ELSEVIER

European Journal of Pharmaceutics and Biopharmaceutics 49 (2000) 41–46

European Journal of Pharmaceutics and Biopharmaceutics

www.elsevier.com/locate/ejphabio

Research paper

Adsorption kinetics of plasma proteins on oil-in-water emulsions for parenteral nutrition

Stephan Harnisch, Rainer H. Müller*

Department of Pharmaceutics, Biopharmaceutics and Biotechnology, The Free University of Berlin, Berlin, Germany

Received 2 February 1999; accepted in revised form 7 September 1999

Abstract

The plasma protein adsorption patterns on colloidal drug carriers are regarded as an important factor for their in vivo fate. In this study the adsorption kinetics on oil-in-water emulsions were determined and compared to the adsorption kinetics on polystyrene particles. In addition, the adsorption kinetics on the same systems after surface-modification with a hydrophilic polymer were also investigated. The protein adsorption was determined by means of two dimensional polyacrylamide gel electrophoresis (2D–PAGE). The determination of the plasma protein adsorption kinetics was carried out using different concentrations of human plasma in the incubation medium to prolong the residence time of the more abundant plasma proteins on the surface. Proteins which are likely to be displaced in a split second are thus accessible to analysis. The oil-in-water emulsion showed a distinctly different adsorption behavior from the one previously described for solid surfaces, where initially adsorbed proteins are displaced by others, having a higher affinity to the surface ('Vroman effect'). No competitive protein adsorption could be observed on the emulsions. Moreover, the predominantly adsorbed apolipoproteins A-I, A-IV, C-II and C-III increase in amount with increasing plasma concentration. The knowledge of the adsorption kinetics of colloidal carriers might be helpful for a better understanding of the in vivo behavior of such systems and for the transfer of principles already known from other carrier systems to the controlled development of emulsions for site specific drug delivery.

Keywords: Two-dimensional electrophoresis; Protein adsorption; Adsorption kinetics; Fat emulsions; Apolipoproteins; Poloxamer 407

1. Introduction

Egg lecithin-stabilized soy oil-in-water emulsions are applied to intensive care patients for parenteral nutrition and are employed as carriers for drugs. The in vivo fate of the emulsion droplets is determined by the physicochemical surface properties (e.g. surface hydrophobicity, charge, nature of functional groups) which affect the protein adsorption patterns on the colloidal carriers [1–3]. Distribution in the body can occur very fast, e.g. recognition by the macrophages of the liver. About 90% of particles recognized by the body as foreign are taken up within the first five minutes after injection [4,5]. Knowledge of the composition of the protein adsorption pattern and its relation to the organ distribution is a prerequisite to utilize parenteral emulsions as drug carriers with high tissue specificity. Due to the fact that the distribution in the body takes place very fast, it is very important to gain knowledge about the proteins adsorbing within the first seconds or minutes on the emulsion droplets. Polystyrene particles have been used to establish two-dimensional polyacrylamide gel electrophoresis (2D PAGE) for the analysis of protein adsorption patterns on particles incubated in human plasma [6–8]. It could be shown that the adsorption patterns depend on the physicochemical surface properties such as hydrophobicity [9]. Time dependence of the adsorption pattern could be shown by kinetics studies [6,10].The adsorption pattern of proteins on solid surfaces has to be regarded as the product of a sequence of adsorption of abundant proteins and their displacement by less abundant proteins with a higher affinity to the investigated surface ('Vroman effect') [11–13].

Blunk et al. [10] demonstrated the displacement of fibrinogen and albumin adsorbed on model particles by less abundant proteins employing strongly diluted plasma. The dilution produces a prolonged residence time of the abundant proteins on the surface, as the concentration of the lower abundance proteins showing a higher affinity to the surface is significantly decreased [14].

This method allows investigation of the protein adsorption within the first seconds or minutes of the incubation, even if particle and droplet separation, respectively, from

* Corresponding author. Department of Pharmaceutics, Biopharmaceutics and Biotechnology, The Free University of Berlin, Kelchstrasse 31, D-12169 Berlin, Germany. Tel.: +49-30-7700-0478; fax: +49-30-7700-047.
E-mail address: mpharm@zedat.fu-berlin.de (R.H. Müller)

0939-6411/00/$ - see front matter
PII: S0939-6411(99)00064-8

excess plasma is as time consuming as in the case of an emulsion [15].

The 2D PAGE technique was previously modified and successfully transferred to oil-in-water emulsions [15]. In this study the adsorption kinetics on emulsions were investigated and the adsorption patterns obtained were compared to those of polystyrene particles of similar size.

2. Materials and methods

2.1. Carrier systems

Two different systems were investigated for protein adsorption kinetics studies. The commercially available oil-in-water emulsion Lipofundin MCT 20%, batch No. 5512A82 (B. Braun Melsungen AG, Germany) was used to investigate the adsorption kinetics on emulsion droplets. The emulsion contained 20% oil, being a 1:1 mixture of long chain triglycerides (LCT) and medium chain triglycerides (MCT), stabilized by egg lecithin.

The second system was a suspension of polystyrene particles (aqueous suspension, 2.69% solid content (w/v)).

The mean diameter of the polystyrene particles (250 nm) and the main droplet population of the oil-in-water emulsion (280 nm) were similar, thus size was expected to have little influence on the adsorption behavior. The polystyrene particles were prepared at the Fraunhofer Institute for Applied Polymer Research (Teltow, Germany).

2.2. Surface modification

The polystyrene particles and the emulsion droplets were surface-modified by adsorption of poloxamer 407. Poloxamer is a ABA block copolymer, consisting of polyoxyethylene (A) and polyoxypropylene (B). Poloxamer 407 (Synperonic F127) was obtained from ICI Surfactants, England.

For modification, four parts of a 5% (w/w) solution of poloxamer were mixed with one part of the 2.69% (w/v) polystyrene particle suspension and incubated at room temperature for 24 h [6]. To obtain the same polymer to surface area ratio for the emulsion droplets a solution containing 10% (w/w) poloxamer 407 was used. One part of the emulsion (20% oil phase (w/w)) was mixed with 16 parts of the poloxamer solution and incubated as with the polystyrene particles.

After incubation the systems were centrifuged at 15 000 × *g* and excess polymer solution was removed by washing with doubly-distilled water. Two washing steps were performed [15]. After the second washing step emulsion and latex suspension were diluted to the original concentration. No increase in particle size of either colloidal system was determined (PCS data not shown).

2.3. Sample preparation

Different dilutions of citrate stabilized human plasma were used to simulate the early stages of protein adsorption onto the colloidal carriers.

With regard to the considerable difference in surface area, the amount of plasma per unit surface area during the incubation was adjusted due to the approx. six fold higher amount of dispersed phase in the emulsion compared to the particle suspension. According to Blunk [10] dilutions of 0.08, 0.8 and 80% plasma were used for the polymeric particles. Taking the different surface areas into account the alignment to the emulsion surface led to values of 1.1, 11 and, due to the feasibility, a maximum of 75% plasma in the dilution. This maximum is explained by the emulsion to plasma ratio of one part emulsion and three parts plasma used for routine analysis.

Incubation of the samples took place at 37°C for 5 min. Separation from excess plasma by centrifugation and the following three washing steps with phosphate buffer pH 7.4 were performed according to [15,16], i.e. the plasma and surfactant solution respectively, was removed by use of a syringe and the emulsion droplets were redispersed in the washing medium. The adsorbed proteins were desorbed by adding a solution containing 10% (w/v) SDS according to [7,17].

For the first dimension immobilized non-linear pH gradients ranging from 3.5 to 10 (Amersham Pharmacia, Sweden) were used. Sample entry was performed by in-sample-rehydration in a custom-made reswelling tray [15,18–20].

All chemicals, devices and running conditions are described elsewhere [6,7,15]. Silver staining of the proteins was performed according to Bjellquist et al. [17]. After scanning of the gels with a laser densitometer the images were analyzed using the Melanie II software (Bio-Rad, Germany). The protein spots were identified by matching the gels to the master map of human plasma which is accessible in the SWISS-PROT database (www.expasy.ch) [21].

3. Results and discussion

3.1. Adsorption kinetics on polystyrene model particles

For solid surfaces Vroman et al. [11] reported that proteins present in plasma in high concentrations are the first to adsorb on the surface. Subsequently they will be replaced by proteins having a higher affinity to the surface but being present in lower concentrations. The replacement of one protein species by another might take place in a split second.

The polystyrene particles with a diameter of 250 nm were incubated in parallel to the emulsion droplets. Fig. 1 shows the amounts of the major proteins involved in the sequence

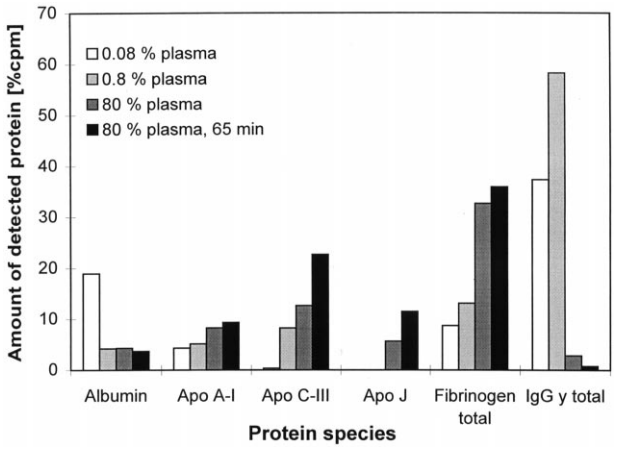

Fig. 1. Amounts of the major adsorbed plasma proteins on polystyrene particles (size 250 nm). CPM (counts per min) is an arbitrary unit for the amount of protein, generated by the evaluation software 'Melanie II'.

of adsorption and desorption. When regarding the adsorption pattern from the dilution containing 0.08% plasma, which corresponds to the very first adsorption phase, an initially high amount of adsorbed albumin seems to be almost replaced by immunoglobulin (Ig) G when compared with the sequence of Vroman and Adams [12]. At the second concentration (0.8% plasma), IgG adsorption increased strongly, while the amount of albumin decreased markedly. At 80% plasma, IgG adsorption strongly decreased while the amount of apoC-III, apoJ and particularly that of fibrinogen increased.

These data confirmed ,in principle, the findings of Vroman and Adams [12] and those of Blunk [10] and will serve in the further discussion as comparison for the kinetics obtained for the oil-in-water emulsion.

3.2. Adsorption kinetics on lecithin stabilized emulsion droplets

The emulsion was incubated in 1.1 and 11% plasma solutions to maintain the same ratio of plasma volume to surface area as used for the polymeric particles. The maximum plasma concentration in the incubation medium reached 75%. In contrast to the solid surface of polymeric model particles, no protein adsorption on emulsion droplets occurred when incubated in a solution of 1.1% plasma and only a few proteins adsorbed during incubation in 11% plasma. Distinct protein adsorption could be detected after incubation in the dilution of 75% plasma. Therefore, intermediate concentrations of 16.5, 22, 33 and 55% of plasma were used as incubation media. At concentrations above 11% the total amount of adsorbed proteins steadily increased (80–1548 cpm).

Fig. 2 shows the 2D gels obtained from incubation with 11.0, 22.0, 33.0 and 75% plasma solution.

The apolipoproteins represented the main type of adsorbed proteins. They accounted for 47.4–68.25% of the overall detected protein on the 2D gels. ApoC-III was the most dominant apolipoprotein; apoA-I, apoA-IV and apoC-II were also present in a relatively high quantity. In addition, IgD δ chain and IgG γ chain were detectable in considerable amounts. The above mentioned proteins were already detectable at the lowest concentration of 11% plasma in the incubation medium.

With increasing plasma concentration few qualitative changes in the adsorption patterns occurred, but gradually more protein spots were detected. The amount of the major proteins increased steadily whilst their percentage, related to the overall amount of adsorbed proteins, remained nearly unchanged (Fig. 3).

As is clearly visible, the adsorption kinetics are not similar to those on solid surfaces. Proteins being present at very high concentrations in the bulk plasma, e.g. albumin, fibrinogen and IgG, showed no preferential adsorption at the beginning of the adsorption process and were present in minor fractions on the 2D pattern even at the highest plasma concentration.

Arai and Norde [22] classified proteins as soft and hard proteins. The soft proteins, easily unfolding on surfaces, will displace the hard ones. The hard proteins, e.g. albumin, show interactions with the surface too weak to compete with any of the apolipoproteins.

Another suggestion is the penetration of protein molecules into the layer of lecithin molecules followed by direct adsorption on the oil droplet surface [23]. It has been described that apolipoproteins, containing multiple α-helical segments which exhibit extensive changes when approaching lipid surfaces, adsorb with high affinity [24]. Apolipoproteins show a relatively flexible molecular structure [25], able to change their conformation readily when adsorbing on surfaces [26]. This ability results in less non-polar residues remaining in contact with water compared to the original state of the protein. The organization of the α-helices in the interface seems to be the decisive property of the proteins for the effective removal of these non-polar residues, which cannot be achieved e.g. in the albumin molecule [23].

A possible conclusion based on the obtained adsorption kinetics is that the intensity of the interaction of the apolipoproteins with the oil-water interface prevents, or at least reduces, the adsorption of other protein species. Even the short residence time might be sufficient for the change in conformation, leading to this lasting adsorption.

3.3. Adsorption kinetics after surface modification of the polystyrene particles and emulsion droplets

The organ distribution of emulsions in the body can be changed by surface modification of the emulsion system [27]. A very simple way of modification is the exchange of lecithin by hydrophilic block copolymers such as poloxamer 188 [27] or poloxamine 908 [28]. The hydrophilic block copolymers lead to reduced uptake of emulsions by

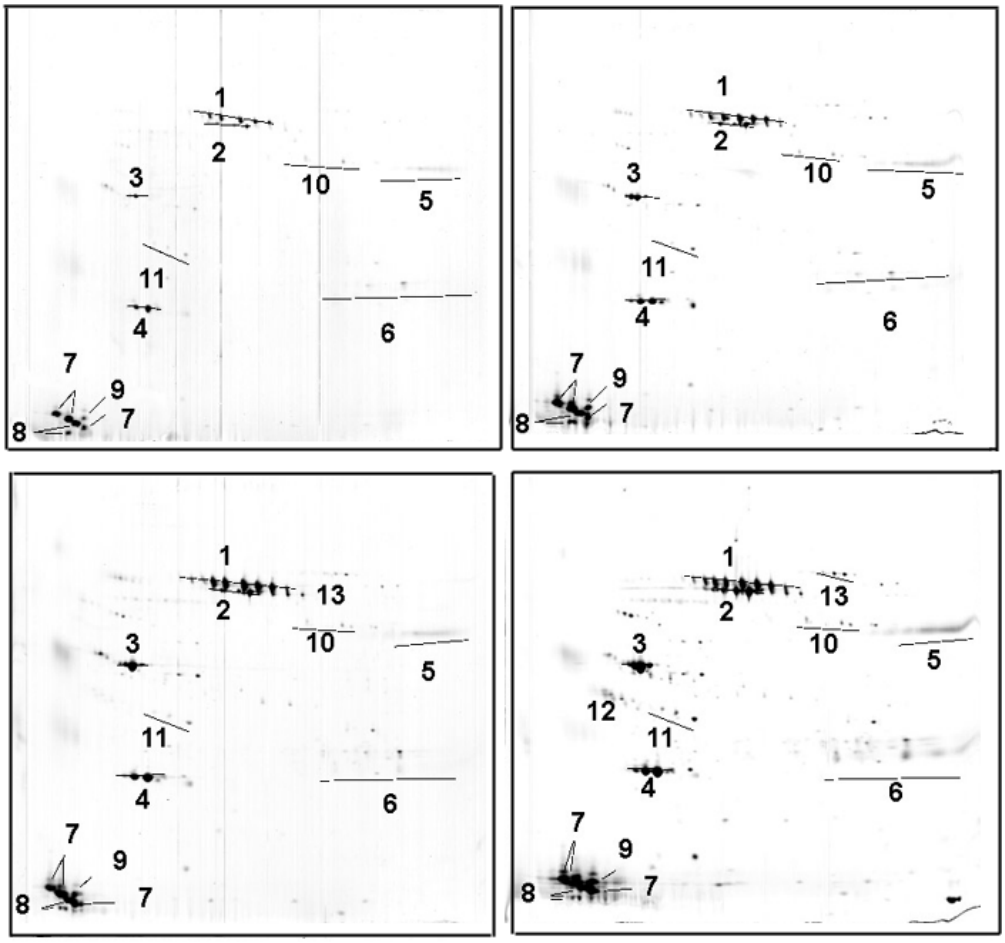

Fig. 2. Protein adsorption pattern on Lipofundin MCT 20% oil-in-water emulsion. Plasma concentration in the incubation media: top left, 11%; top right, 33%; bottom left, 55%; bottom right, 75%. (Original size 16 × 16 cm) 1: Immunoglobulin D δ chain; 2: Albumin; 3: Apolipoprotein (Apo) A-IV; 4: Apo A-I; 5: Immunoglobulin γ heavy chains; 6: Immunoglobulin light chains; 7: Apo C-III; 8: Apo C-II; 9: Apo A-II fragment; 10: Apo H; 11: Apo E; 12: Apo J; 13: Complement factor C3 (β-chain).

the macrophages of liver and spleen and finally to a prolonged circulation time in the blood [27]. To determine a possible effect of surface modification on the protein adsorption pattern, the polymeric reference particles and the lecithin emulsion were surface-modified by adsorption of poloxamer 407. This modification is achieved by incubation of the particles and the emulsion in a poloxamer 407 solution.

The surface-modified systems showed different adsorption kinetics compared to the non-modified systems (Fig. 4). The total amount of proteins adsorbed on the surface was diminished in both systems and the reduction in protein adsorption affected predominantly the larger proteins, e.g. immunoglobulins, fibrinogen or albumin. The poloxamer seemed to shield the surface from the adsorption of larger proteins, thus, mainly proteins with a smaller molecular weight were present in the 2D-pattern. Since the poloxamer covers only parts of the surface, absolute protection against protein adsorption cannot be achieved. This is in accordance with previous studies in this field [10,14].

The surface-modified emulsion again showed a different adsorption behavior compared to the polymeric particles. Increasing plasma concentration was followed by an increasing protein adsorption. In contrast to the non-modified lecithin emulsion, adsorption of proteins was already detected at the very low plasma concentration of 1.1% in the incubation medium (Fig. 5, left). This surprising effect might be attributed to the polyethylene oxide chains of poloxamer, as similar effects are stated in the literature [29].

The first protein adsorbed was apoA-IV; at the higher concentration of 11% more protein species adsorbed (apoA-I, A-II, C-II and C-III) (Fig. 5, middle). Adsorption of apoE and also albumin on the emulsion droplets was detected additionally at a concentration of 75% plasma in the incubation medium (Fig. 5, right). Adsorption of the immunoglobulin D δ chain was completely prevented by the poloxamer layer, this is also one of the major qualitative differences of the two emulsion systems. Comparison with the adsorption kinetics obtained from the unmodified emulsion shows similarities. Although, the amounts of the

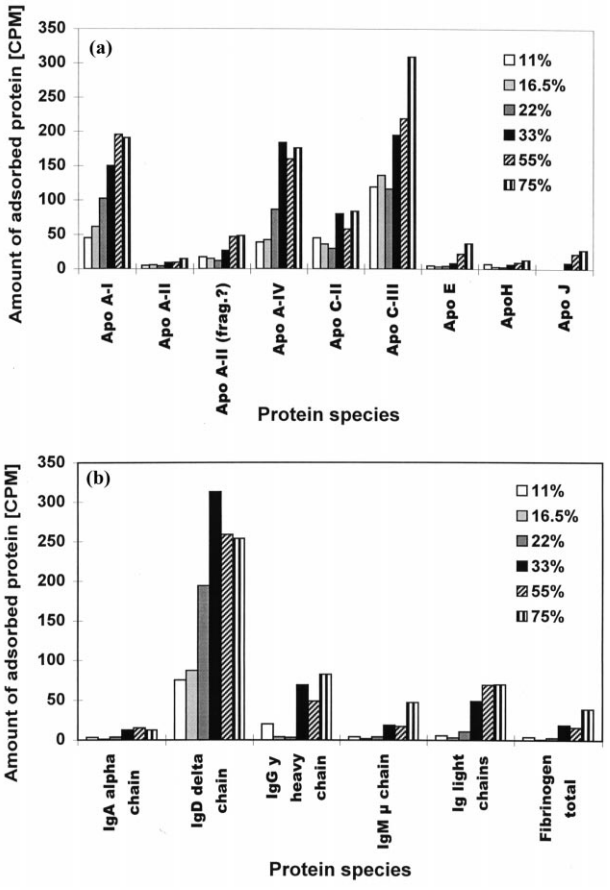

Fig. 3. Amount of major proteins on the 2D gels of plasma proteins adsorbed on Lipofundin MCT 20%, an o/w emulsion for parenteral nutrition. Part (a) shows the increasing adsorption of apolipoproteins with increasing plasma concentration in the incubation medium, part (b) shows the increasing adsorption of certain immunoglobulins and fibrinogen.

adsorbed protein species had changed, the steady increase in adsorption at increasing plasma concentrations remained the same. As ascertained for the unmodified emulsions no desorption of proteins was observed.

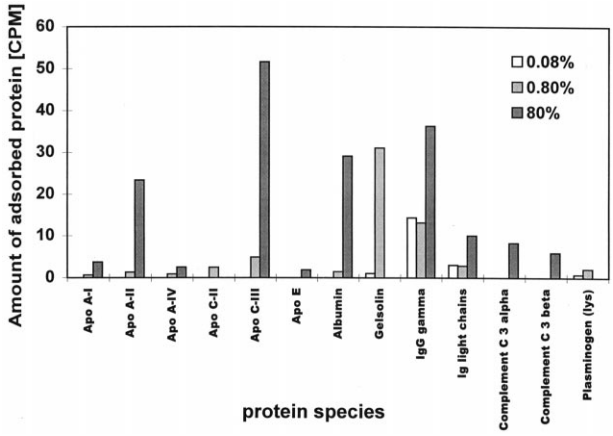

Fig. 4. Amount of major proteins on the 2D gels of plasma proteins adsorbed on poloxamer 407 modified polystyrene particles (diameter 250 nm) in cpm on the gels. Amounts of smaller protein species increased with increasing plasma concentration in the incubation medium (e.g. apolipoproteins), whereas larger proteins decreased in amount (e.g. fibrinogen).

4. Conclusions

The kinetics of protein adsorption on solid polymeric particles are different to those on emulsions. No protein desorption from the emulsion droplets was observed. The differences in the adsorption kinetics can be explained by the different nature of the systems and thus the different binding facilities of the proteins to the surface, that means first of all special arrangements of proteins within the lecithin layer adsorbing to the oily core.

The protein adsorption pattern can be distinctly changed by simple adsorption of surface active molecules onto the oil droplets. This means that alterations in the surface properties will lead to alterations in the adsorption kinetics. A change in kinetics can be different for each single protein. The less pronounced time dependent changes in the adsorption pattern on the emulsion is of high interest for controlled delivery. The adsorption pattern is more stable and may therefore be better utilized for site specific delivery than

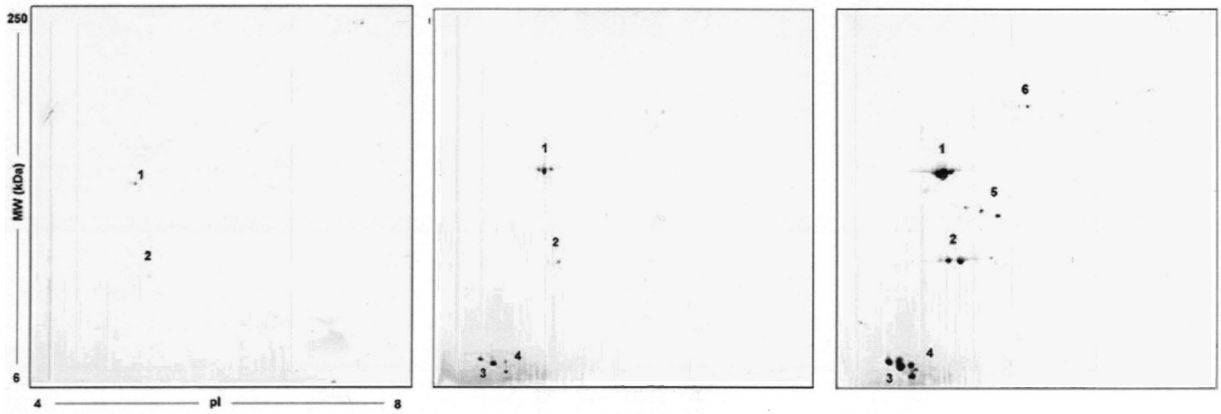

Fig. 5. 2D gels of the adsorbed plasma proteins on poloxamer 407 surface-modified Lipofundin MCT 20%. Plasma concentrations of 1.1% (v/v) (left), 11.0% (v/v) (middle) and 75% (v/v) (right) were used for a 5 min incubation. 1: Apolipoprotein (apo)A-IV; 2: apoA-I; 3: apoC-III; 4: apoA-II; 5: apoE; 6: Albumin.

an adsorption pattern being very dependent on contact time with proteins, especially for systems with a long circulation time in the blood.

Acknowledgements

The authors would like to thank B. Braun Melsungen AG, Germany, and Dr. B.R. Paulke, Fraunhofer Institute for Applied Polymer Research, for kindly providing us the carrier systems and Dr. T. Blunk, MIT, Cambridge, USA, for the helpful scientific discussions. The work in the 2D PAGE laboratory was supported by a grant from the Deutsche Forschungsgemeinschaft. Special thanks are expressed to A. Gessner for putting the finishing touches to this paper.

References

[1] S.S. Davis, P.K. Hansrani, The evaluation of parenterally administered emulsion formulations, in: C.G. Wilson, J.G. Hardy, M. Frier, S.S. Davis (Eds.), Radionucleoide imaging in drug research, Croom Helm, London, 1982.

[2] S.S. Davis, P.K. Hansrani, The influence of emulsifying agents on the phagocytosis of lipid emulsions by macrophages, Int. J. Pharm. 23 (1985) 69–77.

[3] R.H. Müller, Colloidal carriers for controlled drug delivery and targeting – modification, characterization and in vivo distribution, Wissenschaftliche Verlagsgesellschaft Stuttgart, CRC Press, Boca Raton, FL, 1991.

[4] T.R. Billiar, R.D. Curran, Kuppfer cell and hepatocyte interactions a brief overview, J. Parenter. Enteral Nutr., 14 (Suppl.) (1990) 175S-180S.

[5] H.M. Patel, Serum opsonins and liposomes: their interaction and opsonophagocytosis, Crit. Rev. Ther. Drug Carr. Syst. 91 (1992) 39–90.

[6] T. Blunk, Ph.D. thesis, University of Kiel, 1994.

[7] T. Blunk, D. Hochstrasser, J.-C. Sanchez, B.W. Müller, R.H. Müller, Colloidal carriers for intravenous drug targeting: Plasma protein adsorption patterns on surface-modified latex particles evaluated by two-dimensional polyacrylamide gel electrophoresis, Electrophoresis 14 (1993) 1382–1387.

[8] J.-C. Leroux, P. Gravel, L. Balant, B. Volet, B.M. Anner, E. Allemann, E. Doelker, R. Gurny, Internalization of poly (D,L-lacitc acid) by isolated human leukocytes and analysis of plasma proteins adsorbed onto the particles. J. Biomed. Mat. Res, 28 (1994) 28 471-481.

[9] M. Lück, B.-R. Paulke, W. Schröder, T. Blunk, R.H. Müller, Analysis of plasma protein adsorption on polymeric nanoparticles with different surface characteristics, J. Biomed. Mat. Res. 39 (1997) 478–485.

[10] T. Blunk, M. Lück, A. Calvör, D.F. Hochstrasser, J.C. Sanchez, B.W. Müller, R.H. Müller, Kinetics of plasma protein adsorption on model particles for controlled drug delivery and drug targeting, Eur. J. Pharm. Biopharm. 42 (1995) 262–268.

[11] L. Vroman, A.L. Adams, G.C. Fischer, P.C. Munoz, Interaction of high molecular weight kininogen, factor XII and fibrinogen in plasma at interfaces, Blood 55 (1980) 156–159.

[12] L. Vroman, A.L. Adams, Adsorption of proteins out of plasma and solutions in narrow spaces, Colloid Interface Sci 111 (1986) 391–402.

[13] J.L. Brash, Protein adsorption at the solid-solution interface in relation to blood material interactions, in: J.L. Brash, T.A. Horbett (Eds.), Proteins at interfaces, Am. Chem. Soc, Washington D.C., USA, 1987, pp. 490–506.

[14] T.A. Horbett, C.M. Cheng, B.D. Ratner, S.R. Hanson, A.S.J. Hoffman, The kinetics of baboon fibrinogen to polymers: In vitro and in vivo studies, J. Biomed. Mater. Res. 20 (1986) 455–465.

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

[16] K. Thode, Ph.D. thesis, Free University of Berlin, 1996.

[17] B. Bjellquist, C. Pasquali, F. Ravier, J.-C. Sanchez, D.F. Hochstrasser, non-linear immobilized pH gradient for two-dimensional electrophoresis and its definition in a relevant pH scale Electrophoresis 14 (1993) 1357–1365.

[18] T. Rabilloud, C. Valette, J.J. Lawrence, Sample application by in-gel rehydration improves the resolution of two-dimensional electrophoresis with immobilized pH gradients in the first dimension, Electrophoresis 15 (1994) 1552–1558.

[19] J.C. Sanchez, V. Rouge, M. Pisteur, F. Ravier, L. Tonella, M. Moosmayer, M.R. Wilkins, D.F. Hochstrasser, Improved and simplified in-gel sample application using reswelling of dry immobilized pH gradients, Electrophoresis 18 (1997) 324–327.

[20] S. Harnisch, R.H. Müller, Analysis of plasma protein adsorption on commercial emulsions for intravenous parenteral nutrition, Second world congress on emulsions at Bordeaux 1997, Congress Proceedings 3 (No. 4-2-199) (1997).

[21] R.D. Appel, J.C. Sanchez, A. Bairoch, O. Golaz, M. Min, J.R. Vargaz, D.F. Hochstrasser, SWISS-2D–PAGE: a database of two-dimensional gel electrophoresis images, Electrophoresis 14 (1994) 1232–1238.

[22] T. Arai, W. Norde, The behavior of some model proteins at solid-liquid interfaces 2, Sequential and competitive adsorption, Colloids Surfaces 51 (1990) 186–216.

[23] M.C. Phillips, Interactions of apolipoproteins at interfaces, in: M. Rosseneu (Ed.), Structures and Function of Apolipoproteins, CRC Press, Boca Raton, 1992, pp. 186–216.

[24] A. Jonas, Lipid Binding Properties of Apolipoproteins, in: M. Rosseneu (Ed.), Structures and Function of Apolipoproteins, CRC Press, Boca Raton, FL, 1992, pp. 217–250.

[25] G. Camejo, G. Colacicco, D. Rapport, Lipid monolayers: interactions with the apoprotein of high density plasma lipoprotein, J. Lip. Res. 9 (1968) 562.

[26] D.J. Graham, M.C. Phillips, Proteins at liquid interfaces, I. Kinetics of adsorption and surface denaturation, J. Colloid and Interface Sci. 70 (1979) 403.

[27] R. Jeppson, S. Rössner, The influence of emulsifying agents and of lipid soluble drugs on the fractional removal rate of lipid emulsions from the blood stream of the rabbit, Acta Pharmacol. Toxicol. 37 (1975) 134–144.

[28] L. Illum, P. West, C. Washington, S.S. Davis, The effect of stabilizing agents on the organ distribution of lipid emulsions, Int. J. Pharm. 54 (1989) 41–49.

[29] M. Lück, Ph. D. thesis, Free University of Berlin, Berlin, 1997.