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SHORT COMMUNICATION

Poly(ethylene oxide)-coated polyamide nanoparticles degradable by glutathione

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Abstract Poly(adipoyl chloride-*alt*-cystamine) nanoparticles covalently coated with poly(ethylene oxide) (M_w= 2,000 or 5,000) were prepared as a model of glutathione-degradable drug, protein, or deoxyribonucleic acid delivery systems. The polyamide forming the micellar core has disulfide bonds in the main chain and thus its degradation gives low molecular weight products. The degradation of nanoparticles by the action of glutathione in the reduced or oxidized forms was monitored by time dependencies of the light scattering intensity at 37 °C in media modeling intracellular environment. The lifetimes of nanoparticles were determined. The reductive degradation of the nanoparticles by the reaction with reduced glutathione is an order of magnitude faster than their spontaneous degradation. The effect of nanoparticle concentration and solution pH was also investigated.

Keywords Disulfide bond · Nanoparticles · Glutathione · Poly(ethylene oxide) · Light scattering

Introduction

The disulfide bond can be reversibly reduced into two molecules of thiols or may interchange the substituents with other thiols or disulfides [1]. This fact has far-reaching consequences in biological systems. For example, glutathione (α-glutamylcysteinyl glycine), a tripeptide omnipresent in concentrations 0.1–10 mmol l^{-1} inside the living cells [2], serves as a redox buffer maintaining the redox potential of cytoplasma constant due to the reversibility of the disulfide bond formation and also as a radical scavenger. It is not surprising that the chemistry of disulfide bonds attracted considerable attention of polymer chemists developing novel biomaterials. Thiomers containing thiol groups are extensively studied for their mucosa-adhesive and permeation-enhancing properties [1, 3], which are caused by thiol-disulfide exchange and oxidation of thiols to disulfides. Some polymer gel systems utilize disulfide-containing crosslinkers to achieve reversible gel dissolution/gelation in in vivo systems [4]. There were several micellar and polycation–deoxyribonucleic acid (DNA) systems also described [3, 5] with thiol/disulfide groups designed as redox-sensitive DNA delivery systems.

Biodegradability of the disulfide bond with glutathione is especially interesting for the drug, protein, or DNA delivery because the extracellular level of glutathione is much lower (ca. 4.5 μmol l^{-1}) [6] than the intracellular level. This fact can be used in the systems that should be relatively stable during transport outside the cell but should disintegrate after internalization in the target cell. Likewise, Nature used this approach, e.g., in the viral capside assembly during virus maturation and its disruption inside the cell to be infected [7, 8].

In this study, we synthesized and studied poly(adipoyl chloride-*alt*-cystamine) nanoparticles coated with covalently bonded poly(ethylene oxide), which should serve as a model system for glutathione-degradable drug, protein, or DNA delivery systems. The polyamide core has disulfide bonds in the main chain and thus its degradation products are fragments of low molecular weight.

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Experimental

Materials

The chemicals were purchased from Fluka and Aldrich and were used as obtained. The dialysis tubing VISKING (type 27/32, molecular weight cutoff 14 kDa) was obtained from Carl Roth GmbH (Germany) and 0.45 mm poly(vinylidene fluoride) (PVDF) syringe filters were obtained from Whatman (Clifton, NJ, USA).

Preparation of nanoparticles

Poly(ethylene oxide) monomethyl ether (1.00 g) with M_w of 2,000 or 5,000 was azeotropically dried by multiple evaporation of its solution in dry benzene. After the removal of residual benzene in vacuo (25 °C, 13 Pa), it was dissolved in dry dimethylacetamide (15.0 ml) and adipoyl chloride (560 μl, 3.84 mmol) and ethyldiisopropylamine (3.42 ml, 20 mmol) were added. The mixture was stirred at room temperature for 10 min and then cystamine dihydrochloride (865 mg, 3.84 mmol) was added. The slurry was stirred at room temperature. The polymerization mixture was poured into water (300 ml) and dialyzed against excess water for 48 h. Well-defined nanoparticles with sizes dependent on reaction time were observed in aqueous solutions. The suspension of nanoparticles was then filtered through a 0.45-μm PVDF syringe filter and diluted to appropriate concentration with water. The actual concentration of nanoparticles was assayed gravimetrically after lyophilization of an aliquote. The growth of nanoparticle sizes in the course of the reaction (for details, see "Static and dynamic light scattering") was monitored by dynamic light scattering (DLS) method using a ZEN3600. Only the nanoparticles with the hydrodynamic radius R_h comparable with that of viruses (≈50 nm), which is the size suitable for the drug delivery purposes, were used for further investigation. Thus, the nanoparticles obtained after 24 h of polymerization for the system with PEO blocks of M_w=2,000 (**SS2**) and the nanoparticles obtained after 72 h of polymerization for the system with PEO blocks of M_w=5,000 (**SS5**) were selected. **SS2** and **SS5** contained (according to sulfur analysis of lyophilizate) 23.3 and 24.9wt% polyamide, respectively.

Static and dynamic light scattering

Properties of nanoparticles in aqueous suspensions were characterized by static light scattering (SLS) and DLS at 37 °C, using vertically polarized light at λ_o=632.8 nm (He–Ne laser 40 mW) in the angular range of 30–140°. The Zimm plot procedure was used for the evaluation of M_w. The refractive index increments (dn/dc) of the nanoparticles in water were measured with a Brice–Phoenix refractometer at λ_o=632.8 nm; the values obtained are 0.175 and 0.176 ml g^{-1} for the nanoparticles **SS2** and **SS5**, respectively.

DLS was measured with an ALV 5000 autocorrelator. Inverse transform using the REPES [9] method of constrained regularization (program GENDIST) was used for the analysis of time autocorrelation functions. This method uses an equidistant logarithmic grid with fixed components (in this study, a grid of 20 components per decade) and determines their amplitudes. As a result, scattered light intensity distribution function of hydrodynamic sizes was obtained.

The SLS and DLS were also measured at 173° on a Nano-ZS, Model ZEN3600 (Malvern, UK) zetasizer. The apparatus was used mainly for automated particle size (R_h) and scattering intensity (I_s) time measurements at 37 °C. The DTS(Nano) program (the inversion routine CONTIN [10]) was used for data evaluation. The mean positions of the peaks in intensity–hydrodynamic radius (R_h) distribution were taken for data representation.

Spontaneous hydrolytic degradation of the nanoparticles was investigated using the time dependence of light scattering intensity, I_s, measured with a ZEN3600 at a scattering angle of θ=173° with a suspension of nanoparticles in phosphate-buffered saline (pH 7.4 as a model of blood plasma and pH 5.0 as a model of intracellular environment) at 37 °C. Reductive degradation of the nanoparticles by reduced glutathione (model of intracellular environment) and degradation by the interexchange reaction with oxidized glutathione (model of blood plasma) was studied at 37 °C, also using a ZEN3600. Glutathione was added as a stock solution (30 mg ml^{-1}, pH adjusted with NaOH to 7.4 or 5.0) to the suspension of nanoparticles in water; the total glutathione concentration in the sample was 3 mg ml^{-1}. Various nanoparticle concentrations from 0.72 to 3.52 mg ml^{-1} were used for the degradation studies. The degradation was followed.

Atomic force microscopy

Nanoparticles were deposited on mica substrate and characterized by atomic force microscopy (AFM). All measurements were performed under ambient conditions using a commercial atomic force microscope (NanoScope™ Dimension IIIa, MultiMode Digital Instruments, Santa Barbara, CA, USA) equipped with a PPP-NCLR tapping-mode probe with reflex aluminum coating (force constant 41 N m^{-1}, drive frequency 161 kHz). The tapping mode AFM technique was used for all the images. The final image processing was done with software WSxM version 4.0 (Nanotec Electronica: http://www.nanotec.es).

Determination of critical aggregation concentration

The critical aggregation concentration (CAC) values were determined from the fluorescence intensity of the I_1 band ($\lambda_{emission}$=367 nm, using $\lambda_{excitation}$=339 nm) of pyrene fluoroprobes in suspensions with different concentrations of nanoparticles and a constant concentration of pyrene according to method described in [11, 12].

Results and discussion

Nanoparticle parameters

An AFM image of colloidal suspension of **SS5** nanoparticles is shown in Fig. 1. Besides a small portion of small particles, large particles (R_h~50 nm) dominate in the suspension. The parameters of nanoparticles evaluated at 37 °C from light scattering data are shown in Table 1. Because the light scattering intensities of large nanoparticles superimpose those of small ones, the light scattering results in Table 1 are related to dominating larger nanoparticles. The CAC of the nanoparticles water at 37 °C are also shown in Table 1.

The mean weight fractions (ρ_m) of polymers in the nanoparticles (polymer density) are approximately three times lower than those in micelles observed in selective organic solvents [13]. In our opinion, the nanoparticles could be copolymer micelles with polyamide homopolymer chains adsorbed in the cores of the micelles. The polyamide homopolymer probably forms spontaneously during the copolymer preparation. The homopolymer sorption in micellar cores may increase the size of copolymer micelles. The existence of such large particles in ternary solutions of a homopolymer and a diblock copolymer in a selective precipitant for homopolymer was reported previously [14, 15]. The nanoparticles can also be understood as polyamide nanoparticles covered with PEO (PEGylated nanoparticles). Unfortunately, we were not able to assay the polyamide homopolymer content because we have not found a suitable solvent system for chromatography under critical conditions and laser ionization in MALDI-TOF is unable to disintegrate the nanoparticles without chemical degradation. However, due to the polycondensation mechanism, it is highly probable that there exists a fraction of homopolymer

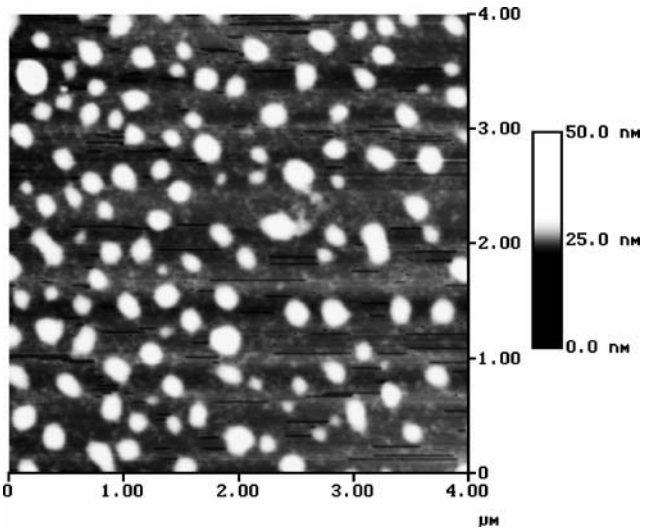

Fig. 1 AFM image obtained from solutions of **SS5** at 37 °C

Table 1 Characteristics of selected MLNs at 37 °C

Nanoparticles	$M_w \times 10^{-7}$	R_h (nm)	PDI[a]	ρ_m (g cm^{-3})	CAC×10^3 (mg ml^{-1})
SS2	2.44	44±1	0.15	0.12	5.5
SS5	6.10	60±2	0.16	0.11	7.5

[a] Polydispersity index

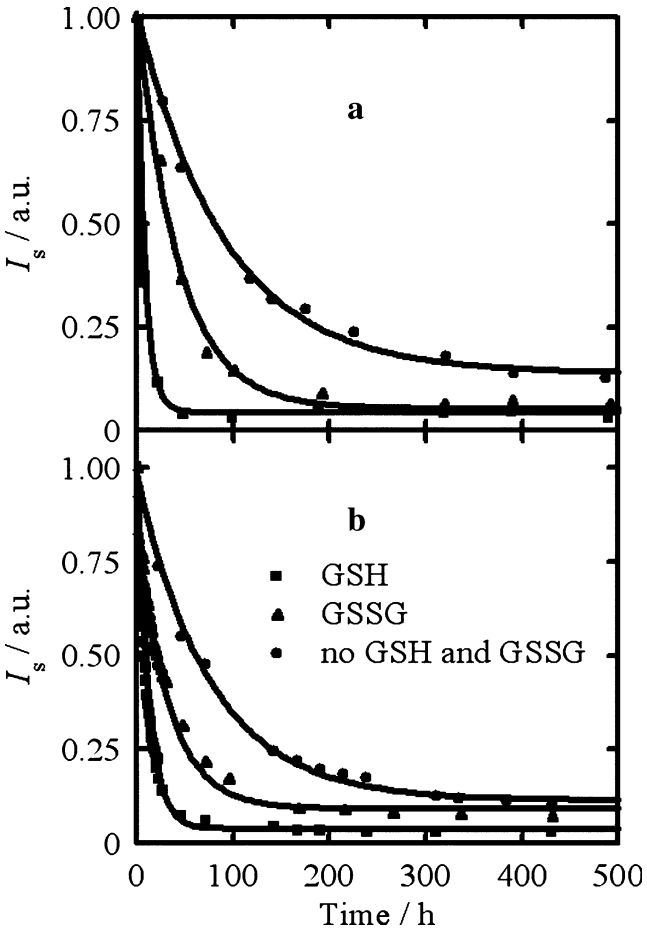

Fig. 2 The time dependencies of normalized I_s for solutions of **SS2** (**a**) and **SS5** (**b**) nanoparticles measured at pH 7.4 without glutathione, with oxidized glutathione (*GSSG*), and with reduced glutathione (*GSH*) measured at 37 °C (c_{MLN}=1.35 mg ml^{-1}, c_{GHS}=3 mg ml^{-1}). The *full lines* are single exponential fits

Table 2 Lifetime, t_{lf}, of MLNs in aqueous solutions at pH 7.4 (c_{MLN} =1.35 mg ml^{-1}, 37 °C)

Solution	t_{lf}/**SS2** (h)	t_{lf}/**SS5** (h)
Phosphate-buffered saline	99	76
Phosphate-buffered saline with oxidized glutathione (GSSG; 3 mg ml^{-1})	43	26
Phosphate-buffered saline with reduced glutathione (GSH; 3 mg ml^{-1})	9.9	9.6

in the core. The investigated nanoparticles are called micelle-like nanoparticles (MLNs) throughout this paper.

Degradation of nanoparticles at pH 7.4

In addition to the spontaneous degradation of MLNs, their degradation in the presence of glutathione in the reduced (GSH) or oxidized (GSSG) form was investigated. The concentration of glutathione $c_{GSH}=c_{GSSG}=3$ mg ml$^{-1}\approx$ 0 mmol l^{-1} was selected to model the interior of the living cells. The time dependencies of normalized I_s for suspensions of **SS2** and **SS5** (MLN concentration $c_{MLN}=1.35$ mg ml^{-1}) are shown in subpanels a and b in Fig. 2, respectively. Because the sizes of MLNs are sufficiently small, I_s variations roughly reflect variations of the weight-average molecular weight of the MLNs.

The time dependencies of $I_s(t)$ can be satisfactorily described by single exponential functions, $I_s(t)=(I_s(0)\exp(-t/t_{lf}))+I_s(\infty)$, where t_{lf} is the characteristic lifetime of MLNs and $I_s(\infty)$ is the residual light scattering intensity. The single exponential fits are shown in Fig. 2a,b as full lines. The lifetimes of MLNs are given in Table 2 for both the investigated systems. The degradation of MLNs in phosphate-buffered saline at pH 7.4 follows the order of the degradation rates GSH>GSSG>no glutathione added (spontaneous degradation). The reproducibility of t_{lf} determination for GSH was better than 1 h. The residual light scattering intensity $I_s(\infty)$ observed at long degradation times will be discussed further.

We suggest a mechanism of MLN degradation shown in Scheme 1. The glutathione molecules interact with copolymers and homopolymers bound in MLNs. In the S–H–S–S interchange reaction (degradation by GSH) or S–S–S–S interchange reaction (degradation by GSSG) with copolymers and homopolymers of MLNs, soluble PEO blocks and less soluble polyamide–glutathione complexes are formed. As a consequence, the weight-average molecular weight of nanoparticles decreases with time, which is reflected in a decrease in $I_s(t)$. The spontaneous degradation of nanoparticles is most likely caused by hydrolysis of the polymer chain and the S–S–S–S interchange reaction.

The effect of nanoparticle concentration

Both the lifetime t_{lt} and residual light scattering intensity $I_s(\infty)$ obtained by the single exponential fit to time dependencies of I_s were found to be strongly dependent on the MLN concentration, c_{MLN}. The results obtained for both the **SS2** and **SS5** MLNs are shown in Table 3. The lifetime of MLNs increases with decreasing c_{MLN} and normalized residual scattering intensity, $I_s(\infty)/(c_{MLN}I_{st})$, where I_{st} is the scattering intensity of toluene, decreases with decreasing c_{MLN}. The residual light scattering intensity originates from partly destroyed MLNs surviving in solution after depletion of glutathione. The residual light scattering intensity of **SS2** nanoparticles is very low at $c_{MLN}=0.72$ mg ml^{-1}. Therefore, we suppose that $c_{MLN}=0.72$ mg ml^{-1} is close the limit concentration suitable for full degradation of **SS2** nanoparticles by the reaction with glutathione ($c_{GSH}=3$ mg ml^{-1}). The limit concentration of c_{MLN} for **SS5** nanoparticles should be similar to that of **SS2**.

Scheme 1 Proposed mechanism of degradation of the MLNs

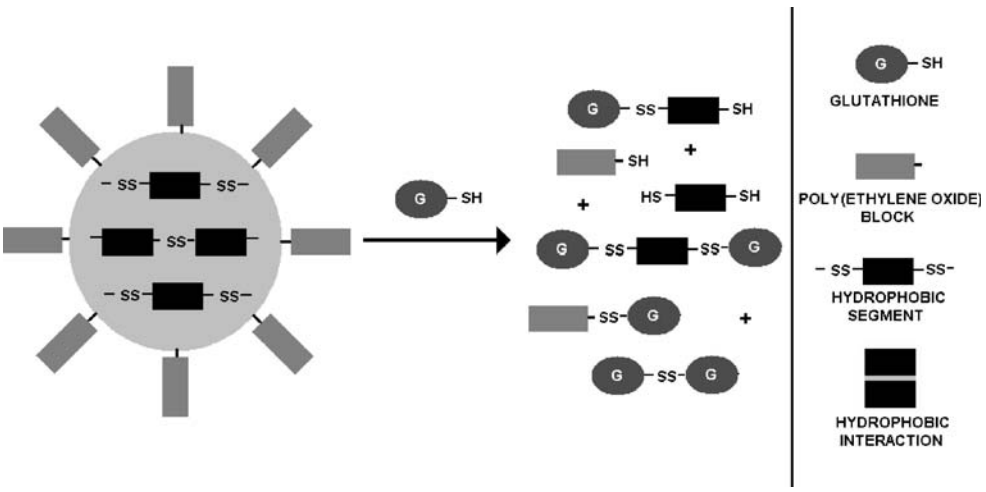

Table 3 Effect of the nanoparticle concentration c_{MLN} on the lifetime t_{lt} and residual light scattering intensity $I_s(\propto)$ normalized by the scattering intensity of toluene I_{st} (37 °C and θ=173°)

Nanoparticles	c_{MLN} (mg ml^{-1}_)	t_{lf} (h)	$I_s(\propto)\times10^{-3}/c_{MLN}I_{st}$ (ml g^{-1})
SS2	3.15	4.5	47
	1.35	9.9	15
	0.72	13.2	2.3
SS5	3.52	3.4	52
	1.35	9.6	23

Hydrodynamic radius of nanoparticles increases with incubation time due to their swelling as a result of their decreasing stability. The most pronounced effect was observed at c_{MNL}=0.72 mg ml^{-1}, where R_h increased from 44 to 95 nm during a 24-h incubation with glutathione.

The effect of pH

In contrast to the results obtained at pH 7.4, I_s and R_h increased at the beginning of the incubation of MLNs with glutathione at pH 5.0. The time dependence of I_s is shown for the suspension of **SS2** nanoparticles (c_{MLN}=1.35 mg ml^{-1}) in Fig. 3. After 2 h, the situation changed and I_s started to decrease to residual intensity $I_s(\propto)/c_{MLN}I_{st}$= 1.13×10^5 ml g^{-1} (I_{st} is the scattering intensity of toluene at θ=173°).

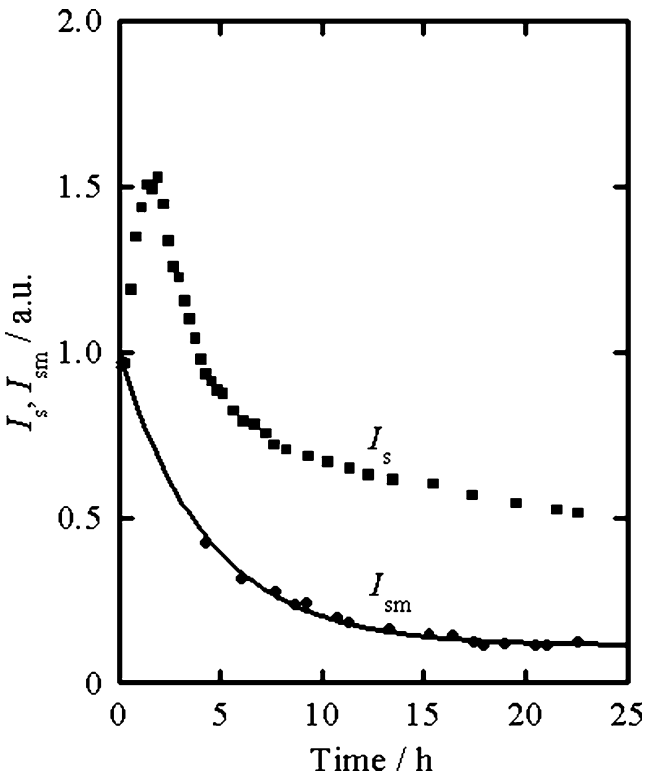

Fig. 3 The time dependencies of normalized I_s and I_{sm} related only to MLNs for solutions of **SS2** measured in the presence of GSH at pH 5 and 37 °C (c_{MLN}=1.35 mg ml^{-1}, c_{GHS}=3 mg ml^{-1}). The *full line* is a single exponential fit

The two dynamic modes could be distinguished in the DLS results after 5 h of incubation; the fast mode is evidently related to MLN diffusion while the slow one corresponds to diffusion of aggregates of the degradation product. The products are less soluble in aqueous solutions at pH 5.0 than at pH 7.4. This is mainly due to lower dissociation of carboxyl groups in the degradation products at pH 5.0 than at pH 7.4. The R_h of aggregates increased from 140 (5 h) to 200 nm (23 h). The relative intensity contribution of aggregates to the total scattering intensity increased with time reaching ca. 80% after 23 h. The aggregates could not be distinguished from the nanoparticles by DLS at the early stages of degradation (the incubation time from 0 to 5 h).

The scattered light intensity I_{sm} from MLNs, calculated as a product of I_s, and the relative intensity contribution of MLNs to the total scattering intensity, fr_m ($I_{sm}=I_s\times fr_m$), is plotted in Fig. 3. The values of fr_m were obtained from DLS measurements by fitting time-autocorrelation functions to two stretched exponential functions. The results of the single exponential fit to the time dependencies of I_{sm} in Fig. 3 are t_{lt}=4.4 h and $I_s(\propto)/c_{MLN}I_{st}=2.0\times10^4$ ml g^{-1}. Thus, the lifetime of MLNs is shorter at pH 5.0 than at pH 7.4 (cf. Table 3).

As to **SS5** nanoparticles, the light scattering of aggregates dominated I_s. Therefore, the above procedure cannot be used for light scattering data evaluation.

Conclusions

Poly(adipoyl chloride-*alt*-cystamine) nanoparticles with poly(ethylene oxide) (M_w=2,000 or 5,000) shell were prepared to serve as a model system for glutathione-degradable drug, protein, or DNA delivery systems. The polyamide micellar core has multiple disulfide bonds in the main chain and thus its degradation products are fragments of low molecular weight. The degradation of nanoparticles by the reaction with glutathione was an order of magnitude faster than their spontaneous degradation. The efficiency of oxidized glutathione was substantially lower. The described properties of PEO-polyamide nanoparticles demonstrate their potential as a model for the construction of DNA and drug delivery systems.

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