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Preparation of surface modified protein nanoparticles by introduction of sulfhydryl groups

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Abstract

The objective of the present study was to establish several methods for the introduction of thiol groups onto the surface of human serum albumin (HSA) nanoparticles. Besides the ε-amino groups of lysine, the carboxyl groups of asparaginic and glutaminic acid, and the carbonyl groups of the cross-linker glutaraldehyde, sulfhydryl groups are possible targets for the covalent linkage of drugs to particle surfaces. In principle, the thiol groups were introduced by the reaction with dithiotreitol (DDT) or 2-iminothiolane, by quenching reactive aldehyde residues with cystaminiumdichloride or by coupling L-cysteine and cystaminiumdichloride by the aqueous carbodiimide reaction. The resulting nanoparticulate systems were characterised concerning the number of available sulfhydryl groups, particle size and particle density. It was shown, that by variation of the reaction conditions, e.g., the concentration of the coupling reagent or the sulfhydryl containing component as well as the reaction time, the proposed methods enabled the preparation of HSA nanoparticles with a well defined surface characteristic. Stability studies showed that the introduced thiol groups were relatively stable and lost their reactivity with a half-life of 28.2 days independently of the method used for the sulfhydryl group introduction. Besides the quantification of free sulfhydryl groups the covalent attachment of cystaminiumdichloride by the carbodiimide reaction was used to calculate the amount of free carboxyl groups on the surface of the nanoparticles. The toxicity of the modified nanoparticles was evaluated in cell culture experiments.

Keywords: Nanoparticles; Human serum albumin; Sulfhydryl group introduction; Surface characterisation

1. Introduction

In the past covalent linkage of drugs to soluble proteins like human serum albumin (HSA) and various antibodies was used for the preparation of a variety of drug delivery systems (Sezaki and Hashida, 1984; Kratz et al., 1999). On the other hand colloidal drug delivery systems such as nanoparticles enable the modification of the body distribution (Kreuter, 1983) as well as the enhancement of the cellular uptake of the bound drug (Schäfer et al., 1992). Particularly, colloidal

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systems based on proteins may be very promising since they combine the advantages of nanoparticles with the advantages of covalent protein–drug conjugates. Moreover, they are biodegradable and non-antigenic (Rubino et al., 1993), relatively easy to prepare, and their size distribution can easily be monitored (MacAdam et al., 1997). In the present study, HSA was used for the preparation of nanoparticles because of its well-defined primary structure combined with the advantage of enabling surface modifications and covalent drug attachment under stoichiometric conditions.

HSA offers several target sites for covalent modification such as the ε-amino groups of lysine, the carboxyl groups of asparaginic and glutaminic acid as well as the hydroxyl groups of tyrosine. For glutaraldehyde-stabilised HSA nanoparticles the remaining carbonyl residues of the cross-linker can be used as a further site.

Protein conjugation most often is achieved by the use of established bifunctional cross-linkers, characterised by two different specific binding sites, one for primary amino groups and the other for sulfhydryl groups. Examples for such bifunctional cross-linkers are SIAB (Weltman et al., 1983), SMCC (Partis et al., 1983), SMPB (Martin and Papahadjopoulos, 1982) or SPDP (Neurath and Strick, 1981). In order to attach an amino group-containing compound to protein based nanoparticles by the use of such cross-linkers, it is imperative that the nanoparticles expose reactive sulfhydryl groups on their surface (Langer et al., 278). However, as the primary structure of HSA reveals, there are only a negligibly small number of reactive sulfhydryl groups available in the genuine protein. Moreover the introduction of free sulfhydryl groups may improve the mucoadhesive properties of the nanoparticulate drug delivery system as previously outlined for soluble polymers (Bernkop-Schnürch et al., 1999).

In our study, we have evaluated several methods for the introduction of sulfhydryl groups onto the surface of HSA nanoparticles, using the above mentioned target sites of the HSA molecule. Additionally, the nanoparticles were characterised with regard to the particle size, density, and toxicity. Finally, a method was proposed for the quantification of carboxyl residues on the nanoparticle surface, which is based on the carbodiimide reaction with cystaminiumdichloride.

2. Materials and methods

2.1. Reagents and chemicals

HSA (fraction V), glutaraldehyde 8% solution, L-cysteine hydrochloride monohydrate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (Steinheim, Germany). Cystaminiumdichloride was purchased from Merck-Schuchardt (Hohenbrunn, Germany) and hydroxylamine hydrochloride was obtained from Fluka (Buchs, Germany). The BupH™ MES buffered Saline Pack, 2-iminothiolane hydrochloride (Traut's reagent), 5,5′-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent) and dithiotreitol (DTT) (Cleland's reagent) were purchased from Pierce (Rockfort, IL, USA). Dulbecco's modified eagle's medium (DMEM), fetal calf serum (FCS) and other cell culture supplements were provided by Biochrom KG (Berlin, Germany). All reagents were of analytical grade and used as received.

Unless otherwise stated all incubation steps were performed at 25°C for 24 h and all purification steps of the nanoparticles consisted of a 5-fold centrifugation (20 000 × g, 8 min) and redispersion in an equal volume of water by ultrasonication (Elma Transsonic Digital, Singen, Germany).

2.2. Preparation of human serum albumin nanoparticles

HSA nanoparticles were prepared using a previously outlined desolvation technique (Marty et al., 1978; Weber et al., 2000). HSA (200 mg) was dissolved in 2.0 ml purified water followed by desolvation of the 10% protein solution by the dropwise addition of 6.0 ml ethanol. Unless otherwise stated 235 μl of 8% aqueous glutaraldehyde solution were added to achieve particle cross-linking. After stirring over 24 h, reactive glutaralde-

hyde residues were inactivated by the addition of 400 mg hydroxylamine hydrochloride followed by further stirring over 2 h. The resulting nanoparticles were purified as described above.

2.3. Determination of free sulfhydryl groups at the human serum albumin nanoparticle surface

The particle preparation (150 μl) was diluted with 1315 μl phosphate buffer pH 7.5, containing 1 mM EDTA and 35.0 μl of 0.4% 5,5′-dithio-bis-(2-nitrobenzoic acid) aqueous solution (Ellman's reagent) were added. After 15 min incubation, the particles were separated from the supernatant by centrifugation at 20 $78 \times g$ for 15 min. The supernatant was spectrophotometrically assayed for 2-nitro-5-thiobenzoic acid (TNB) at 412 nm (Hitachi U-3000 spectrophotometer, Berkshire, UK). L-cysteine standards were prepared and treated in the same manner as the particle preparations and the number of sulfhydryl groups was calculated relative to the standard values. Unmodified HSA nanoparticles prepared as decribed above served as negative control.

2.4. Introduction of sulfhydryl groups

Method 2.4.1: Introduction of sulfhydryl groups with Cleland's reagent. The HSA nanoparticles were prepared as described previously. Aliquots of 500.0 μl HSA nanoparticle suspension were mixed with volumes of 10.0, 20.0, 60.0, 100.0, 200.0, 400.0, and 750.0 μl of a dithiotreitol (DTT, Cleland's reagent) solution (60 mg ml^{-1}). The volume was adjusted with water. The samples were incubated and purified by repeated centrifugation and redispersion.

Method 2.4.2: Quenching of reactive glutaraldehyde residues with cystaminiumdichloride. The HSA nanoparticles were prepared as described previously except for the final quenching with hydroxylamine and the purification procedure. Two hours after the glutaraldehyde addition, volumes of 0.0, 1.6, 3.2, 6.2, 12.5, 25.0, and 75.0 μl of a cystaminiumdichloride solution (25 mg ml^{-1}) were added to 500.0 μl aliquots of the nanoparticle suspension. The volume was adjusted with water. The samples were incubated and purified. Afterwards, 100 μl DTT solution (30 mg ml^{-1}) were added to each sample. The particles were incubated and purified again.

Method 2.4.3: Modification of carboxyl groups with EDC and cystaminiumdichloride

Evaluation of reaction time. The nanoparticles were prepared as described previously. Aliquots (250.0 μl) of the nanoparticle suspension were diluted with 500.0 μl MES buffer pH 4.7, 100.0 μl cystaminiumdichloride solution (110 mg ml^{-1}) and 100.0 μl EDC solution (130 mg ml^{-1}). After 15 min, 30 min, 45 min, 2 h, 6 h, and 24 h the reaction was terminated by the addition of 100 μl hydroxylamine hydrochloride solution (125 mg ml^{-1}). The samples were purified followed by reduction of the disulfide linkages with 100 μl DTT solution (30 mg ml^{-1}) and further purification.

Evaluation of EDC concentration. The nanoparticles were prepared as described previously. The particle suspension (500.0 μl) was diluted with 700 μl MES buffer pH 4.7 and 100.0 μl of the cystaminiumdichloride solution (90 mg ml^{-1}) were added. Volumes of 0.0, 20.0, 30.0, 40.0, 50.0, 100.0, 150.0, and 200.0 μl of EDC (110 mg ml^{-1}) were mixed to the particle preparations. The volume was adjusted with water and the samples were incubated and purified. Afterwards, aliquots of an aqueous DTT solution (100 μl, 30 mg ml^{-1}) were added to the samples followed by a further incubation and purification procedure.

Evaluation of cystaminiumdichloride concentration. The nanoparticles were prepared as described previously. Aliquots (250.0 μl) of the nanoparticle suspension were diluted with 500 μl MES buffer pH 4.7. Volumes of 0.0, 12.5, 25.0, 50.0, 100.0, 200.0, and 500.0 μl of an aqueous cystaminiumdichloride solution (26 mg ml^{-1}) were added and 100.0 μl of EDC (110 mg ml^{-1}) were mixed to each sample. The volume was adjusted and the samples were incubated and purified. The reduction of the disulfide linkages with DTT was performed as described above.

Method 2.4.4: Modification of carboxyl groups with EDC and L-cysteine. The samples were prepared as described for the coupling of cystaminiumdichloride with the exception of using 100.0 μl of a cysteine solution (50 mg $m^{-1}l$) instead of the cystaminiumdichloride solution. The EDC solu-

tion (60 mg ml^{-1}) was added in aliquots of 0.0, 50.0, 100.0, 200.0, 300.0, and 400.0 μl, respectively.

Method 2.4.5: Introduction of sulfhydryl groups with 2-iminothiolane (Traut's reagent)

Evaluation of 2-iminothiolane concentration. The nanoparticles were prepared as described previously. Aliquots (500.0 μl) of the nanoparticle suspension were diluted with 1.0 ml TRIS buffer pH 8.5 and mixed with volumes of 0.0, 12.5, 25.0, 50.0, 75.0, 100.0, 250.0, and 500.0 μl of an aqueous 2-iminothiolane solution (26 mg ml^{-1}). After adjusting the volume with water the samples were incubated and purified.

Evaluation of the reaction time. The nanoparticles were prepared as described above. For each sample, aliquots (250.0 μl) of the nanoparticle suspension were diluted with 1 ml TRIS buffer pH 8.5 and mixed with 250.0 μl of an aqueous 2-iminothiolane solution (25.5 mg ml^{-1}). After 15 min, 30 min, 45 min, 1 h, 2 h, 6 h, and 24 h the reaction was terminated by the addition of 100.0 μl hydroxylamine hydrochloride solution (125 mg ml^{-1}) followed by the purification procedure.

2.5. Storage stability of the introduced sulfhydryl groups

The HSA nanoparticles treated with DTT and Traut's reagent, as well as the particles coupled with cystaminiumdichloride and L-cysteine in the presence of EDC were investigated for the degradation rate of the introduced sulfhydryl groups. The particle samples were stored at 4°C in Eppendorf tubes without any addition of stabilising agents for a period of 8 weeks. After predefined time intervals the nanoparticle suspensions were assayed for reactive sulfhydryl groups.

2.6. Determination of particle size and particle density

The particle size was measured by photon correlation spectroscopy (PCS) using a BI-200 SM Goniometer Version 2 (Brookhaven Instruments Corp., Holtsville, NY, USA). The samples were diluted 1:400 with purified water and measured at a temperature of 25°C and a scattering angle of 90°.

The particle density was calculated based on the density of the aqueous nanoparticle suspension in comparison to the density of the dispersion medium water using a DMA 48 density meter (AP Paar, Graz, Austria). Calculations were based on the following reduced equation:

$$\rho_P = \frac{\rho_S \times c}{\rho_L + c - \rho_S}$$

with ρ_P as the nanoparticle density (g cm^{-3}), ρ_S as the density of the aqueous nanoparticle suspension (g cm^{-3}), ρ_L as the density of the dispersion medium (water) (g cm^{-3}), and c as the nanoparticle concentration in suspension determined by gravimetry (g cm^{-3}).

2.7. MTT assay

HeLa cells were seeded into 24-well-plates at a density of 1×10^5 cells per well and cultured for one day at 37°C and 7% CO_2 in Dulbecco's modified eagle's medium (DMEM) containing 10% (v/v) FCS, supplemented with 4 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin. The serum was heat inactivated at 65°C for 1 h.

The cells were incubated with 1 ml suspension of the different nanoparticle formulations diluted to the desired final concentration with DMEM cell culture medium over a period of 4 h. Cells were further cultured for 24 h, then washed using phosphate buffered saline (PBS) pH 7.4 and incubated with 1 ml DMEM medium and 250 μl MTT solution (1 mg ml^{-1} in PBS) (Mosmann, 1983). Cells were kept at 37°C and 7% CO_2 for additional 2 h in a cell culture incubator model BB 6220 CU (Heraeus, Germany). The supernatant was removed carefully and the resulting blue dye was washed out by the treatment with 0.06 N HCl in isopropyl alcohol. Quantification of the formazane product was performed by photometric measurement with an ELISA Reader Spektra Max 340 (Molecular Devices, Sunnyvale, USA) at a wavelength of 540 nm.

The measured extinction of the blue dye correlated to the cell viability. Untreated cells served as reference and were taken as 100% viability.

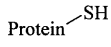
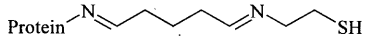
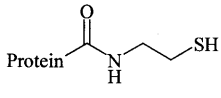
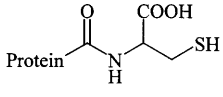
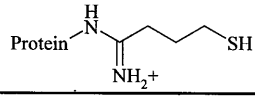
3. Results and discussion

In this paper, we present several methods for the introduction of sulfhydryl groups onto the surface of HSA nanoparticles, which can be used to couple drugs via thiol-reactive cross-linkers such as maleinimide group containing substrates. One mol of the protein HSA consists of 585 mol amino acids including 58 mol lysine, 23 mol serine, 28 mol threonine, 18 mol tyrosine, 38 mol asparaginic acid, 59 mol glutaminic acid, and 35 mol cysteine. However, 34 of these cysteine residues are fixed as 17 disulfide linkages (Carter and Ho, 1994). About 30% of the single free SH-group (Cys 34) are coupled to cysteine or glutathione. Apart from that, HSA preparations contain up to 20% of dimerised albumin (Carter and Ho, 1994). The free amino, carboxyl and aldehyde groups remaining from the cross-linking process of the protein nanoparticles as well as disulfide bonds on the surface of protein nanoparticles provide an appropriate target for the introduction of sulfhydryl groups. The preparation of HSA nanoparticles are based on our previous study which described the influence of the desolvation and cross-linking process on the physicochemical characteristics such as particle size, surface charge and amount of free amino groups on the surface of the nanoparticles (Weber et al., 2000). An overview over the five methods for the introduction of sulfhydryl groups is given in Table 1.

3.1. Methods for the introduction of sulfhydryl groups

Method 1: Reductive introduction of sulfhydryl groups with dithiotreitol. It has to be assumed that

Table 1
Overview over the five sulfhydryl introduction methods[a]

Method	Target sites	Reagents	Introduced residue	Maximum SH-group content [mol/mol HSA]	Evaluation of	Time constant of degradation [d^{-1}]
1	-S-S-	DTT		24	DTT conc.	0.02601
2	$-NH_2$	glutaraldehyde cystamine (DTT)		5 (21)	cystamine conc.	n.d.
3	-COOH	EDC cystamine (DTT)		21 (44)	cystamine conc. EDC conc. reaction time	0.02429
4	-COOH	EDC cysteine (DTT)		7 (23)	cysteine conc.	0.02164
5	$-NH_2$	2-iminothiolane		15	2-iminothiolane conc. reaction time	0.02631

[a] For methods 2–4, the total number of sulhydryl groups including the DTT deriving sulfhydryl groups is given in brackets. n.d.: not determined.

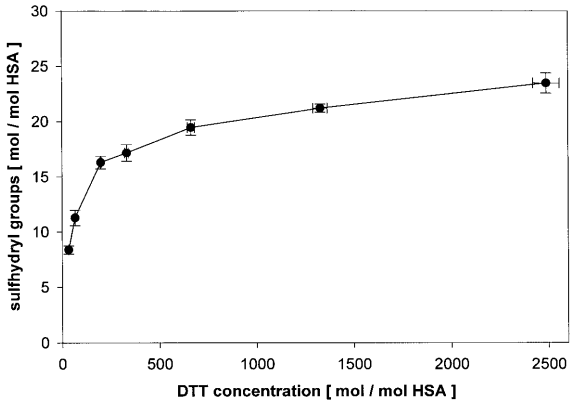


Fig. 1. Introduction of sulfhydryl groups using DTT: number of introduced reactive sulfhydryl groups per HSA molecule in correlation to the amount of DTT added for HSA disulfide bond reduction (mean ± SD; $n = 3$).

after the preparation of HSA nanoparticles by the desolvation procedure a certain amount of disulfide linkages of the HSA molecules remains on the surface of the particulate system. In order to reduce these disulfide linkages on the particle surface, the strong reducing agent DTT (Cleland's reagent) was used (Cleland, 1964). Raising concentrations of DTT were added to the cross-linked nanoparticles. After an intensive washing procedure, the number of free sulfhydryl groups on the particle surface was determined with 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB = Ellman's reagent) (Ellman, 1959). In contrast to protein solutions a direct photometric determination of the nanoparticle samples after reaction with DTNB was impossible. Therefore, following the DTNB reaction the particles were separated from the supernatant by centrifugation and the supernatant was assayed spectrophotometrically for the reduced form TNB. The results of the sulfhydryl group determinations after the treatment with raising amounts of DTT are shown in Fig. 1. Over the concentration range from 34.2 to 2564.1 mol DTT per mol HSA a significant increase in the sulfhydryl group content was observed, achieving an introduction of up to 23.5 ± 0.9 ($n = 3$) mol sulfhydryl groups per mol HSA. Further investigations revealed that even higher DTT concentrations led to no further significant improvement of the results (data not shown).

Method 2: Quenching of free aldehyde residues with cystaminiumdichloride. Quenching of free aldehyde residues with primary amines is a commonly used method for the inactivation of the aldehyde residues or the covalent coupling of primary amines to the particle surface. Due to the desired surface characteristics of the particles in earlier studies ethanolamine or glycine were used for the introduction of hydroxyl or carboxyl groups (Longo et al., 1982; Roser and Kissel, 1993; Irache et al., 1994; Latha and Jayakrishnan, 1995; MacAdam et al., 1997). Moreover, antibodies and other drugs such as doxorubicin and daunorubicin were coupled to the surface of protein particles by quenching free aldehyde residues (Akasaka et al., 1988; Golightly et al., 1988; Cummings et al., 1991). In the present study, the diamine cystaminiumdichloride was employed for the quenching experiments. The cystaminiumdichloride sulfhydryl group is protected by a disulfide bond. Therefore, after the quenching reaction and prior to the determination of the sulfhydryl groups, the disulfide bonds of cystaminiumdichloride had to be reduced with DTT. At a fixed glutaraldehyde concentration of 100%

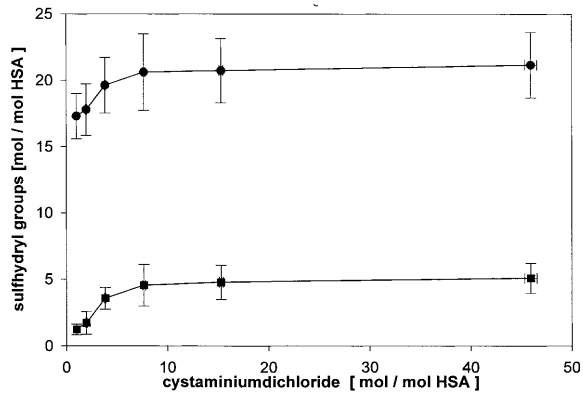


Fig. 2. Quenching of reactive aldehyde residues deriving from the cross-linker glutaraldehyde with raising amounts of cystaminiumdichloride: number of 2-aminoethanethiol groups introduced via aldehyde quenching (-■-) and the total number of sulfhydryl groups introduced via aldehyde and DTT (-●-) in correlation to the amount of cystaminiumdichloride added to the reaction mixture (mean ± SD; $n = 3$).

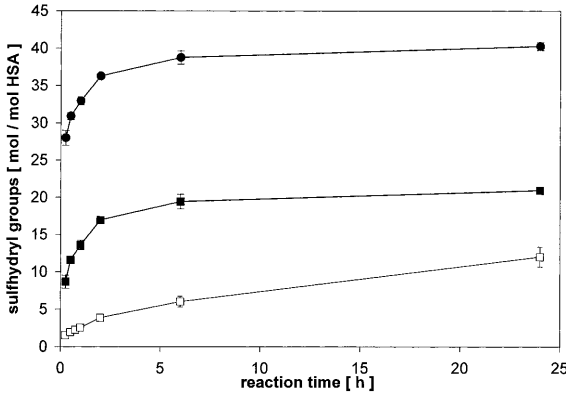


Fig. 3. Evaluation of reaction time: (A) EDC-coupling reaction of cystaminiumdichloride: (-■-) 2-aminoethanethiol groups introduced by amide linkage, (-●-) total number of sulfhydryl groups introduced via amide linkage and DTT in correlation to the reaction time (mean ± SD; $n = 3$).(B) 2-Iminothiolane-coupling reaction: (-□-) sulfhydryl groups introduced in correlation to the reaction time (mean ± SD; $n = 3$).

referred to a monovalent binding of the aldehyde to all 59 amino groups present in the HSA molecule, increasing amounts of cystaminiumdichloride were added. Besides the 2-aminoethanethiol residues introduced to the nanoparticle surface deriving from the reduced cystaminiumdichloride further sulfhydryl groups due to the reduction of HSA disulfide linkages were generated by the reaction with DTT. The amount of introduced 2-aminoethanethiol groups was calculated as the difference between the total amount of sulfhydryl groups on the modified nanoparticles and the amount of sulfhydryl groups on the unmodified HSA nanoparticles both after the DTT treatment (Fig. 2). The data revealed a significant increase from 1.2 to 4.5 mol per mol HSA between the addition of 1 and 7.5 mol cystaminiumdichloride per mol HSA, and a further slight increase to 5 mol sulfhydryl groups per mol HSA up to the addition of 46 mol cystaminiumdichloride per mol HSA. Higher amounts of cystaminiumdichloride in the reaction mixture did not lead to any further increase of the sulfhydryl group content (data not shown). The same was true with increasing glutaraldehyde concentrations in the reaction mixture.

Further quenching experiments with the amino acid L-cysteine, a method previously described by Coester et al. for the preparation of modified gelatin nanoparticles (Coester et al., 2000), only led to a negligibly small number of reactive sulfhydryl groups (1–2 mol per mol HSA) on the particle surface. This may be attributed to the attachment of the nucleophilic cysteine-sulfhydryl group instead of the primary amino group to the glutaraldehyde-carbonyl residue.

Method 3: Modification of carboxyl groups with EDC and cystaminiumdichloride. The third general method for the introduction of sulfhydryl groups to the surface of HSA nanoparticles was the coupling of SH-group containing substrates to the surface carboxyl groups of the HSA nanoparticles by the well-established carbodiimide reaction. Prior to the covalent attachment of the sulfhydryl containing substrate, the carboxyl groups on the surface of the nanoparticles were activated with the coupling reagent EDC at a pH of 4.7.

For this coupling reaction, cystaminiumdichloride was selected again as an appropriate substrate, because of its well-protected sulfhydryl group and the lack of other functional groups besides the amino residues. To obtain free sulfhydryl groups the disulfide bonds were re-

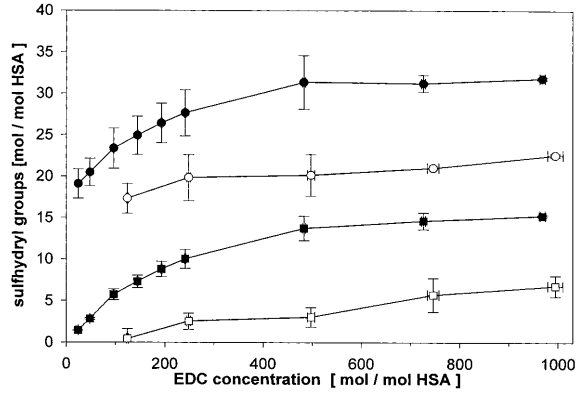


Fig. 4. Modification of the surface carboxyl groups using raising amounts of EDC and (A) Cystaminiumdichloride: (-■-) 2-aminoethanethiol groups introduced via amide linkage, (-●-) total number of sulfhydryl groups introduced via amid linkage and DTT in correlation to the amount of EDC added to the reaction mixture (mean ± SD; $n = 3$). (B) L-Cysteine: (-□-) cysteine thiol groups introduced via amide linkage, (-○-) total number of sulfhydryl groups introduced via amid linkage and DTT in correlation to the amount of EDC added to the reaction mixture (mean ± SD; $n = 3$).

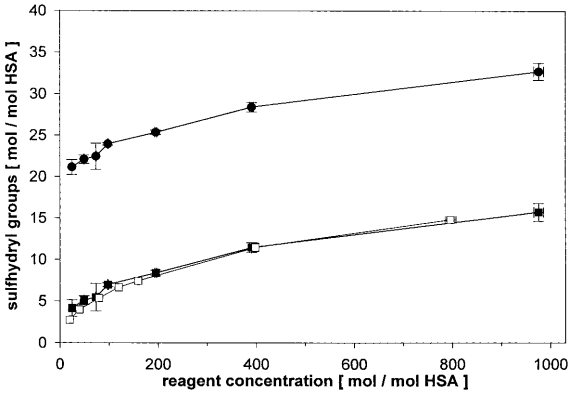


Fig. 5. Modification of the nanoparticle surface using raising amounts of (A) Cystaminiumdichloride: (-■-) 2-aminoethanethiol groups introduced via amide linkage, (-●-) total number of sulfhydryl groups introduced via amid linkage and DTT in correlation to the amount of cystaminiumdichloride added to the reaction mixture (mean ± SD; $n = 3$). (B) Traut's reagent: (-□-) sulfhydryl groups per HSA molecule in correlation to the amount of 2-iminothiolane added to the reaction mixture (mean ± SD; $n = 3$).

duced using DTT as already described. In general, this method was already used by Lin et al. (1990) for the introduction of sulfhydryl groups into the soluble proteins carbonic anhydrase and adrenocorticotropic hormone. Nevertheless, slight modifications concerning the reaction time, pH and substrate concentration had to be carried out for the coupling to the nanoparticulate system. The evaluation of the reaction time revealed that the main part of the coupling reaction occurred during the first 6 h (Fig. 3), but nevertheless there was a further detectable increase in the number of sulfhydryl groups between 6 and 24 h reaction time. Consequently for all EDC coupling reactions the reaction time was fixed to 24 h.

At a fixed cystaminiumdichloride concentration of 340 mol cystaminiumdichloride per mol HSA, different EDC concentrations ranging between 25 and 970 mol EDC per mol HSA were employed. A remarkable increase in the number of introduced 2-aminoethanethiol groups up to 15.2 ± 0.1 mol per mol HSA was observed (Fig. 4). Under these conditions and due to the reduction with DTT the total number of sulfhydryl groups available on the particle surface showed a maximum value of 31.8 ± 0.1 mol per mol HSA. As the DTT concentration (165 mol DTT per mol HSA) used for the reduction of cystaminiumdichloride was relatively low compared to the concentration used in method 1, the maximum value of about 24 sulfhydryl groups introduced with DTT (Fig. 1) was not achieved.

In the following step, the cystaminiumdichloride concentration was varied at a constant EDC level of 980 mol EDC per mol HSA and cystaminiumdichloride concentrations ranging between 24 and 980 mol per mol HSA (Fig. 5). Over the whole cystaminiumdichloride concentration range a markedly increase of 2-aminoethanethiol groups from 4.1 ± 1.0 to 15.7 ± 1.1 mol per mol HSA was observed.

The number of 2-aminoethanethiol groups that were introduced by covalent attachment of cystaminiumdichloride by means of EDC may also be considered as a measure for the number of available carboxyl groups on the surface of HSA nanoparticles. As a result of the preliminary studies concerning the reaction time and the concentrations of EDC as well as cystaminiumdichloride the coupling procedure was performed with maximum concentrations of EDC and cystaminiumdichloride as well as 24 h incubation time. A maximal coupling of 20.9 ± 0.4 mol 2-aminoethanethiol groups per mol HSA was detected on the nanoparticle surface. Therefore, we postulate a carboxyl group content of about 21 mol per mol HSA on the surface of HSA nanoparticles prepared under the described conditions.

Method 4: Modification of carboxyl groups with EDC and L*-cysteine*. In comparison to the EDC reaction with cystaminiumdichloride the coupling reaction was also performed with L-cysteine as the amino and sulfhydryl containing component at a fixed cysteine concentration of 230 mol cysteine per mol HSA. The quantitation of the sulfhydryl groups revealed, that there were nearly no sulfhydryl groups detectable on the nanoparticle surface (data not shown). This was probably due to the competitive reaction of the cysteine sulfhydryl groups with EDC, a phenomenon that has already been described by Carraway and Triplett (Carraway and Triplett, 1970). An improvement of the results was achieved by the

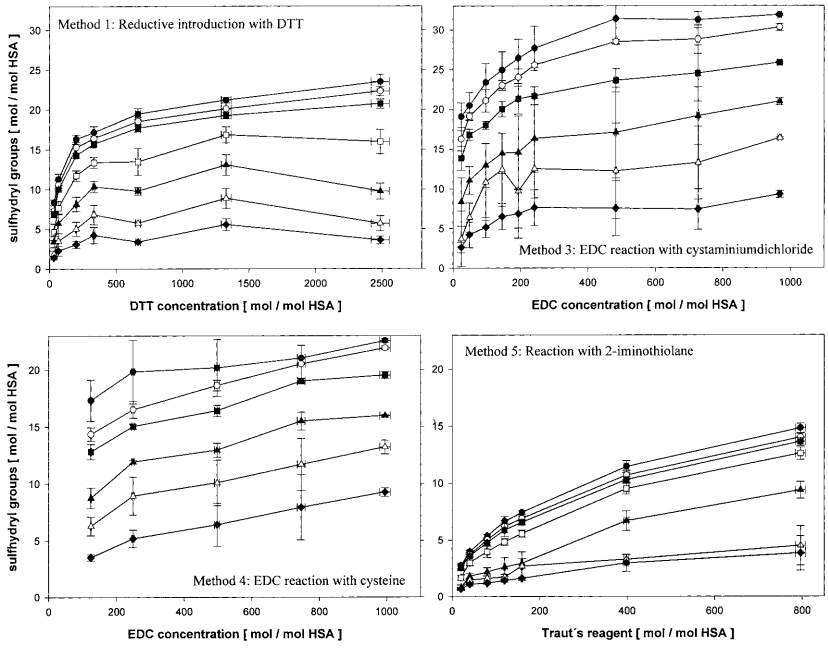


Fig. 6. Influence of the storage time on the amount of sulfhydryl groups on the nanoparticle surface after introduction using 4 different modification methods. Quantification of thiol groups with Ellman's reagent after 0 day (-●-), 2 days (-○-), 5 days (-■-), 7 days (-□-), 14 days (-▲-), 28 days (-△-), and 56 days (-◆-), respectively (mean ± SD; $n = 3$).

addition of DTT in order to reduce conceivable oxidation and EDC conjugation products of the sulfhydryl groups (Fig. 4). The increase in sulfhydryl groups over the EDC concentration range is slight but nevertheless significant. Comparing the maximum sulfhydryl group content of 6.8 ± 1.3 mol per mol HSA to the number of 10.0 ± 0.1 mol sulfhydryl groups at a corresponding cystaminiumdichloride concentration, the EDC coupling procedure with L-cysteine was significantly less effective than the coupling of cystaminiumdichloride. This may be due to the formation of cysteine thioesters instead of the formation of amide bonds with the carboxyl groups on the nanoparticle surface as well as the intermolecular amide formation of cysteine with EDC.

Method 5: Introduction of sulfyhydryl groups with 2-iminothiolane (Traut's reagent). The last method under evaluation is the reaction of 2-iminothiolane (Traut's reagent) with the primary amino groups present on the surface of HSA nanoparticles (Traut et al., 1973). The results revealed a significant increase of the sulfhydryl group content over the whole concentration range of 2-iminothiolane (Fig. 5). Using this method, a maximum value of 14.8 ± 0.4 mol sulfhydryl

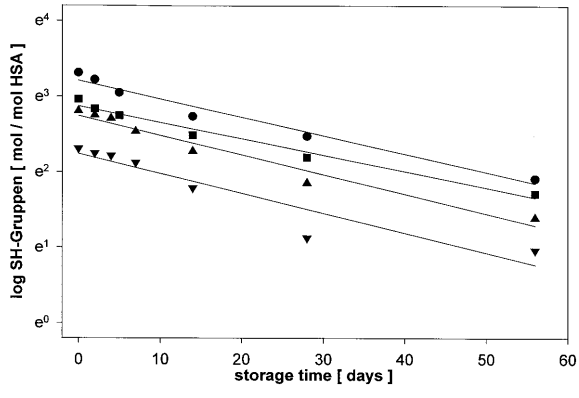


Fig. 7. Degradation of the introduced sulfhydryl groups: logarithmic number of sulfhydryl groups on DTT-nanoparticles (-▲-), cystaminiumdichloride-DTT-nanoparticles (-●-), cysteine-DTT-nanoparticles (-■-), and 2-iminothiolane-nanoparticles (-▼-) in correlation to the storage time at 4°C.

groups per mol HSA was achieved. For evaluation of the reaction time, the reaction was terminated after different time intervals by the addition of an excess of hydroxylamine hydrochloride (Fig. 3). It was obvious that the coupling procedure of 2-iminothiolane with the amino groups on the nanoparticle surface required a long reaction time, since there was a drastic increase in the number of sulfhydryl groups over the whole time range up to the 24 h. In order to achieve an effective coupling, in the present study, the reaction time was fixed to 24 h. These results are in contrast with the data of Singh et al. (1996), who postulated an initially thiol adduct, which is unstable and decays by a first-order process with half-lifes between 0.3 and 3 h at 23°C.

3.2. Stability of sulfhydryl groups

For the stability investigations, the content of sulfhydryl groups on the nanoparticle surface was determined over a time period of 8 weeks and in dependence of the method used for the introduction of the sulfhydryl groups (Fig. 6). There was a significant reduction of the sulfhydryl group content over the total storage time independent of the introduction method. For the determination of the degradation rate constant and the order of reaction the mean value of the amount of sulfhydryl groups was calculated for each method at three different reagent concentrations. The results were plotted at a base e logarithmic scale against the storage time (Fig. 7). The degradation appeared to follow a pseudo-first-order kinetic. Based on this assumption the degradation rate constants k were calculated as the slopes of the linear regressions (Table 1) resulting in a mean degradation rate constant of $k = 0.0246 \pm 0.0021$ (d^{-1}). Therefore the half-life time $t_{1/2}$ of sulfhydryl groups on the nanoparticle surface was 28.2 days.

3.3. Density of human serum albumin nanoparticles

The HSA nanoparticles used in this study were prepared by the desolvation of an aqueous HSA solution with ethanol, resulting in a system with a mean nanoparticle diameter of 341.2 ± 28.6 nm and a density of 1.31 ± 0.025 g cm^{-3} ($n = 3$). The HSA nanoparticles exhibited a slightly lower density than particles prepared by an emulsion technique with a density of 1.48 g cm^{-3} (MacAdam et al., 1997). The determination of the particle density was used for a rough calculation of the number of HSA molecules involved in particle formation and therefore for an estimation about the total number of reactive groups on the surface of one HSA nanoparticle. Based on the density data and the fact, that HSA nanoparticles were of spherical shape (SEM results, data not shown) and the HSA molecular weight of 65 kDa, a HSA nanoparticle consisted of about 2.5×10^5 molecules HSA. A typical HSA nanoparticle prepared by the described method therefore presented about 1.8×10^6 amino groups (Weber et al., 2000) and 5.0×10^6 carboxyl groups on its surface. Thiol contents of up to 1.1×10^7 per HSA nanoparticle were achieved using the method of coupling cystaminiumdichloride with EDC (Fig. 4).

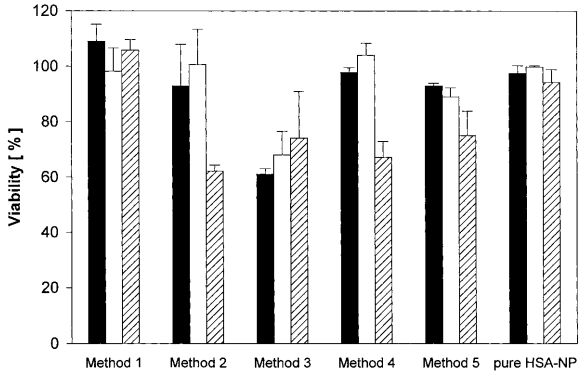


Fig. 8. Cytotoxicity of the nanoparticles modified by method 1: DTT, method 2: glutaraldehyde/cystaminiumdichloride, method 3: EDC/cystaminiumdichloride, method 4: EDC/cysteine, method 5: Traut's reagent in comparison to pure HSA nanoparticles. Nanoparticle concentrations of 0.005% (black bars), 0.05% (white bars) and 0.375% (hatched bars) were used (mean $\pm$ SD; $n = 3$).

3.4. MTT assay

The results in Fig. 8 show that pure HSA nanoparticles possessed nearly no toxicity for all particle concentrations. Similar to these findings,

cells treated with nanoparticles prepared by methods 1 and 5 using DTT and 2-iminothiolane showed no severe toxicity with a slight dose dependent effect on the viability of the cells. At low concentrations, the nanoparticles with cysteine surface modification (method 4) as well as cystaminiumdichloride/glutaraldehyde-modified nanoparticles (method 2) were nearly untoxic. However, at the highest concentration employed, remarkable toxicity could be observed. Due to the extremely high EDC concentration used for the particle preparation following method 3 and the resulting positive surface charge, significant toxicity occurred within all applied concentrations.

4. Conclusion

In the present study, several effective methods for the introduction of sulfhydryl groups onto the surface of HSA nanoparticles were evaluated. The most effective method is represented by the coupling of cystaminiumdichloride with EDC followed by the reduction of the disulfide bonds of cystaminiumdichloride and HSA with DTT. A maximum sulfhydryl group content of about 44 mol per mol HSA, roughly estimated 1.1×10^7 thiol groups per nanoparticle, was achieved. Unfortunately, these nanoparticles exhibited the highest cytotoxic effect of the preparations under evaluation. The EDC coupling with cysteine was less effective, probably because of undesired side reactions. The simplest sulfhydryl creating process is the exclusive reduction of HAS–disulfide-linkages with DTT, resulting in values of about 24 mol sulfhydryl groups per mol HSA. The use of 2-iminothiolane is an easy to handle and appropriate method for the introduction of lower amounts of about 14 mol sulfhydryl groups per mol HSA. Quenching of free glutaraldehyde residues with cystaminiumdichloride led to the introduction of a slight amount of about 5 mol sulfhydryl residues per mol HSA. The degradation of the sulfhydryl groups showed a rate constant of $k = 0.0246 \pm 0.0021$ (d^{-1}) independent of the method used for the modification.

All methods were easy to monitor by varying the concentration of the coupling reagent, the sulfhydryl component or the reaction time, respectively. Therefore, the methods represent a rational basis for further protein conjugations to nanoparticles by the use of sulfhydryl-reactive cross-linkers. Additionally, improved mucoadhesive properties may be expected due to the interaction of the surface modified nanoparticles with disulfide bonds of the mucus gel layer.

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