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Protein rejecting properties of PEG-grafted nanoparticles: Influence of PEG-chain length and surface density evaluated by two-dimensional electrophoresis and bicinchoninic acid (BCA)-proteinassay

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Poly (ethylene glycol) (PEG)-grafted nanoparticles have been described as potential intravenously injectable, long-circulating drug carriers. The *in vivo* behaviour of intravenous administered nanoparticles is decisively influenced by the interaction of the particles with the blood proteins. Two-dimensional electrophoresis (2-DE) was employed to study the protein rejecting properties of PEG-grafted polymer nanoparticles, possessing PEG-200 and PEG-400 chains, respectively. The calculated PEG-chain distances varied between 0.39/0.31 nm (PEG-200) and 0.39/0.34 nm (PEG-400), therefore it was possible to study the influence of high chain densities attained by the use of short PEG chains on the protein adsorption. Apart from a stronger protein rejection of small-MW proteins achieved by PEG-chain distance diminution, the affinity of several proteins for the PEG-chains are shown and discussed. Beside the study of protein adsorption patterns, the total protein mass adsorbed to the particles, as well as the extent of protein desorption prior to 2-DE, was investigated using the bicinchoninic acid (BCA)-protein assay.

1. Introduction

A major problem in intravenous drug targeting is particle uptake by macrophages of the mononuclear phagocytic system (MPS) due to opsonic proteins adsorbing onto the particles (Douglas et al. 1986; Senior 1987). Several publications deal with the prolonged circulation time and low plasma protein adsorption on PEG-grafted nanoparticles and liposomes (Allen et al. 1991; Gref et al. 1994; Norman et al. 1993; Peracchia et al. 1999; Vandorpe et al. 1997; Verrecchia et al. 1995; Zalipsky et al. 1996). First attempts to understand the ability of a grafted polymer to prevent the adsorption of proteins on a sterical basis were made in the nineties of the last century (Jeon and Andrade 1991; Jeon et al. 1991). Mathematical modelling showed that long PEG-chains (i.e. high molecular weight, MW) and primarily a high surface density of PEG attached to hydrophobic surfaces in a brush-like arrangement, are optimal conditions for the protein resistance of the surface. In contrast to that, Szleifer and co-workers discussed that the conditions for most experimental observations of PEG on hydrophobic surfaces are not in the brush regime (Szleifer 1996; Szleifer and Carignano 1996). Later publications deal with the surface coverage of grafted polymers and interactions between the monomers of the polymer and the surface, as two of the most important variables to determine the ability of a polymer layer to reduce protein adsorption (Carignano and Szleifer 2000; Szleifer 1997). There is a distinction between the thermodynamic and kinetic control of protein adsorption. A kinetic prevention, i.e. a delay in protein adsorption, is sufficient for e.g. particulate drug carriers that need to be in the blood stream for a limited time (hours to a few days). Prevention of unspecific protein adsorption should be thermodynamic in the case of e.g. artificial organs; i.e. the amount of adsorbed protein in the equilibrium is ideally near zero. For the case of polymer to surface attraction, the polymer chains are not very efficient to form a long range steric barrier to the approaching proteins (low kinetic control). The prevention from protein adsorption is therefore controlled thermodynamically. Firstly, because of the polymer to surface attraction, there is a large local concentration of the polymer segments close to the particle surface. This leads to a strong sterical repulsion of the proteins at the surface. Secondly, both the polymer and proteins compete for adsorption sites on the particle surface (gain of energy). These two effects result in a lower equilibrium amount of protein adsorbed to the surface of particles that attract the polymer compared to those that do not, and are valid for the same surface coverage. When the monomers of the grafted polymers have a preferential attraction to the surface, the longer the polymer ($MW > 2{,}000$), the more effective is the prevention of protein adsorption at fixed surface coverage.

In previous 2-DE studies, investigation of the PEG influence on the protein rejecting properties of particulate surfaces concentrated on nanoparticles possessing long PEG-chains ($MW > 2{,}000$); the PEG content was compara-

Table 1: BCA-protein assay: Total protein amounts in µg in the different protein samples obtained from protein adsorption on PEG-grafted polystyrene nanoparticles

a: PEG-200

PEG-chain distance (nm)	0.39	SD	0.37	SD	0.33	SD	0.32	SD	0.31	SD
A (µg)	43	1.5	36	2.1	32	2.1	36	2.1	38	1.2
B′ (µg)	40	2.0	31	1.5	30	1.0	32	2.1	34	2.1
B″ (µg)	2	0.6	3	1.0	2	0.6	2	0.6	3	1.0

b: PEG-400

PEG-chain distance (nm)	0.39	SD	0.37	SD	0.34	SD
A (µg)	69	2.6	83	2.1	52	1.5
B′ (µg)	63	2.1	73	2.5	44	1.5
B″ (µg)	4	1.0	10	2.1	6	0.8

The protein amounts refer to an incubated surface area of approx. 0.3 m^2, according to the incubated particle surface for the 2-DE experiment
A: Particle pellets with adsorbed proteins (total protein amount after plasma incubation)
B′: Desorbed proteins
B″: Particle pellets after desorption (proteins that remain at the particles' surface after desorption)
The values are the mean of three experiments

tively low resulting in broader PEG chain distances (Gref et al. 2000; Peracchia et al. 1999). A prevention of protein adsorption should be achieved by one PEG-2000 segment per 100 $Å^2$, which means a maximum PEG-chain distance of 1 nm (Carignano and Szleifer 2000). To investigate the effect of high surface densities achieved with very short PEG-chains on the protein adsorption, well-defined PEG-grafted polystyrene model particles were used in this study. The grafted particles possessed PEG of MW 200, 400, and chain distances between 0.39 and 0.31 nm, 0.39 and 0.34 nm, respectively.
Evaluation of the protein adsorption patterns was carried out by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE, 2-DE). Additionally, the total protein amounts adsorbed on the particles as well as degree of protein desorption prior to 2-DE, were determined by a bicinchoninic acid (BCA)-protein assay.

2. Investigations, results and discussion

2.1. Protein quantification by BCA-assay

A series of PEG-grafted polystyrene nanoparticles was synthesized to study the influence of short PEG-chains (MW 200 and 400) on the protein adsorption from human plasma. To determine total protein amounts in diverse samples, a range of protein assays is commercially available. However, in contrast to other protein assays, e.g. Bradford or Lowry assay, the BCA-assay is suitable to measure even proteins covalently bound to surfaces (Smith et al. 1985; Stich 1990). Since the purple complex formed is water-soluble, it is released into the supernatant instead of remaining associated with the proteins and polymer surfaces.
The particle suspensions were incubated in human plasma according to sample preparation prior to 2-DE (see "Experimental"). Afterwards, each sample was parted, resulting in samples A and B. In samples B, the plasma proteins adsorbed to the particles' surface were desorbed according to Blunk et al. (1993). Then, the solutions containing the desorbed proteins (samples B′) were carefully removed from the particle pellets (samples B″) and aliquots of the samples were used for the protein assay.

Table 1 lists the total protein amounts given in µg, for the range of PEG-200 and for the PEG-400 modified particles obtained after quantification using the BCA-protein assay. The extent of protein desorption was about 90%, i.e. most of the adsorbed proteins are recovered after protein desorption and are therefore accessible to the later performed analysis by 2-DE. Apart from the determination of the extent of protein desorption, another goal of this study was the quantification of the protein mass adsorbed to the particles. The total protein amount adsorbed on PEG-400 particles with calculated chain distances of 0.39 nm was 69 µg. Compared to that, PEG-200 particles with chain distances of 0.39 nm show less proteins adsorbed to the surface (total amount: 43 µg protein). Even on particles with maximum chain density of PEG-400 chains (0.34 nm; 52 µg protein) a higher amount of proteins is adsorbed to the surface, as compared to the PEG-200 particles possessing the lowest chain density (0.39 nm; 43 µg protein). Despite of an increasing surface density on PEG-200 and -400 grafted nanoparticles, no or only minor changes in the protein amount adsorbed could be detected, with regard to a reduction in the total protein adsorption (Table 1).

2.2. Analysis of protein adsorption by 2-DE

After protein quantification using the BCA-proteinassay, 2-DE analysis gives evidence about the protein adsorption patterns on the particles observed. It is also possible to gain information about the composition of the protein pattern on a semiquanitative basis (Gessner et al. 2003; Poehling and Neuhof 1981). Figs. a (PEG-200) and b (PEG-400) show the effect of increasing PEG-surface density on the protein adsorption patterns, in each case the patterns on the particles with lowest (left) and highest (right) PEG-chain density are shown.
Table 2 lists the percentage of proteins detected on the 2-DE gels, the qualitative composition of the plasma protein adsorption patterns on the different PEG-grafted nanoparticles are very similar. Table 2a lists the major proteins adsorbed on the surface of the PEG-200 modified nanoparticles. As discussed in the preceding section, there is no considerable change in the total amount of protein adsorption, despite the increased PEG surface density. Immunocompetent proteins such as fibrinogen, Immunoglobulin (Ig) G and IgM are by far the dominant proteins of the adsorption patterns, representing over 70% of the total detected protein amounts. Increase of the PEG surface density does not lead to a diminished adsorption of large molecular weight (MW) proteins such as albumin, the immunoglobulins and fibrinogen (MW above 100,000). Obviously, a maximum decrease of adsorption of high MW proteins is already attained within a PEG-200 chain distance of 0.39 nm. A decrease in protein adsorption is only recorded for the apolipoproteins (A-I, A-IV, J), in the course of diminution of the PEG-chain distance from 0.39 to 0.33 nm. Whilst the decrease in the amount of these proteins within this interval is up to 80% (ApoJ), further increase of PEG surface density resulted only in a minor reduction of adsorption. Consequently, a threshold of a PEG-chain distance resulting in a minimum protein adsorption is achieved between 0.39 and 0.33 nm, concerning proteins of MW below 50,000. Because of the comparatively small protein amounts, the decrease in apolipoprotein adsorption has no effect on the total protein amount.
Table 2b lists the major proteins adsorbed on the surface of the PEG-400 modified nanoparticles. As for the PEG-

Table 2: 2-DE experiment: Protein adsorption on PEG-grafted polystyrene particles

a: PEG-200

	PEG-chain distance [nm]									
	0.39		0.37		0.33		0.32		0.31	
	% VOL	SD	% VOL	SD	% VOL	SD	% VOL	SD	% VOL	SD
albumin	3.48	2.31	2.37	0.69	3.26	1.86	2.24	0.62	2.84	0.74
ApoA-I	1.56	1.29	0.67	0.29	0.68	0.21	0.61	0.10	0.58	0.26
ApoA-IV	0.81	0.25	0.65	0.27	0.50	0.28	0.50	0.25	0.52	0.31
ApoJ	2.60	0.51	0.87	0.57	0.57	0.56	0.42	0.3	0.27	0.31
fibrinogen	37.91	5.17	42.51	5.85	44.32	2.97	39.76	10.22	52.40	14.85
IgGγ	14.44	3.97	10.56	0.44	9.98	2.29	10.05	2.52	11.01	2.83
IgM	12.27	0.04	11.26	8.27	7.04	5.10	7.64	4.84	6.66	4.63
Ig-light chains	8.07	4.84	9.60	2.19	8.71	1.23	8.52	1.89	8.39	2.02
total amount (VOL)	27.51	5.53	28.88	11.96	35.86	6.88	38.66	9.40	31.50	1.70

b: PEG-400

	PEG-chain distance (nm)					
	0.39		0.37		0.34	
	% VOL	SD	% VOL	SD	% VOL	SD
albumin	3.15	0.23	3.37	0.35	2.07	0.76
ApoA-I	1.33	0.60	0.61	0.44	0.56	0.24
ApoA-IV	1.54	0.72	1.31	0.56	0.63	0.36
ApoJ	3.75	0.99	0.95	0.18	0.44	0.53
Fibrinogen	41.89	6.55	37.53	2.91	46.38	8.61
IgGγ	18.58	0.86	15.73	0.26	19.14	1.89
IgM	11.03	1.55	12.01	1.21	10.47	2.55
Ig-light chains	11.54	1.36	9.36	3.43	7.48	5.97
total amount (VOL)	39.53	8.23	41.87	7.96	31.53	6.64

The values are the mean of three experiments, given as percentage of the overall amount of proteins adsorbed on the particles (%VOL); except total protein amount: values given in VOL (arbitrary unit). Fibrinogen is given as a sum of its α-, β- and γ-chains

200 grafted particles, there is no considerable change in the total protein amount in the course of PEG-400 chain distance diminution from 0.39 to 0.34 nm. Again, fibrinogen, IgG and IgM are dominating the adsorption patterns, representing about 80% of the total detected protein amounts. Reduced adsorption is recorded in the apolipoproteins (ApoA-I, A-IV, J), as for PEG-200 grafted particles. The decrease in the amount of these proteins within diminution of the PEG-chain distance from 0.39 to 0.34 nm is up to 90% (ApoJ). A threshold of PEG-400 chain distance resulting in a minimum protein adsorption is achieved between 0.39 and 0.34 nm (as for PEG-200) concerning proteins of MW below 50,000.

Comparison of PEG-200 and 400 nanoparticles show only minor differences in the total adsorption. The differences in the total protein amounts detected on the 2-DE gels [VOL] are negligible. A direct comparison of total protein amounts obtained from silver-stained 2-DE gels and the BCA-assay is difficult, due to the totally different concept of protein detection and the semi-quantitative character of the data obtained by 2-DE. The differences in the protein amounts detected by BCA-assay might be caused by the higher percentage of IgG adsorbed to the PEG-400 particles. Due to the protein-to-protein variation of the BCA-assay, as is with other protein assays, there is a stronger color response with IgG than with e.g. albumin (Smith et al. 1985). Therefore a sample containing a higher amount of IgG (i.e. protein adsorption on PEG-400), compared to another sample (i.e. protein adsorption on PEG-200), might lead to differences in the detected protein content.

Theoretical calculations by Jeon et al. (1991) concerning the optimal conditions for protein rejection by graft polymers (MW $>$ 2,000), suggested a minimum chain distance of about 1 nm for small proteins, and 1.5 for larger proteins (proteins supposed as spheres of $r = 2$ nm and $r = 6-8$ nm, respectively). As shown in the present study, adsorption of proteins is not completely prevented, even on particles possessing the highest PEG-chain densities. This might be due to an interaction of certain proteins with the PEG segments. A lead for this assumption is the constant amount of proteins, such as albumin, fibrinogen and IgG found to be adsorbed on the particles, despite the increasing PEG-chain density. Several protein adsorption studies on PEG-modified nanoparticles lead to the same assumption. Despite increasing the PEG-surface densities, several proteins (albumin, IgG) showed no considerable decrease (Gref et al. 2000; Harnisch 1998; Peracchia et al. 1999). In the present study, the PEG-chain distances of PEG-200 and 400 are directly comparable. The higher amount of total protein (BCA-assay) adsorbed to the PEG-400 particles compared to the PEG-200 particles is possibly explained by a higher portion of IgG adsorbed to the PEG-400 particles. This finding corroborates the assumption that the PEG-chains are involved in the adsorption of single proteins, i.e. despite the same PEG-chain densities, more IgG is adsorbed to the PEG-400 particles, due to the longer PEG chains. This is possibly caused by a higher number of adsorption sites the longer chain segments offer. As Carignano and Szleifer (2000) concluded in a previous publication, prevention of protein adsorption by grafted polymers is simply due to the exclusion of proteins from the surface, but a very high surface coverage can modify the surface chemistry and may result in a protein attractive surface.

However, further studies will be necessary to estimate the involvement of graft PEG-segments in protein adsorption and the following consequences for their *in vivo* behaviour. In the long term, the knowledge gained from such basic studies should be applicable to the controlled development of i.v. injectable, site-specific drug carriers.

3. Experimental

3.1. Materials

For 2-DE all chemicals were of analytical grade. *N,N,N′,N′*-tetramethylenediamine (TEMED), ammonium persulfate and piperazine diacrylamide (PDA) were purchased from BioRad (Munich, Germany). Immobiline DryStrips/immobilized pH gradients (IPG, pH 3.5–10, nonlinear) were from Amersham Pharmacia (Uppsala, Sweden). All other chemicals (Blunk et al. 1993; Hochstrasser et al. 1988) were supplied either from Sigma/Fluka

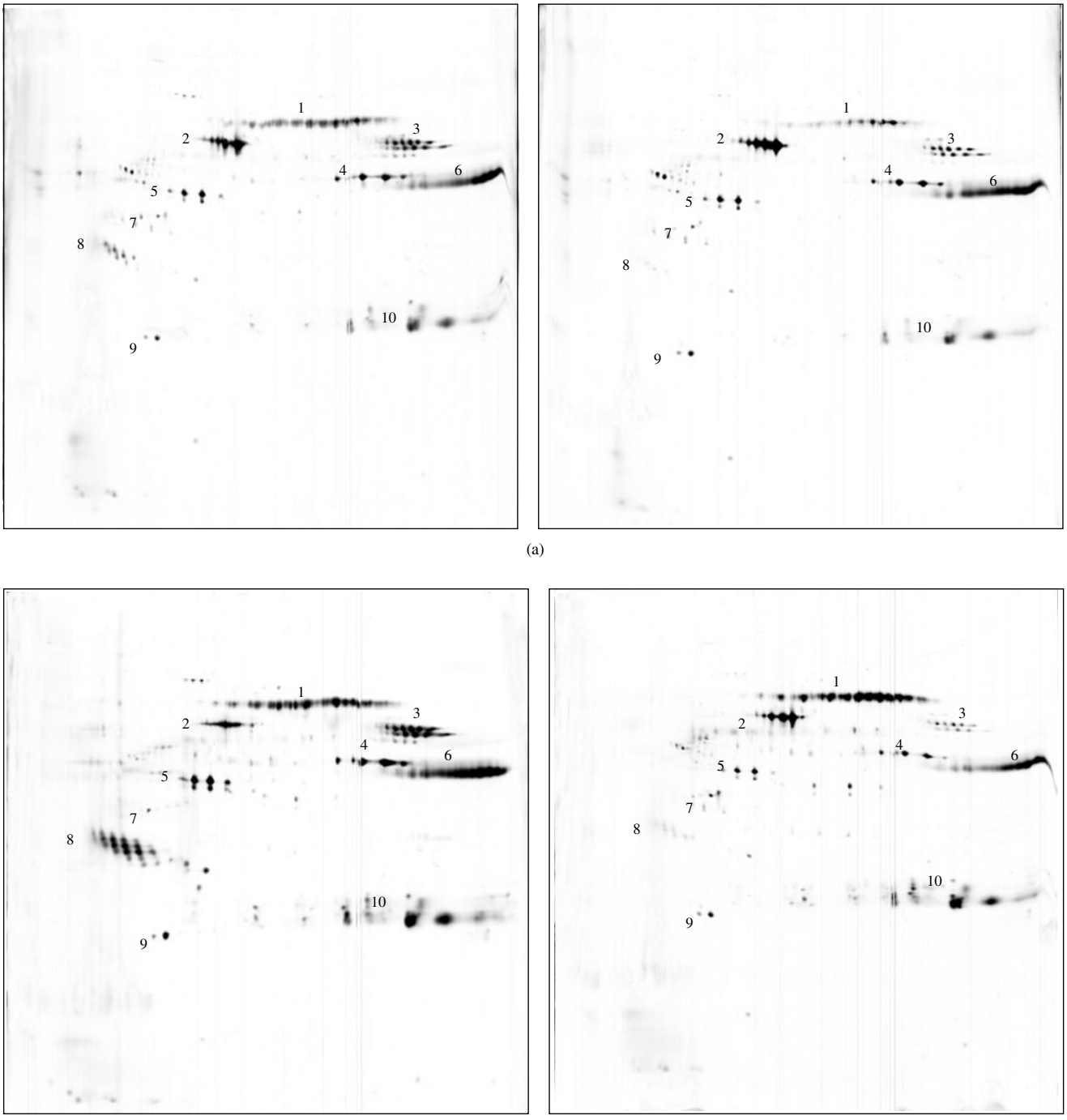

Fig.: Protein adsorption patterns on PEG-grafted polystyrene particles
The entire gels are shown, pI 4–9 (left to right gel side), MW 250–6 kDa (upper to lower)
(1) IgM, (2) albumin, (3) fibrinogen α, (4) fibrinogen β, (5) fibrinogen γ, (6) IgGγ, (7) ApoA-IV, (8) ApoJ, (9) ApoA-I, (10) Ig light-chains
a: Protein adsorption patterns on PEG-200 grafted polystyrene particles: PEG-chain distances 0.39 nm (left) and 0.31 nm (right)
b: Protein adsorption patterns on PEG-400 grafted polystyrene particles: PEG-chain distances 0.39 nm (left) and 0.34 nm (right)

(Deisenhofen, Germany) or Merck (Darmstadt, Germany). Frozen human plasma was purchased from the German Red Cross (Berlin, Germany) and stored at −70 °C. The BCA reagent-kit for protein quantification was obtained from PIERCE (Rockford, USA), sodium-deoxycholate and trichloracetic acid were from Merck (Darmstadt, Germany).

3.2. Methods

3.2.1. Preparation and characterization of the model nanoparticles

Polystyrene model latex particles with covalently fixed polyethylene glycol (PEG) chains on the surface were prepared at the Fraunhofer Institute by a free radical emulsion polymerization of styrene in water. In a batch polymerization series (Wang et al. 1994), different amounts of PEG mono methacrylates (PEG-MA 400, 200) were copolymerized with styrene using a standard recipe (water, styrene, emulsifier SDS, buffer). Temperature (60 °C) and stirring conditions were also kept constant. The emulsion polymerization was started by means of a PEG-modified azo-initiator (PEGA 200 (Tauer and Kosmella 1993)). In this series, particle sizes of 50–60 nm (PEG-MA 400) and 45–55 nm (PEG-MA 200) were obtained. Emulsifier, salt and rest monomer traces were removed by dialysis against deionized water and subsequent ultrafiltration. All latices were sterically stabilized by PEG chains on their surface. For PEG-200 grafted nanoparticles the calculated distance between two PEG chains on the surface was approximately 0.39, 0.37, 0.33, 0.32 and 0.31 nm, respectively. Calculated chain distance for PEG-400 grafted nanoparticles was 0.39, 0.37 and 0.34 nm, respectively.

3.2.2. Sample preparation and 2-DE

The aqueous particle suspensions (100 µL) containing constant surface areas (3 m^2 per mL) were incubated in 2.2 mL human plasma for 5 min at 37 °C. The particles were separated from plasma by centrifugation and afterwards washed four times with bidistilled water. Finally adsorbed proteins were desorbed by protein solubilizing solutions containing 10% sodium-dodecyl sulfate (SDS) (Blunk et al. 1993). The samples were analyzed by means of 2-DE as described previously (Blunk et al. 1993; Gessner et al. 2000; Harnisch and Müller 1998). Briefly, isoelectric focusing (IEF) was carried out in a Multiphore II from Amersham Pharmacia, equipped with an E752 power supply from Consort (Turnhout, Belgium). IPG-strip equilibration was performed according to Bjellqvist et al. (1993). For SDS-PAGE, a Model 395 gradient former, the Multi-gel casting chamber and Protean II Multi-Cells equipped with 1000/500 power supplies were used (BioRad). After second dimension the gels were silver-stained according to Bjellqvist et al. (1993), and scanned with a laser densitometer from Molecular Dynamics (Krefeld, Germany). Each protein spot has its own characteristic coordinates on the gel (pI and MW), therefore identification can be carried out by comparison of the obtained 2-DE gels with reference maps (Golaz et al. 1993). Analysis of the 2-DE gel images was carried out using the MELANIE II software (BioRad); the values for the protein amounts are given in arbitrary units (VOL). 2-DE analysis for each particle suspension was run in triplicate.

3.2.3. Protein quantification

The samples were prepared according to the sample preparation prior to 2-DE. After the last washing step, each sample was parted, resulting in samples A (total protein amount after plasma incubation) and B. Each sample contained a particle amount representing a total surface area of about 1 m^2. The later discussed protein amounts are calculated for surface areas of about 0.3 m, according to the 2-DE sample preparation protocol. The above mentioned protein solubilizing solution (Blunk et al. 1993) was added to the samples B. After centrifugation, the solutions containing the desorbed proteins (samples B′) were carefully removed from the particle pellets (samples B″). To remove desorbed proteins and remains of solubilizing solutions, the samples B″ were extensively washed. The solubilized proteins in samples B′ and the washing solutions were precipitated using sodium-deoxycholate and trichloracetic acid. According to Brown et al. (1989) this is a suitable method to obtain quantitatively solubilized proteins and to remove chemicals interfering the BCA-assay. After precipitation each protein sample was collected using 0.1 N NaOH containing 5% SDS. Due to the same treatment of samples, alkaline SDS solution was also added to the samples A and B″. Aliquots of the samples were used for the protein assay.

The standard BCA-assay was performed as described in the manufacturer's instruction: 100 µL sample solution were mixed with 2 mL BCA working-reagent. After incubation for 30 min at 60 °C the samples A and B″ were centrifuged in order to remove the particles (20,000 g/15 min/7 °C). During centrifugation of samples A (particle pellets with adsorbed proteins) and B″ (particle pellets after desorption), the samples B′ (desorbed proteins) and BSA-standards were kept in a refrigerator at 7 °C in order to slow down the color reaction. There was no considerable change in the absorption of the complex formed during the time of centrifugation or storage (Gessner 2001). The absorptions of the samples and a range of BSA-standard solutions were measured at $\lambda = 562$ nm with an UV-VIS-spectrophotometer (Uvikon 940, Kontron Instruments, Eching/Germany). The range of the standard curve was 5–250 µg/ml.

References

Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A (1991) Liposomes containing synthetic lipid derivates of poly(ethylene glycol) show prolonged circulating half-lives *in vivo*. Biochim Biophys Acta 1066: 29–36.

Bjellqvist B, Pasquali C, Ravier F, Sanchez JC, Hochstrasser DF (1993) A nonlinear wide-range immobilized pH gradient for two-dimensional electrophoresis and its definition in a relevant pH scale. Electrophoresis 14: 1357–1365.

Blunk T, Hochstrasser DF, Sanchez J-C, Müller BW, Müller RH (1993) Colloidal carriers for intravenous drug targeting: plasma protein adsorption patterns on surface-modified latex particles evaluated by two-dimensional polyacrylamide gel electrophoresis. Electrophoresis 14: 1382–1387.

Brown RE, Jarvis KL, Hyland KJ (1989) Protein measurement using bicinchoninic acid: elimination of interfering substances. Anal Biochem 180: 136–139.

Carignano MA, Szleifer II (2000) Prevention of protein adsorption by flexible and rigid chain molecules. Colloids Surf B Biointerfaces 18: 169–182.

Douglas SJ, Davis SS, Illum L (1986) Biodistribution of poly(isobutyl-cyanoacrylate) nanoparticles in rabbits. Int J Pharm 34: 145–152.

Gessner A, Paulke BR, Müller RH (2000) Analysis of plasma protein adsorption onto polystyrene particles by two-dimensional electrophoresis: comparison of sample application and isoelectric focusing techniques. Electrophoresis 21: 2438–2442.

Gessner A (2001) Untersuchungen zur Proteinadsorption auf kolloidalen Modellpartikeln und Arzneistoffträgern zur parenteralen Anwendung. PhD Thesis, Freie Universität Berlin.

Gessner A, Lieske A, Paulke BR, Müller RH (2003) Functional groups on polystyrene model nanoparticles: influence on protein adsorption. J Biomed Mater Res 65A: 319–326.

Golaz O, Hughes GJ, Frutiger S, Paquet N, Bairoch A, Pasquali C, Sanchez JC, Tissot JD, Appel RD, Walzer C (1993) Plasma and red blood cell protein maps: update 1993. Electrophoresis 14: 1223–1231.

Gref R, Minamitake Y, Peracchia MT, Trubetskoy V, Torchilin V, Langer R (1994) Biodegradable long-circulating polymeric nanospheres. Science 263: 1600–1603.

Gref R, Lück M, Quellec P, Marchand M, Dellacherie E, Harnisch S, Blunk T, Müller RH (2000) 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. Colloids Surf B Biointerfaces 18: 301–313.

Harnisch S (1998) Vergleichende Untersuchungen zur Plasmaproteinadsorption auf O/W-Emulsionen und Polymer-Partikeln zur parenteralen Anwendung. PhD Thesis, Freie Universität Berlin.

Harnisch S, Müller RH (1998) Plasma protein adsorption patterns on emulsions for parenteral administration: establishment of a protocol for two-dimensional polyacrylamide electrophoresis. Electrophoresis 19: 349–354.

Hochstrasser DF, Harrington MG, Hochstrasser AC, Miller MJ, Merril CR (1988) Methods for increasing the resolution of two-dimensional protein electrophoresis. Anal Biochem 173: 424–435.

Jeon SL, Andrade JD (1991) Protein-surface interactions in the presence of polyethylene oxide, II. Effect of protein size. J Coll Interf Sci 142: 159–166.

Jeon SL, Lee JH, Andrade JD, De Gennes PG (1991) Protein-surface interactions in the presence of polyethylene oxide, I. Simplified theory. J Coll Interf Sci 142: 149–158.

Norman ME, Williams P, Illum L (1993) In vivo evaluation of protein adsorption to sterically stabilised colloidal carriers. J Biomed Mater Res 27: 861–866.

Peracchia MT, Harnisch S, Pinto-Alphandary H, Gulik A, Dedieu JC, Desmaele D, d'Angelo J, Müller RH, Couvreur P (1999) Visualization of in vitro protein-rejecting properties of PEGylated stealth polycyanoacrylate nanoparticles. Biomaterials 20: 1269–1275.

Poehling H-M, Neuhof V (1981) Visualization of proteins with a silver "stain": a critical analysis. Electrophoresis 2: 141–147.

Senior JH (1987) Fate and behavior of liposomes in vivo: a review of controlling factors. Crit Rev Ther Drug Carrier Syst 3: 123–193.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150: 76–85.

Stich TM (1990) Determination of protein covalently bound to agarose supports using bicinchoninic acid. Anal Biochem 191: 343–346.

Szleifer I (1996) Statistical thermodynamics of polymers near surfaces. Curr Opin Colloid Interface Sci 1: 416–423.

Szleifer I, Carignano MA (1996) Tethered polymer layers. Adv Chem Phys 44: 165–260.

Szleifer I (1997) Protein adsorption on surfaces with grafted polymers: a theoretical approach. Biophys J 72(2 Pt 1): 595–612.

Tauer K, Kosmella S (1993) Synthesis, Characterization and Application of Surface Active Inititators. Polymer international 30: 253–256.

Vandorpe J, Schacht E, Dunn S, Hawley A, Stolnik S, Davis SS, Garnett MC, Davies MC, Illum L (1997) Long circulating biodegradable poly(phosphazene) nanoparticles surface modified with poly(phosphazene)-poly(ethylene oxide) copolymer. Biomaterials 18: 1147–1152.

Verrecchia T, Spenlehauer G, Bazile DV, Murry-Brelier A, Archimbaud Y, Veillard M (1995) Non-stealth (poly(lactic acid/albumin)) and stealth (poly(lactic acid-polyethylene glycol)) nanoparticles as injectable drug carriers. J Contr Rel 36: 49–61.

Wang Q, Fu S, Yu T (1994) Emulsion Polymerization. Progress in Polymer Science 19: 703–753.

Zalipsky S, Hansen CB, Lopes de Menezes DE, Allen TM (1996) Long-circulating, polyethylene-glycol-grafted immunoliposomes. J Contr Rel 39: 153–161.