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Research paper

Influence of surface charge density on protein adsorption on polymeric nanoparticles: analysis by two-dimensional electrophoresis

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Abstract

Plasma protein adsorption is regarded as a key factor for the in vivo organ distribution of intravenously administered colloidal drug carriers, and strongly depends on their surface characteristics, e.g. surface hydrophobicity or charge. A range of polymeric nanoparticles with a steep variation of the surface charge density was synthesized as model drug carriers. Physicochemical parameters, i.e. particle size, surface charge density, hydrophobicity and surface topography were determined. Two-dimensional electrophoresis (2-DE) was employed for determination of particle interactions with human plasma proteins. Increasing surface charge density showed an increase in plasma protein adsorption, but did not show differences in the detected protein species. For the first time it was possible to show plasma protein adsorption patterns on a range of nanoparticles with variation of only one parameter, i.e. the charge, while size and surface hydrophobicity remain practically unchanged. The knowledge about the interactions of proteins with particulate surfaces can be exploited for the future controlled design of colloidal drug carriers and possibly in the controlled creation of biocompatible surfaces of other devices that come into contact with proteins (e.g. microparticles and implants). © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Nanoparticles; Surface charge density; Protein adsorption; Two-dimensional electrophoresis; Colloidal drug carriers

1. Introduction

Particulate colloidal carriers for intravenous administration are one approach to the site-specific delivery of drugs. A major obstacle for their application is the immunological recognition by cells of the mononuclear phagocyte system (MPS). Many attempts have been made to modify the physicochemical properties of the particles, e.g. surface hydrophobicity, chemical composition of the surface and particle charge, and to relate these properties to phagocytosis and the in vivo organ distribution. In these previous studies, only a few particle parameters like size and charge were determined and related to the uptake by the MPS. Increasing MPS clearance with increasing particle size, charge and hydrophobicity of the surface were established as general rules [1–8]. A few particulate systems have been found empirically, which avoid, or at least clearly reduce, uptake by the cells of the MPS. A prolonged circulation time in the bloodstream was achieved for liposomes and polymeric particles surface modified with polyoxyethylene (PEG) chains [9–12].

A simple determination of the physicochemical properties did not lead to an explanation of the in vivo behavior of particulate systems, e.g. particles coated with block co-polymers poloxamine 908/poloxamer 407 possessed identical physicochemical properties (i.e. size, charge, coating layer thickness, etc.), but showed completely different in vivo behavior [13]. This shows the limitations of physicochemical characterization, therefore determination of plasma protein adsorption on particulate systems has been established [14]. The protein adsorption pattern is regarded as a key factor for the explanation of the recognition or non-recognition by the MPS, and interaction with specific target cells via receptors [13]. Considerable differences in protein adsorption on polymer particles were shown in several studies [14–17], but in vivo particle behavior is still far from being reliably predicted.

Studies dealing with the influence of charge on protein adsorption were mostly carried out by the use of polymeric films or other plain surfaces. One approach is the use of a fixed surface charge and variation of protein charge by change of the solution pH [18,19]. Other research groups used electrodes to induce different surface charges and

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studied the effects on protein adsorption [20,21]. A general finding of these studies was an increase in protein adsorption with increasing surface charge, but most of these studies were carried out by the use of single proteins, e.g. albumin. Kapur et al. [21] used human serum, but due to the application of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the analysis of protein adsorption patterns was limited. The results presented in the current study account for the protein adsorption on nanoparticles from a natural mixture of proteins (plasma) and a highly sensitive analyzing technique (2-DE). Therefore, not only the change in amounts of protein adsorbed to the particles can be shown, but also the identification of the proteins is possible.

Change of only one particle parameter is often difficult to achieve, e.g. change of surface hydrophobicity by use of different core materials also affects other parameters (e.g. surface charge, functional groups) [15,17]. A previous study by Lück et al. [15] showed less protein adsorption on particles with increasing surface charge density. What has to be taken into account is the fact, that the particles with higher surface charge density also showed a strongly diminished hydrophobicity, due to the different core materials (styrene, 2,3-epoxypropylmethacrylate, respectively). Additional spacers possessing hydroxylic groups also influenced the protein adsorption. Consequently, it is difficult to draw general conclusions about the influence of certain parameters on protein adsorption. Instead of broad variations of particle properties, steep variation of only one parameter should lead to manageable changes in protein adsorption and may help to understand the in vivo behavior of intravenously injected particulate drug carriers.

In this study, the focus concerning protein adsorption on model particles is on the effect of increasing surface charge density on the adsorption patterns. A range of model latex nanoparticles was synthesized, possessing constant particle parameters, i.e. particle diameter and surface hydrophobicity were in the same order of magnitude. The only variable parameter was the surface charge density in the range of -3.7 to -8.2 µC/cm^2.

2. Materials and methods

2.1. *Model nanoparticles: synthesis and physicochemical characterization*

Polystyrene latex particles were prepared at the Fraunhofer Institute. The goal was to synthesize a variety of nanoparticles with different surface charge densities, but constant parameters such as particle size or hydrophobicity. A simple system of persulfate/styrene/water would only be sufficient to obtain surface charge densities in a range of 2–7 µC/cm^2. Another problem is the instability during purification after polymerization of the formed latices possessing low surface charge densities (<2–4 µC/cm^2). Therefore, the particles were prepared in a batch polymerization series [22], using Na-styrenesulfonate as a co-monomer to styrene (particles 1–5). This system has been described by several authors as an useful approach to synthesize model colloids possessing high densities of ionic groups on the surface [23,24]. Latices possessing different surface charge densities were obtained under variation of the process temperature (60–80°C) that had the function to rule radical formation as well as styrenesulfonate incorporation into the particle interface (approximate particle diameter: 60 nm).

Particle size was determined by photon correlation spectrometry. Surface charge density was titrated using a cationic (poly-diallyl-dimethyl-ammoniumchloride) polyelectrolyte standard. Point of zero surface charge was detected via streaming potential of the dispersion (Particle Charge Detector, MÜTEK, Germany). Further description of characterization methods for particle sizing and determination of the surface charge can be found in Ref. [25].

The surface hydrophobicity of the latices was evaluated by adsorption of the hydrophobic dye Rose Bengal (Sigma, Deisenhofen) as described previously [13]. Briefly, a fixed amount of dye (40 µg/ml) was added to nanoparticle suspensions of increasing concentration. Rose Bengal undergoes partitioning between the particle surface and the dispersion medium. After incubation and centrifugation, the amount of Rose Bengal was determined by absorption measurement of the supernatant at 542.7 nm using a UV spectrophotometer (Uvikon 940, Kontron). The partitioning quotient (PQ) was calculated according to PQ = amount of Rose Bengal bound on surface/amount of Rose Bengal in dispersion medium (for each particle concentration). PQ was plotted as a function of the total surface area resulting in straight lines (Fig. 1). The slopes were taken as a measure of the degree of surface hydrophobicity. Latex particle characterization data are given in brief in Table 1.

Electrophoretic mobility measurements were carried out for further particle surface characterization. Measurements of the variation of the ionic strength of the aqueous medium give evidence about the surface condition, with regard to a

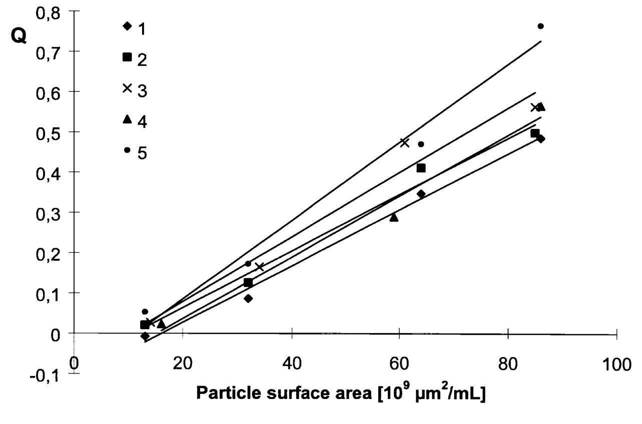

Fig. 1. Rose Bengal partitioning method. Plot of the PQ *Q* versus the surface area of the particle suspensions. The slope *S* of the straight line is a measure of the surface hydrophobicity of the latex.

Table 1
Physicochemical characterization data of model nanoparticles (latex 1–5)

Latex	1	2	3	4	5
Surface charge density (μC/cm^2)	− 3.7	− 5.2	− 6.0	− 6.7	− 8.2
Particle size (nm) (SD 0.01)	54	61	64	57	60
Hydrophobicity: slope from Rose Bengal partitioning experiment (ml/m^2)	7	7	8	8	10

smooth or a tentacle-bearing surface. Hydrophilic tentacles on hydrophobic particles can be regarded as polyelectrolyte chains. Depending on the ionic strength of the surrounding medium, these chain structures undergo changes in their conformation [26]. With respect to particles bearing tentacles on their surface, this should lead to changes in the hydrodynamic diameter and the electrophoretic mobility. Due to an expected greater effect, the focus of the physicochemical measurements was on the electrophoretic mobility (complementary measurements of the hydrodynamic diameter by dynamic light scattering were carried out in random sample). The electrophoretic mobility of the particles was measured during titration with 0.5 N NaCl in a Zetamaster (Malvern Instruments, UK). Polystyrene latices with smooth surfaces (particle suspensions A, B) and tentacles on the surface (Particle suspension C) were used as standards. Standard A (mean diameter 80 nm, surface charge density −2.6 μC/cm^2) and standard B (250 nm, −4.6 μC/cm^2) were smooth styrene beads with sulfate groups directly on the surface. Standard C (100 nm, −17.9 μC/cm^2) consisted of a styrene/styrenesulfonate copolymer with sulfate/sulfonate groups and styrenesulfonate tentacles on the surface. The electrophoretic mobility profiles are shown in Fig. 2.

2.2. Two-dimensional electrophoresis (2-DE) of plasma protein adsorption

Two hundred micro liters of the aqueous particle suspensions containing constant surface areas (0.3 m^2 per ml) were incubated in 2.2 ml citrate stabilized human plasma for 5 min at 37°C. The particles were separated from plasma by centrifugation, washed four times with double-distilled water, finally adsorbed proteins were desorbed by protein solubilizing solutions [14]. The samples were analyzed by the means of 2-DE with equipment from BioRad (Munich, Germany), Amersham Pharmacia Biotech (Uppsala, Sweden) and Consort (Turnhout, Belgium) as described in detail previously [27–29]. Briefly, 2-DE is divided into two steps for protein separation: isoelectric focusing (IEF) in the first dimension, and a SDS-PAGE in the second dimension. During IEF using Immobiline DryStrips (pH 3.5–10, nonlinear) from Amersham Pharmacia Biotech, the proteins are separated solely according to their isoelectric points (pI). In the second dimension (SDS-PAGE), separation of proteins takes place in gradient slab gels due to their molecular weights (MW). After SDS-PAGE the gels were silver-stained according to Refs. [27,28], and scanned with a laser densitometer (Personal Densitometer from Molecular Dynamics, Krefeld, Germany). Since each protein spot has its own characteristic coordinates (pI and MW), identification can be carried out by comparison of the obtained 2-DE gels with reference maps [30, or accessible via internet at http://www.expasy.ch]. 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 (cpm). For data assessment it has to be taken into account, that the slope obtained from silver-staining (staining density vs. protein amount) is characteristic for each protein [31,32]. Thus the data can only be regarded as semi-quantitative when different proteins are quantified. Nevertheless, it can be used for a reliable approximation to the amounts of protein adsorbed on the particles, when identical protein spots are compared. 2-DE analysis for each particle suspension was run in triplicate.

3. Results and discussion

3.1. Electrophoretic mobility

The synthesized latices represent a steep variation of surface charge density whereas other parameters, i.e. parti-

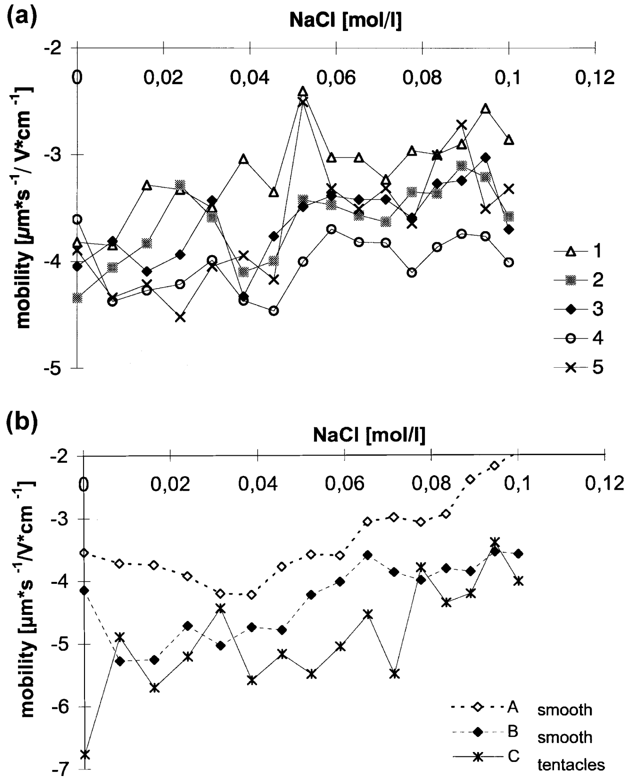

Fig. 2. Electrophoretic mobility profiles at pH 6 of (a) latex 1–5 and (b) standard latices A–C as a function of electrolyte concentration (NaCl), SD 0.05–0.1.

cle diameter and surface hydrophobicity, are kept constant. Fig. 2a shows the electrophoretic mobility profiles of the investigated latices 1–5, Fig. 2b of the particle standards A–C. The mobility of smooth polystyrene particles (A and B) increases from the first addition of NaCl up to a maximum of mobility at concentrations between 10^{-3} and 10^{-2} mol/l NaCl. This can be explained by adsorption of additional anions into the Helmholtz layer surrounding the particles, resulting in a higher actual particle charge. Due to the compression of the electrochemical bilayer and the masked surface charge with increasing salt concentrations (>0.04 mol/l NaCl), the Coulomb effect and therefore the mobility of the particles decreases. This initial increase followed by a decrease of the electrophoretic mobility results in a local maximum of mobility.

Latex 1, possessing the lowest surface charge density, follows this scheme (Fig. 2a). The suspensions 2–5 show a different behavior, comparable to that of the polystyrene standard C with tentacles on the surface. With the first addition of NaCl, the mobility is constant or even decreases, due to a conformational change of the tentacles. The tentacles are stretched with low, and random coiled with high ionic strength of the surrounding medium. The effect of the tentacles' conformational changes on the mobility is greater than that of additionally adsorbed ions. The electrophoretic mobility maximum occurs delayed or does not take place. In the range of high ionic concentrations (10^{-2}–10^{-1} mol/l NaCl), the decrease of mobility is much slower as compared to the smooth particles, or is nearly constant. This is due to the fact, that the compression of the electrochemical bilayer does not occur to the same extent as for smooth particles, owing to the tentacles the shear plane at the diffuse double layer cannot measure up to the Helmholtz layer. It is assumed, that the tentacles of the latices 1–5 consist almost entirely (>90%) of styrenesulfonate units.

The low degree of surface hydrophobicity obtained with the Rose Bengal partitioning method, was in the same order of magnitude for all investigated particles (average of the slope: 8 ml/m^2). The constant surface hydrophobicity is evidence for the assumption, that the increase of the surface charge density of the latices 1–5 is due to an increasing tentacle length. An increase of the number of tentacles would cause a greater decrease in surface hydrophobicity. As a matter of fact, a higher surface charge density achieved by higher concentrations of Na-styrenesulfonate, probably comes down to longer and possibly less tentacles on the particle surface.

3.2. Protein adsorption

Fig. 3 shows typical 2-DE gels of human plasma (left), protein adsorption patterns on the particle suspensions with lowest (latex 1, middle) and highest surface charge density (latex 5, right). The gel of human plasma is given as a reference, major spots are indicated. Since albumin is the most abundant plasma protein, the gel is dominated by its expanded spot. In previous studies a preferential adsorption of proteins from human plasma could be shown, i.e. enrichment of certain proteins on particulate surfaces while others are diminished as compared to their concentration in the bulk medium [14–17,28,29]. Since the in vivo fate of intravenously administered nanoparticles is strongly influenced by interactions with blood components, enrichment of certain proteins might be able to direct the particles to specific target cells or tissues. In Fig. 4 the percentages of the major proteins detected on the 2-DE gels are compared. The proteins represent over 80% of the overall protein amount detected as being adsorbed on the particles. With increasing surface charge density in the interval from −3.7 to −8.2 μC/cm^2, the qualitative protein adsorption pattern did not change. Fibrinogen was by far the dominant protein of the adsorption patterns, followed by IgGγ and the Ig light chains. Apart from these proteins mediating the particle clearance by the MPS [33–35], albumin was found, and several apolipoproteins were detected as being preferentially adsorbed on the particles (i.e. increased compared to the presence in plasma). Increase in the total protein amount, expressed in cpm (Fig. 5), of about 40% with

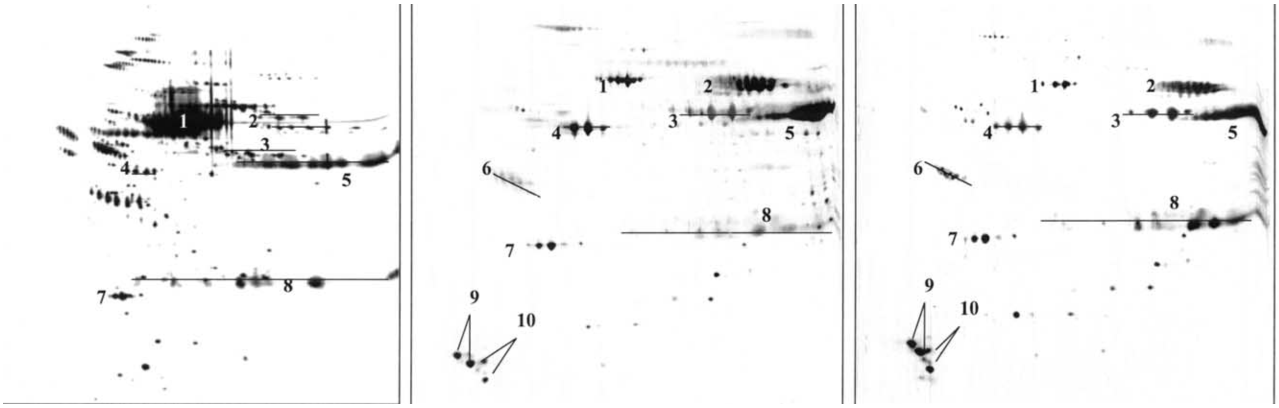

Fig. 3. 2-DE gels. Left, human plasma; middle, protein adsorption pattern on latex 1; right, protein adsorption pattern on latex 5. The entire gels are shown, pI 4.0–10.0 (from left to right, non-linear), MW 250–6 kDa (top to bottom, non-linear). (1) albumin, (2) fibrinogen α, (3) fibrinogen β, (4) fibrinogen γ, (5) IgGγ, (6) ApoJ, (7) ApoA–I, (8) Ig light chains, (9) ApoC-III, (10) ApoA-II.

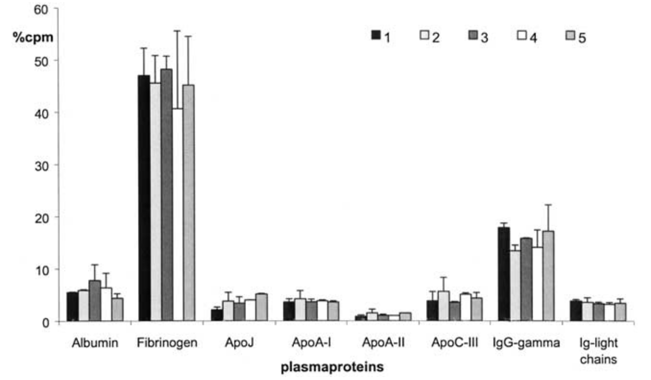

Fig. 4. Major proteins adsorbed on latices 1–5. The protein amounts are expressed in %cpm (percentage of the overall detected protein pattern), error bars represent the standard deviation ($n = 3$).

increasing surface charge density is mainly attributed to a higher protein adsorption in general. The qualitative composition of the adsorption pattern is identical, the quantitative composition shows no or very little change for most proteins (e.g. ApoA-I, C-III). That means increasing the number of charged groups (i.e. increase of the surface charge density), increases the total amount of adsorbed proteins, while the qualitative and quantitative composition of the pattern is less affected. The only exception is the greater increase in the amount of ApoJ (75%) that was detected beside the increase of other proteins adsorbed to the particles, which was in general in the range of about 35% (cpm data not shown). The greater increase of ApoJ adsorption is also reflected in its proportion of the total protein adsorption (Fig. 4). The amount of ApoA-II on latex 1 is only 0.8% and on latex 5 1.8%, which means the relative amount has doubled, but it has to be taken into account, that the percentage is less than the threshold of 1% normally chosen for proteins to be considered as relevant in the pattern.

The proportion of particular proteins with respect to the entire particle adsorption pattern might be of importance with regard to the in vivo behavior of the particles. The relevance of single proteins adsorbed on particulate surfaces possibly depends on amount and type of other proteins present on the surface. One might speculate about an influence of increasing ApoJ adsorption on the in vivo fate of the particles. Since proteins that promote the removal of the particles from systemic circulation are present on the surface in very high proportions, the in vivo fate should be influenced decisively by fibrinogen and IgG.

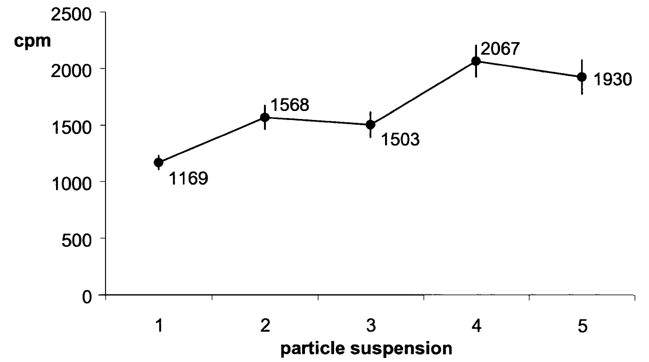

Fig. 5. Total protein amounts of the adsorption patterns detected on latices 1–5 expressed in arbitrary units (cpm). The values are the mean of three experiments, error bars represent the standard deviation.

The increase in protein adsorption is attributed to the increasing surface charge density of the particles, and may therefore be mainly driven by Coulomb forces and less by van der Waals interactions with the core material. Since the surface hydrophobicity is in the same order of magnitude, the proteins seem to be attracted to the particles solely by the influence of the styrenesulfonate tentacles. The plasma proteins approaching will find highly loaded, strong acidic tentacles as an outer sphere of the particles. As proteins are still ampholytes, an association with the tentacles conditioned by charge seems to be probable, since it is even possible for negatively charged proteins (e.g. albumin) to adsorb on negatively charged surfaces due to co-adsorption of ions from solution that annihilate the antagonistic charge [36].

4. Conclusion

A range of model nanoparticles was synthesized to study the influence of the surface charge density on protein adsorption. Detailed physicochemical characterization was followed by qualitative and semi-quantitative analysis of the protein adsorption using 2-DE. A dependence of protein adsorption on the particle surface charge density was shown: the higher the particle surface charge density, the more proteins are adsorbed. In the range of increasing surface charge, no qualitative change of protein adsorption was detected. A distinct protein adsorption pattern staying constant with moderate change of charge density, should be useful for the design and production of variable parenteral products, e.g. particulate drug carriers (nano- and microparticles), implants or artificial organs. The results show as well, that 2-DE for the analysis of protein adsorption should be a useful tool for the design of these devices getting in contact with body proteins. That means improving biocompatibility of the surface by controlled surface modification avoiding or minimizing adsorption of body proteins triggering an immune response. The knowledge about protein adsorption behavior and the subsequent body distribution would be an undeniable advantage for the development of carriers for site-specific drug delivery. Data from experiments as presented in this study could serve as a basis for the establishment of these correlations.

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