactive with thiol-reactive functions, such as maleimide, it has been recently demonstrated that TCEP can rapidly react with maleimide functions (Shafer et al., 2000) thus rendering elimination of TCEP from the sample compulsory before further conjugation.

The mean size of the nanoparticles after thiolation was also measured and the results (282.4 nm) were encouraging since only a slight increase in the size was observed compared to the plain nanoparticles (258.9 nm).

Three months after thiolation of the nanoparticles the concentration of sulfhydryl functions did not significantly change (104.3 mmol/mol PLA). This confirms that thiol functions on the surface of lyophilised nanoparticles (stored at -20°) seems to be stable for a sufficient period of time.

4. Conclusions

In the present study it has been demonstrated that surface modification by coupling L-cysteine hydrochloride and cystamine dihydrochloride by the carbodiimide reaction on PLA nanoparticles is feasible. The introduction of thiol functions by cysteine (method #1) has a number of advantages over the cystamine technique (method #2 end #3); it allows a simple surface modification procedure without the inconvenience of an additional reaction step (reduction of disulfide bound). However, the method #1 allows the introduction of a relatively small number of sulfhydryl groups per nanoparticle. On the contrary, the two other methods using cystamine dihydrochloride as substrate generate more sulfhydryl functions on the nanoparticle surface (up to 1.3×10^4 functions per nanoparticle). The presence of DTT is the major drawback of the second method since it can interfere, by way of its thiol functions, in further conjugations. The third approach involving TCEP represents an interesting alternative to the two previous methods since it permits the introduction of more thiol functions without the problem of undesired DTT reagent.

Further research is ongoing aiming to add moieties such as antibodies for active drug targeting via bifunctional cross-linkers.

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