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Research paper

Poly(lactic acid) nanoparticles labeled with biologically active Neutravidin™ for active targeting

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Abstract

In this study, NeutrAvidin™ was covalently bound to the surface of poly(DL-lactic acid) (PLA) nanoparticles, with the aim of attaching targeting compounds, such as proteins, to their surface. Sulfhydryl groups were first introduced on the surface of PLA nanoparticles through a carbodiimide reaction. NeutrAvidin™ was then bound to the thiolated nanoparticles via a bifunctional cross-linker, which offers two binding sites, one for primary amine groups and one for thiol functions. The amount of sulfhydryl groups obtained on the surface of the nanoparticles was determined. The NeutrAvidin™-labeled nanoparticles were evaluated with respect to particle size, protein concentration and biotin binding capacity. The concentration of thiol functions on the surface of nanoparticles was 105 ± 15 mmol/mol PLA. The quantification of NeutrAvidin™ coupled to the nanoparticles revealed that a significant amount of the protein was covalently bound to the nanoparticles. The concentration of NeutrAvidin™ bound to the nanoparticles could be controlled by varying the amount of protein during the coupling reaction. The maximum concentration of NeutrAvidin™ attached to the particles was 6 mmol per mol of PLA. The specific activity of NeutrAvidin™ bound to the nanoparticles was also evaluated and results revealed that the protein maintained the capacity to bind biotin. The activity of the NAR-labeled nanoparticles was lower than expected, due to the undesired aggregation of the native NeutrAvidin™. Altogether, the results suggest that other proteins, such as antibodies could be coupled to the nanoparticles for active targeting. Furthermore, PLA nanoparticles bearing NeutrAvidin™ are interesting candidates for active targeting with biotinylated antibodies using the biotin–avidin interaction in a two step procedure.

Keywords: Nanoparticles; Poly(DL-lactic acid); Sulfhydryl functions; Surface chemistry; NeutrAvidin™; Drug targeting

1. Introduction

Poly(α-hydroxy carboxylic acid) nanoparticles have shown great potential as carrier systems for an increasing number of active molecules, largely due to the excellent biocompatibility and the controlled biodegradability properties of these polymers. However, after intravenous administration, PLA nanoparticles are rapidly taken up by the cells of the mononuclear phagocyte system (MPS) [1,2]. In order to overcome this problem, many studies have been devoted to enhancing the circulation time of the nanoparticles in the blood stream. Several research groups have investigated the possibility of achieving longer circulating nanoparticles by coating them with hydrophilic and flexible polymers such as poly(ethylene glycol) (PEG) [3,4]. 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 [5,6].

Although capable of enhanced accumulation in the target tissue compared to plain particles, the PEG-coated nanoparticles cannot provide specific targeting. Therefore, the need of exploring new strategies for the development of particles that are able to deliver active compounds to specific sites in the organism, is the challenge in this field. The active targeting of particles to organs other than the liver and the spleen (passive targeting) can be achieved by using nanoparticles exhibiting a ligand, such as antibodies

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or peptides, which recognize and bind specifically to target cells. To this end, antibodies have been adsorbed onto the particle surface [7–10], but with this approach, a potential competitive displacement of the adsorbed antibodies by blood components can happen [8,9]. An alternative approach to physical adsorption is the covalent binding of the antibody to the particle surface [11,12]. Frequently, antibodies cannot be directly attached to the surface of nanoparticles since these latter, do not offer functional groups for coupling reactions. For this reason, it is essential to develop methods that enable functional groups to be generated on the nanoparticle surface thus allowing covalent binding of substrates (e.g. antibodies, specific peptides, etc). Commonly, antibody or protein conjugation is realized by using bifunctional cross-linkers such as sulfo-*m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (sulfo-MBS) [13] that offer two binding sites, one for primary amino groups (antibodies, active drugs, specific peptides) and one for thiol functions. Since PLA does not have such functions, the end groups of the polymer must first be modified to obtain accessible thiol groups. Owing to the presence of these latter, various compounds can then be bound to the nanoparticles.

The purpose of the present work was to demonstrate the feasibility of binding a protein to the surface of PLA nanoparticles. NeutrAvidin™ was chosen as a model protein since the avidin–biotin interaction has proved to be an efficient tool for active targeting using a multi-step targeting approach [14,15]. Furthermore, the avidin-labeled nanoparticles can be a starting point for the binding of a whole series of biotinylated compounds besides antibodies, such as drugs or fluorescent dyes. The binding of avidin to the nanoparticles was achieved thanks to the presence of thiol functions. These latter were formerly added to the nanoparticle surface by a carbodiimide reaction in the presence of cystamine. This method has been selected for optimal properties among three different method explored [16]. The coupling reaction between thiolated nanoparticles and the protein was achieved via the bifunctional cross-linker sulfo-MBS.

2. Materials and methods

2.1. Materials

Poly(DL-lactic acid) (PLA) (100DL 4A, Mw 57 kDa) was a gift from Alkermes (Cincinnati, Ohio, USA), cystamine dihydrochloride was purchased from Fluka (Buchs, Switzerland). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), D-biotin and 2-(*N*-Morpholino)ethanesulfonic acid (MES) were obtained from Sigma (Buchs, Switzerland). Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), *m*-Maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (sulfo-MBS), avidin, Immunopure NeutrAvidin™ biotin binding protein (NA) and D-Salt™ dextran plastic desalting columns were supplied by Pierce (Rockford, IL, USA). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) was purchased from Aldrich (Buchs, Switzerland). NeutrAvidin™ Rhodamine Red™-X (NAR) and biotin-4-fluorescein were obtained from Molecular Probes (Leiden, The Netherlands). All reagents were of analytical grade.

2.2. Introduction of thiol functions on the surface of the nanoparticles

Plain nanoparticles having free carboxylic functions available were prepared by a salting-out process according to a previously described method [17]. Thiolated nanoparticles were prepared by a two-step reaction in the presence of EDAC [16]. Typically, plain 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 5 ml of a solution of cystamine dihydrochloride (71 mg/ml). The final suspension was completed to 25 ml with the same buffer. The mixture was stirred under mild conditions for 24 h at room temperature. Thereafter, EDAC and the unbound cystamine dihydrochloride were removed from the mixture by 4 successive centrifugations ($20{,}000 \times g$ for 10 min) and the nanoparticles were re-suspended in the same 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. The concentration of thiol functions on the surface of nanoparticles was determined by a spectrophotometric technique (Hewlett Packard, Model 8453, Germany) using Ellman's reagent [18]. Details of the method have been previously described [16]. The size of nanoparticles was characterised by photon correlation spectroscopy using a Zetasizer 5000 (Malvern Instruments Ltd, UK).

2.3. Coupling Neutravidin™ Rhodamine Red™-X to thiolated nanoparticles

A solution of NeutrAvidin™ Rhodamine Red™-X (NAR) (1 mg) was prepared in 160 μl PBS (pH 7.4) and activated by 40 μl of a solution of sulfo-MBS (8 mg/ml) for 60 min at room temperature. The excess of sulfo-MBS was removed from the mixture by size exclusion chromatography using a D-Salt™ dextran plastic desalting column. The fractions containing the maleimide-activated NAR were collected and stored for further conjugation with thiolated nanoparticles. A volume of 500 μl of the activated NAR solution (0–339 μg/ml) was added to an equal volume of thiolated nanoparticles (20 mg/ml) and the resulting mixture was gently shaken for 60 min at room temperature. Thereafter, the uncoupled protein was removed by centrifugation at $20{,}000 \times g$ for 10 min until no free NAR was detected in

the supernatant. Finally, NAR conjugated nanoparticles were either lyophilised and kept at −20 °C, or stored in solution at 4 °C.

The number of NAR functions generated on the surface of a single nanoparticle was determined using a particle density of 1.5 g/cm^3 and the mean nanoparticle radius.

$$n = aN(d4/3\pi r^3) \quad (1)$$

n, number of NAR per nanoparticle
a, mol of NAR per g PLA
d, density of nanoparticles
r, mean radius of nanoparticles
N, 6.022×10^{23} (Avogadro Number)

2.4. Measurement of the specific activity of NeutrAvidin™ Rhodamine Red™-X before and after the coupling reaction

The specific activity of the protein was measured using a spectrofluorimetric assay (Spex, Fluoromax®, UK) and a biotin–fluorescein conjugate [19]. A solution of NAR (7.5 μg/ml) was prepared in a phosphate buffer (100 mM NaCl, 50 mM NaH_2PO_4, 1 mM EDTA, pH adjusted to 7.5 with NaOH). A suspension of NAR-labeled nanoparticles (2 mg/ml) was also prepared in the same buffer. A sample (2 ml) containing either the solution of NAR or the suspension of NAR-labeled nanoparticles was added to a cuvette for fluorescence monitoring. To this sample, aliquots of 5 μl of a solution of biotin-4-fluorescein (2.94–15.9 μM) were successively added at 1-min intervals. The sample was excited at 494 nm and the emission was measured at 523 nm after each addition of biotin-4-fluorescein. Some trials have been also performed with unlabeled NA.

3. Results and discussion

3.1. Preparation of PLA nanoparticles bearing thiol functions on the surface

Plain nanoparticles were prepared by a salting-out process according to a previously described method [17]. PLA having free carboxylic acid end groups was chosen since these groups are essential for the covalent attachment of thiol functions. It is assumed that the free carboxylic acid groups are located on the surface of the formed particles due to their hydrophilicity. Thiolation of the particles was initiated by activation of the carboxylic acid groups by EDAC and by the covalent binding of cystamine (Fig. 1). Directly available thiol functions were obtained with the reduction reaction using TCEP; this approach was recently described by our group [16]. The result of the binding reaction was evaluated by a spectrophotometric method using Ellman's reagent. The thiolation reaction was found to be very reproducible with an incorporation of up to 105 ± 15 mmol of thiol functions per mol of PLA, corresponding to approximately 14,000 functions per nanoparticle. The average size of the particles after the reaction was also measured and, as shown in Table 1, only a slight increase was observed compared to plain nanoparticles.

3.2. Coupling NeutrAvidin™ Rhodamine Red™-X to thiolated nanoparticles

NA is a modified avidin with low non-specific binding properties due to the absence of carbohydrates. It has, however, maintained the strong and stable interaction with biotin [20]. Furthermore, NA, unlike avidin, is not rapidly taken up by liver and kidney and consequently has an increased circulation time in the organism.

The binding of NAR to the thiolated nanoparticles was carried out using a bifunctional cross-linker, sulfo-MBS. This cross-linking agent offers two binding sites, one (succinimidyl ester) for primary amines (NAR) and one (maleimide group) for thiol functions (thiolated nanoparticles). This coupling reaction, which we have adapted for PLA nanoparticles, was recently described by Langer et al. for the preparation of human serum albumin nanoparticles [21]. The first step consisted of binding sulfo-MBS to NAR to form a stable amide bond (Fig. 2). The unreacted sulfo-MBS was eliminated using D-Salt™ dextran plastic desalting column, and the fractions containing the maleimide-activated intermediate were collected. The concentration of NAR in each fraction was determined by a spectrofluorimetric method (absorption 573 nm, fluorescence emission 591 nm). Since maleimide groups are labile in aqueous solution, the maleimide-activated protein fractions were immediately added to the thiolated nanoparticles. Different reaction times of 30 min to 17 h were evaluated for the coupling between NAR and thiolated nanoparticles. No significant difference among the various reaction times was observed (data not shown). Consequently, for all subsequent experiments, a reaction time of 60 min was chosen. Unbound NAR was removed from the suspension by ultracentrifugation (20,000 × g for 10 min) until no free protein was detectable by fluorescence monitoring. Subsequently, nanoparticles were either freeze-dried or stored in solution at 4 °C. The amount of NAR coupled to the nanoparticles was quantified by measurement of the fluorescent emission of Rhodamine Red™-X conjugated to NA. The assay was carried out on lyophylised nanoparticles after hydrolysis (NaOH 1 M) to avoid all background interferences. Results revealed that it is possible to bind NAR to the surface of the nanoparticles and to control the amount of protein coupled to the particles (Fig. 3) and that an increase in concentration of the protein during the coupling reaction led to a related increase in the amount of the protein obtained on the surface of the nanoparticles. Subsequent use of higher concentrations of NAR (30 mmol/mol PLA)

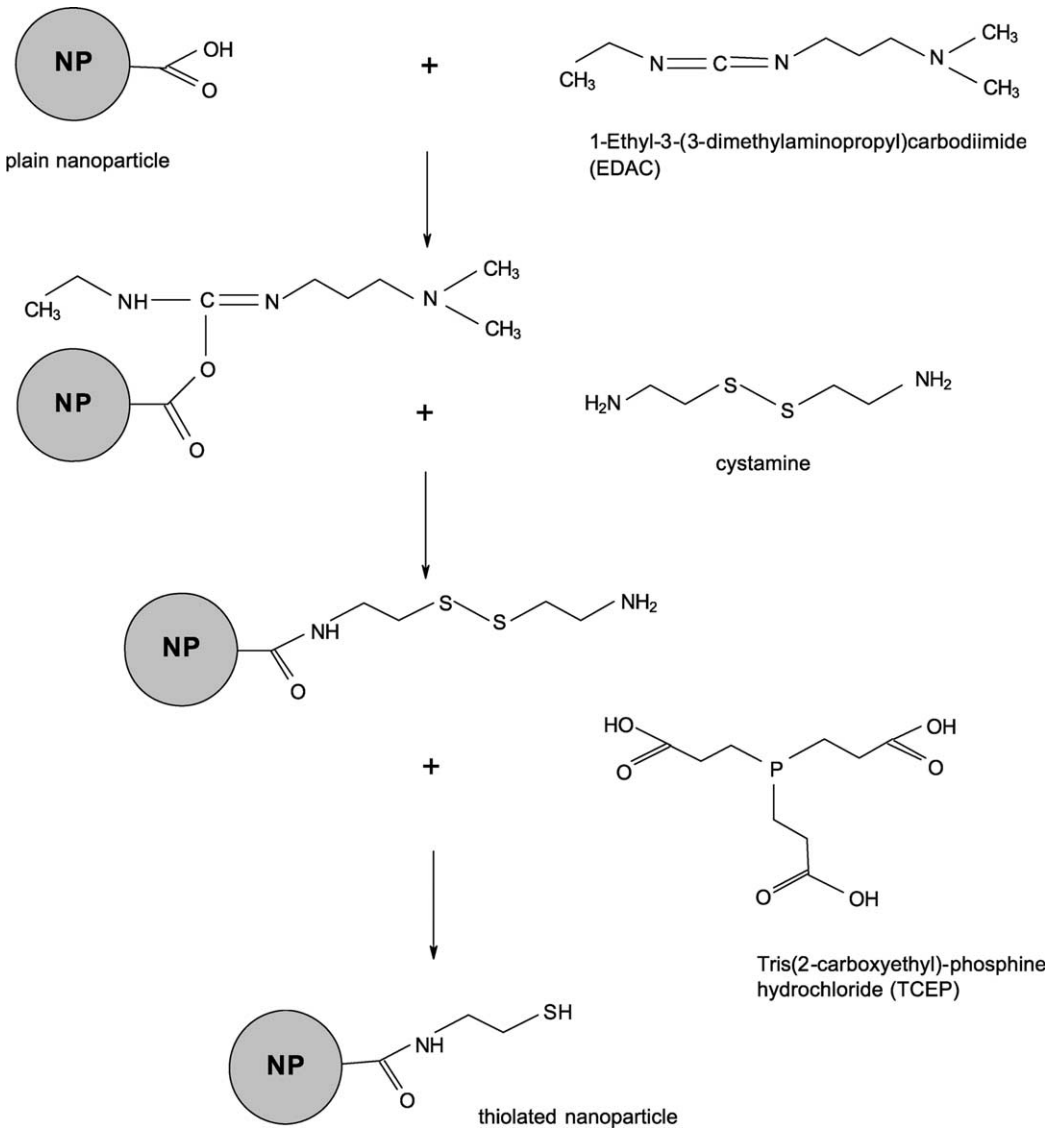

Fig. 1. Schematic presentation (not at scale) of the coupling of thiol groups on nanoparticle (NP) using cystamine as substrate.

for the coupling reaction did not lead to an increase in the amount of protein bound to the nanoparticles, as shown in Table 2. This suggested that all the thiol functions available on the surface of the nanoparticles were already saturated.

To demonstrate that the coupling of NAR to the thiolated nanoparticles was effectively due to covalent linkage, control reactions on plain nanoparticles as well as on thiolated nanoparticles were performed in the absence of sulfo-MBS. The results showed (Fig. 4) that only a small amount of NAR was adsorbed onto the surface of the nanoparticles in the absence of the bifunctional cross-linker. The amount of protein non-specifically adsorbed on the surface of thiolated nanoparticles was higher than that on plain particles. This might be due to a stronger interaction between thiol functions and the protein. These results provide strong evidence that the coupling reaction is highly efficient and specific allowing the creation of a stable amide bond between NAR and the nanoparticles.

Up to 975 molecules of NAR were present on the surface of each nanoparticle. Theoretically, considering the total number of available thiol functions, 14,000 molecules of

Table 1
Mean size of the nanoparticles

Batch #	Plain NP (nm)[a]	Thiolated NP (nm)[a]	NAR-labeled NP (nm)[a]	NAR-labeled NP (after freeze-drying) (nm)[a]
1	276 (0.01)	305 (0.13)	293 (0.08)	524 (0.46)
2			392 (0.31)	629 (0.48)
3			322 (0.21)	599 (0.51)
4			279 (0.08)	599 (0.29)
5	270 (0.16)	297 (0.10)	305 (0.10)	543 (0.38)
6			314 (0.11)	603 (0.32)

[a] Diameter of NP; in parenthesis the polydispersity index (0.00–1.00).

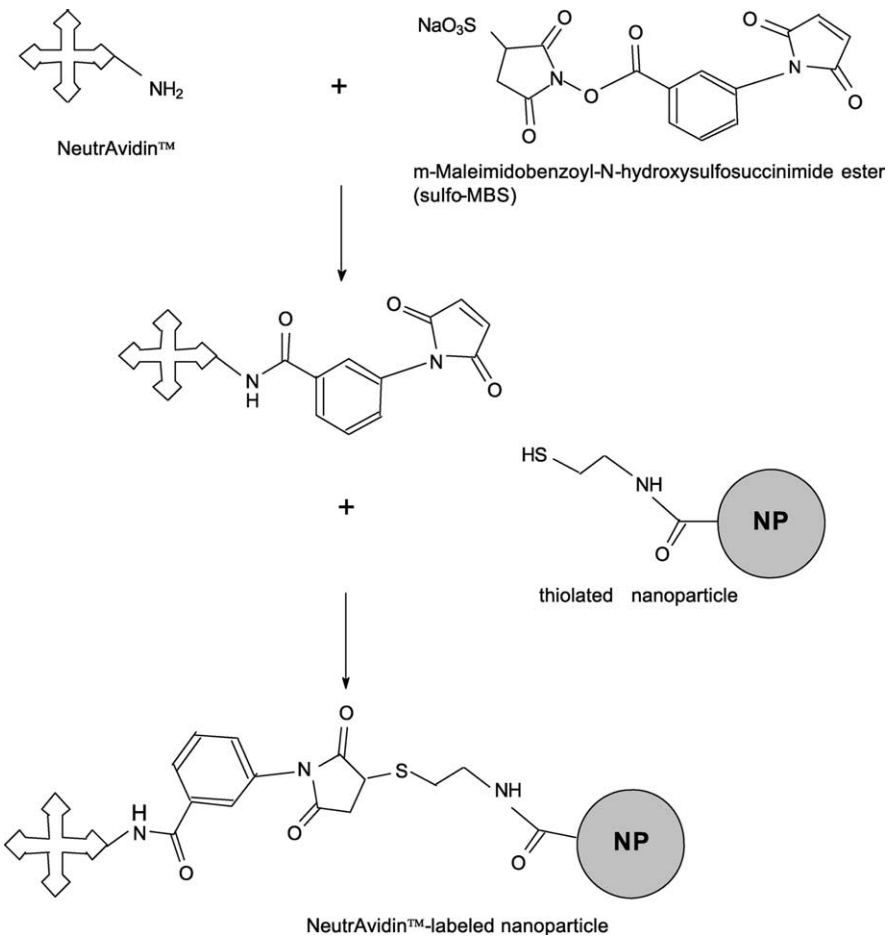

Fig. 2. Schematic presentation (not at scale) of the coupling of NAR on nanoparticle (NP).

NAR would have been expected to bind to the surface of each nanoparticle. Most probably, this significant difference is due to the steric hindrance of NAR, which reduces the accessibility to thiol functions.

The mean size of NAR-labeled nanoparticles was measured before and after freeze-drying. As shown in Table 1, a significant increase in the Z average size of the particles was observed after freeze-drying. This may be due to the formation of aggregates of nanoparticles as a result of the presence of NAR on their surface. The formation of aggregates after freeze-drying is frequently observed with proteins [22]. Since these aggregates are not desired, especially considering that it can affect the biotin binding activity, all further NAR-labeled-nanoparticles were stored as aqueous suspensions at 4 °C rather than being freeze-dried.

3.3. Measurement of the specific activity of NeutrAvidin™ Rhodamine Red™-X

A quantitative measurement of avidin and streptavidin using a specrofluorimetric technique has been described by Kada et al. [19]. This method allows the concentration of functional avidin to be determined even at very low concentrations using the strong and stable interaction with biotin-4-fluorescein. The accurate determination of the concentration of avidin is possible thanks to the strong quenching due to the binding of biotin-4-fluorescein to avidin. After the complete occupation of all biotin-binding

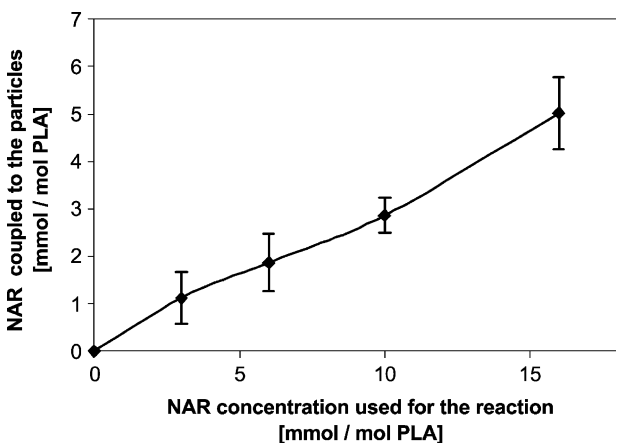

Fig. 3. Influence of the concentration of NAR (0–16.1 mmol/mol PLA) during the coupling reaction on the amount of bound protein obtained at the surface of nanoparticles (mean ± SD; $n = 5$).

Table 2
Quantification of NAR on the surface of nanoparticles and comparison between the total amount and the active amount of NAR

Batch #	NAR used for the reaction (mmol per mol of PLA)	Total concentration of NAR on the surface of NP (mmol per mol of PLA)	Concentration of NAR having active sites on the surface of NP* (mmol per mol of PLA)
1	13.0	5.1	3.6
2	30.0	4.7	2.1
3	30.0	4.6	3.4
4	30.0	6.0	4.0

Values are expressed as the means of two trials; *Assay with biotin-4-fluorescein.

sites on avidin, a sudden increase in the fluorescence emission is observed owing to the excess of free biotin-4-fluorescein. The breakpoint in the fluorescence determines the amount of biotin bound to the protein, and thus the specific activity of the protein.

Avidin is known as a tetramer that possesses four biotin binding sites. This should also be the case for NA [20] and consequently for NAR. Since the specific activity of NAR was unknown, its measurement was essential to evaluate the effect of the coupling reaction on the specific activity of the protein. It appeared that only 1.8 molecules of biotin bound to one molecule of NAR, which corresponds to a specific activity of 7.6 ± 0.3 units/mg. An example of a titration is given in Fig. 5a. This amount is very low compared to the usual value of 10–15 units/mg reported for NA. There are two possible hypotheses for such a low binding: (i) the labeling of NA with Rhodamine Red™-X reduced the biotin binding capacity of the protein; (ii) the protein is partially aggregated which would reduce the accessibility of the biotin binding site. Considering that only a mean of two molecules of Rhodamine Red™-X are conjugated to each molecule of NA, the second hypothesis

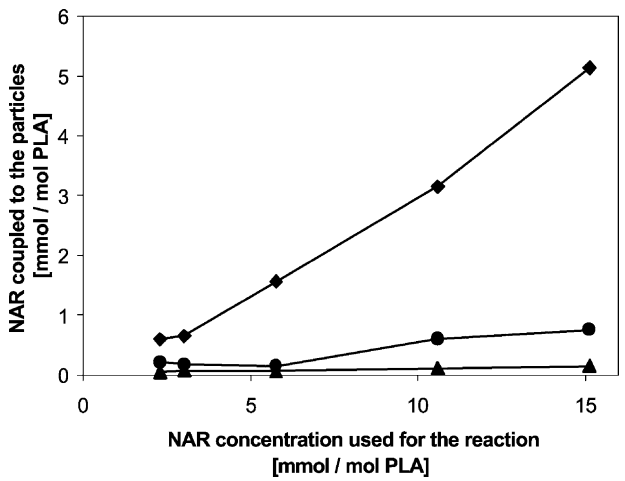

Fig. 4. Influence of the concentration of NAR (2.3–15.1 mmol/mol PLA) used during the coupling reaction on the amount of NAR coupled to the nanoparticles (◆, NAR covalently bound to thiolated particles; controls: ▲, plain nanoparticles; ●, thiolated nanoparticles).

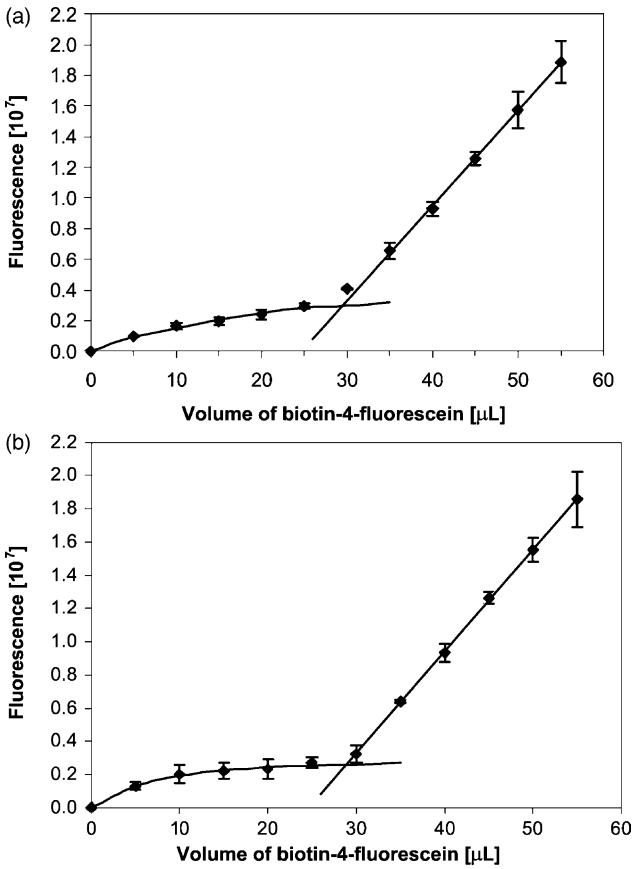

Fig. 5. Titration of the biotin-binding sites in NAR (125 nM) with biotin-4-fluorescein. The titration was carried out either in the absence (a) or in the presence (b) of nanoparticles. The fitting of the first part of the curve was achieved by a second degree polynomial fit, and the second part of the curve by a linear regression with offset. (a) 28.8 μl of biotin-4-fluorescein (15.9 μM) were added to reach the breakpoint in the fluorescence, corresponding to a specific activity of NAR of 7.6 ± 0.3 units/mg (mean ± SD; $n = 3$). (b) 28.3 μl of biotin-4-fluorescein (15.9 μM) were necessary to reach the breakpoint in the fluorescence, corresponding to a specific activity of NAR of 7.4 ± 0.2 units/mg (mean ± SD; $n = 3$).

seemed to be more plausible, taking into account that the protein alone is subject to partial aggregation [20]. This was confirmed by the measurement of the specific activity of NA. Results (Fig. 6a) showed that the specific activity of NA was also very low (8.0 ± 0.2 units/mg). The biotin binding activity of NA was also measured using D-biotin instead of 4-biotin–fluorescein [23]. In this case, the breakpoint in the fluorescence was determined by monitoring the tryptophan fluorescence of the protein [24] while adding successive aliquots of 5 μl of D-biotin solution (16.1 μM). Here again, a very low specific activity was found, confirming that the expected biotin binding sites are not all available, due most probably to partial aggregation of the protein (Fig. 6b). This latter hypothesis was confirmed by size-exclusion chromatography (data not shown) which demonstrated that more than 50% of the resolubilised NA protein was aggregated.

The biotin binding activity of the NAR bound to the nanoparticles was also measured in order to evaluate

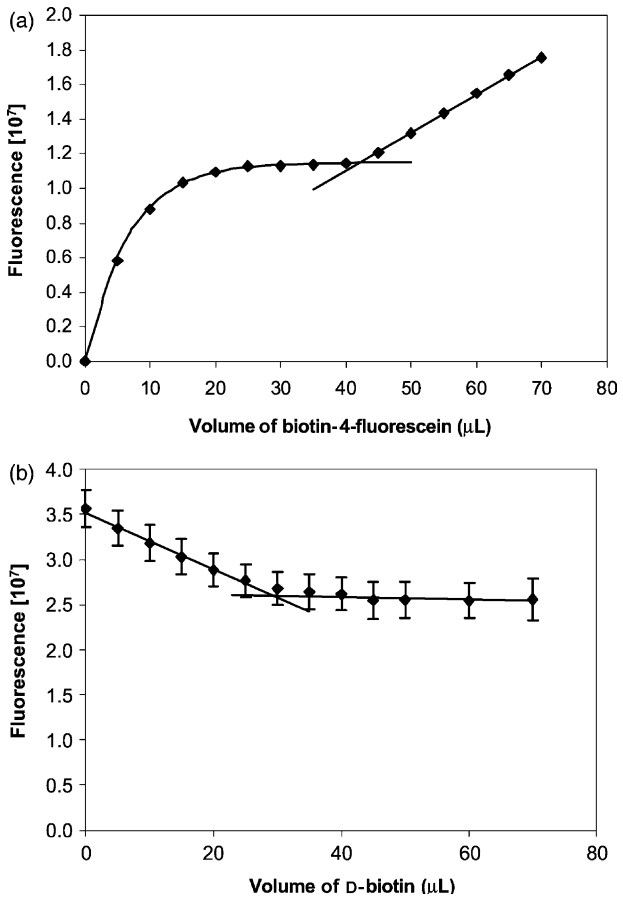

Fig. 6. Titration of the biotin-binding sites in NA with biotin-4-fluorescein (a) and D-biotin (b). The concentration of NA was, respectively, of 165 nM (a) and of 111 nM (b). (a) 41.9 μl of biotin-4-fluorescein (15.9 μM) were necessary to reach the breakpoint in the fluorescence, corresponding to a specific activity of NA of 8.0 units/mg. (b) 29.8 μl of D-biotin (16.1 μM) were added to reach the breakpoint in the fluorescence, corresponding to a specific activity of NA of 8.4 ± 0.4 units/mg (mean ± SD; $n = 4$).

the influence of the coupling reaction on the biotin binding capacity of the protein. To be sure that the presence of nanoparticles would not interfere in the assessment, the specific activity of free NAR was measured in the presence of thiolated nanoparticles. A specific activity of 7.4 ± 0.2 units/mg was found demonstrating that the nanoparticles do not interfere in the quenching and in the monitoring of the fluorescence of biotin-4-fluorescein. An example of the experiment is given in Fig. 5b. The same test was undertaken with NAR-labeled nanoparticles to quantify the amount of active protein bound to the particles. The results are given in Table 2 and an example of the monitoring of the fluorescence of biotin-4-fluorescein is shown in Fig. 7. The breakpoint in the fluorescence is quite pronounced which allowed precise determination of the amount of biotin-4-fluorescein required to occupy all available sites of the protein. Furthermore, the results obtained for each batch were reproducible in a narrow range. For comparison, the total amount of NAR bound to the nanoparticles was also assessed by measuring

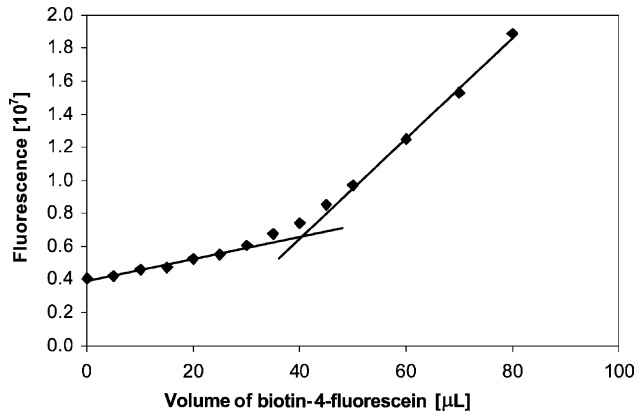

Fig. 7. Titration of the biotin-binding sites in NAR-labeled nanoparticles (batch #2, 35.1 μM) with biotin-4-fluorescein. 43 μl of biotin-4-fluorescein (2.94 μM) were added to reach the breakpoint, corresponding to a concentration of 2.1 mmol/mol PLA.

the fluorescence of Rhodamine Red™-X. This method does not discriminate between the active NAR, which has biotin binding sites available, and the non-active protein. As shown in Table 2, there is a significant difference between the amount of bound NAR measured by the spectrofluorimetric method using a linear regression and the concentration obtained by the approach developed by Kada et al. Indeed, only between 44 and 75% (mean 64%) of the protein coupled to the nanoparticles was found to be active. At present, one cannot distinguish between at least three possible explanations for this observation: (i) a relatively significant fraction of the protein sites are hidden in the aggregates, (ii) a significant number of linkage with sulfo-MBS have taken place with a lysine residue situated close to a binding site reducing its biotin binding capacity, (iii) not all the proteins coupled to the particles are appropriately oriented. Since we have shown evidence that the free NAR protein forms aggregates in solution, it is very likely that these aggregates are responsible, at least partly, for this difference. The two remaining explanations contribute certainly but to a lesser extent.

Overall, we have shown that NAR was covalently bound to the surface of nanoparticles and that a high number of biotin binding sites are available.

4. Conclusions

The present work illustrates an efficient and reproducible method for the covalent attachment of NeutrAvidin™ to the surface of PLA nanoparticles. The described approach has several promising attributes: (i) efficient conjugation reaction under mild conditions; (ii) control of the amount of NAR bound to the surface of nanoparticles; and (iii) preservation of the biotin binding capacity of the protein after the coupling reaction. Up to 975 active NAR molecules were bound to the surface of each nanoparticle having a mean size of 320 nm.

It has to be noticed that we have encountered problems regarding the physical stability of NAR, and more precisely its aggregation. In fact, NA and NAR formed aggregates in solution, which cannot be dissociated by moderate ultrasonication. These aggregates are responsible for a somewhat reduced specific activity of the native protein and of the NAR attached to the surface of the nanoparticles.

The preparation of NAR-labeled nanoparticles can be considered as a first step for the binding of a whole battery of biotinylated compounds for both diagnostic and therapeutic appliaction.

Currently investigations are underway to add biotinylated monoclonal antibodies to the nanoparticles via the stable and strong interaction between NA and biotin, which open interesting prospects for the use of polymeric nanoparticles for the active targeting of tumoral cells.

Acknowledgements

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