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The role of plasma proteins in brain targeting: species dependent protein adsorption patterns on brain-specific lipid drug conjugate (LDC) nanoparticles

A. Gessner [a], C. Olbrich [a], W. Schröder [b], O. Kayser [a], R.H. Müller [a,*]

[a] *Department of Pharmaceutics, Biopharmaceutics and Biotechnologie, Free University of Berlin, Kelchstr. 31, D-12169 Berlin, Germany*

[b] *Department of Biochemistry, Free University of Berlin, Fabeckstr. 36a, D-14195 Berlin, Germany*

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Abstract

The in vivo organ distribution of particulate drug carriers is decisively influenced by the interaction with plasma proteins after i.v. administration. Serum protein adsorption on lipid drug conjugate nanoparticles, a new carrier system for i.v. application, was investigated by 2-dimensional electrophoresis (2-DE). The particles were surface-modified to target them to the brain. To assess the protein adsorption pattern after i.v. injection in mice prior to in vivo studies, the particles were incubated in mouse serum. Incubation in human serum was carried out in parallel to investigate similarities or differences in the protein patterns obtained from men and mice. Distinct differences were found. Particles incubated in human serum showed preferential adsorption of apolipoproteins A-I, A-IV and E. Previously, preferential adsorption of ApoE was reported as one important factor for targeting of Tween®80 modified polybutylcyanoacrylate nanoparticles to the brain. Preferential adsorption of ApoA-I and A-IV took place after incubation in mouse serum, adsorption of ApoE could not be clearly confirmed. In vivo localization of the LDC nanoparticles at the blood-brain barrier and diffusion of the marker Nile Red into the brain could be shown by confocal laser-scanning microscopy. Differences of the obtained adsorption patterns are discussed with regard to their relevance for correlations of in vitro and in vivo data obtained from different species.

Keywords: Nanoparticles; Protein adsorption; Two-dimensional electrophoresis; Drug targeting; Trypanosomiasis

Colloidal drug carriers are used as an approach to controlled drug delivery and drug targeting to specific body organs. The organ distribution is strongly influenced by interactions with blood components. Carriers with different protein adsorption patterns acquired after i.v. administration will interact with different tissue specific receptors or will be recognized by different macrophage subpopulations (concept of 'differential adsorption', Müller and Heinemann, 1989).

* Corresponding author. Tel.: +49-30-77000478; fax: +49-30-770004101.

E-mail address: mpharma@zedat.fu-berlin.de (R.H. Müller).

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Depending on the particles surface properties, certain proteins are preferentially adsorbed after incubation in serum or plasma, i.e. some proteins are enriched on the particles surface while others are diminished, as compared to the bulk serum or plasma.

Analysis of the protein adsorption patterns is carried out by 2-dimensional electrophoresis (2-DE). 2-DE was previously established to determine plasma protein adsorption patterns on model nanoparticles for intravenous drug targeting (Blunk et al., 1993). The protein separation is carried out according to two independent parameters: the isoelectric point (pI) and the molecular weight (MW) of the proteins.

To analyze the protein adsorption on the particles, suspensions of the particles containing constant surface areas are incubated in the desired medium such as plasma or serum. The particles are separated from the bulk medium by centrifugation and washed (Blunk et al., 1993). The adsorbed proteins are desorbed by protein solubilizing solutions (Blunk et al., 1993), and the sample is applied to the 2-DE and processed like described previously (Bjellqvist et al., 1993; Harnisch and Müller, 1998). After 2-DE, the gels are silver stained and scanned with a laser densitometer. Spot identification takes place by comparison of the obtained 2-DE gels with reference maps. It is possible to identify so far unknown protein spots by N-terminal microsequencing, which is facilitated by enrichment of the proteins on particulate surfaces (Lück et al., 1997). The gel images are analyzed by employing an automated computer analysis system (Blunk et al., 1993; Harnisch and Müller, 1998).

In the present study, serum protein adsorption patterns on lipid drug conjugate (LDC) nanoparticles were investigated. In contrast to solid lipid nanoparticles (SLN), which release the drug by diffusion, drug release from LDC nanoparticles occurs and is controlled via matrix degradation. The particle matrix consists of stearic acid and a drug base, the emulsifier is Tween®80 (Olbrich et al., 2000). The drugs used are Diminazene and SIPI, a structural analogon to Diminazene (*p*,*p*′-diamidinodiazoaminobenzene). Both drugs are effective against *Trypanosoma brucei gambiense*/*rhodesiense*, the causal agents of trypanosomiasis, which are transmitted by the tsetse fly.

Trypanosomiasis is also called African sleeping sickness. The course of the disease is divided into two stages. At first, a trypanosomal chancre is developed at the site of the insects' bite, followed by the haemolymphatic stage (stage I). Subsequently, after invasion of the central nervous system (CNS), the disease leads up to the meningoencephalitic stage (stage II), with symptoms like headache, somnolescence and later coma. If untreated, trypanosomiasis leads to death. A problem is the persistance of the trypanosomes in the CNS (stage II), that is why a penetration of the blood-brain barrier (BBB) is required for the treatment of trypanosomiasis. Most drugs for the treatment of this disease, e.g. Suramin, do not or only to a limited extent penetrate the BBB. Those penetrating the BBB are mostly neurotoxic, e.g. arsenicals such as Melarsoprol. The BBB consists of the endothelium of the brain vessels, the basal membrane and neuroglial cells. It is only permeable for small, lipid-soluble molecules. Larger molecules, e.g. regulators of the brain function such as transferrin and LDL, pass via active transport processes the BBB. One approach to penetrate the BBB is the use of intravenous injectable particulate drug carriers.

ApoE has been reported as a key factor for the BBB passage. Besides the liver as a major site of ApoE receptors, there are also ApoE receptors present at the BBB (Kim et al., 1996). An in vivo study previously carried out by Alyautdin et al. (1995), demonstrated the BBB passage of Dalargin using Dalargin-loaded polybutylcyanoacrylate (PBCA) nanoparticles modified with Tween®80. Dalargin is a hexapeptide, it possesses central analgesic activity, but it does not penetrate the BBB in a sufficient amount. A preferential adsorption of ApoE on these particles was shown by 2-DE (Lück, 1997; Müller et al., 1997). In this study, human plasma was used for the protein adsorption studies. The role of ApoE as key factor was verified by pre-adsorbing ApoE on the negative control used in the experiment by Alyautdin et al., i.e. non-modified Dalargin-

loaded PBCA particles with pre-adsorbed ApoE. An analgesic effect in mice was detected by the tail-flick test, i.e. the ApoE surface-modified particles delivered Dalargin to the brain. A pre-dosing with Naloxone lead to a non-analgesic effect, as expected.

In the present study, 2-DE was conducted for a pre-selection of promising nanoparticulate carriers for brain targeting, i.e. prediction of the behaviour of the nanoparticles after i.v. injection in mice. Therefore analysis of the protein adsorption was performed. The incubation media used were human and mouse serum. Mouse serum incubation was carried out to assess the protein adsorption pattern after i.v. injection in mice prior to in vivo studies. Incubation in human serum was carried out to investigate similarities or differences in the protein patterns obtained from the two species.

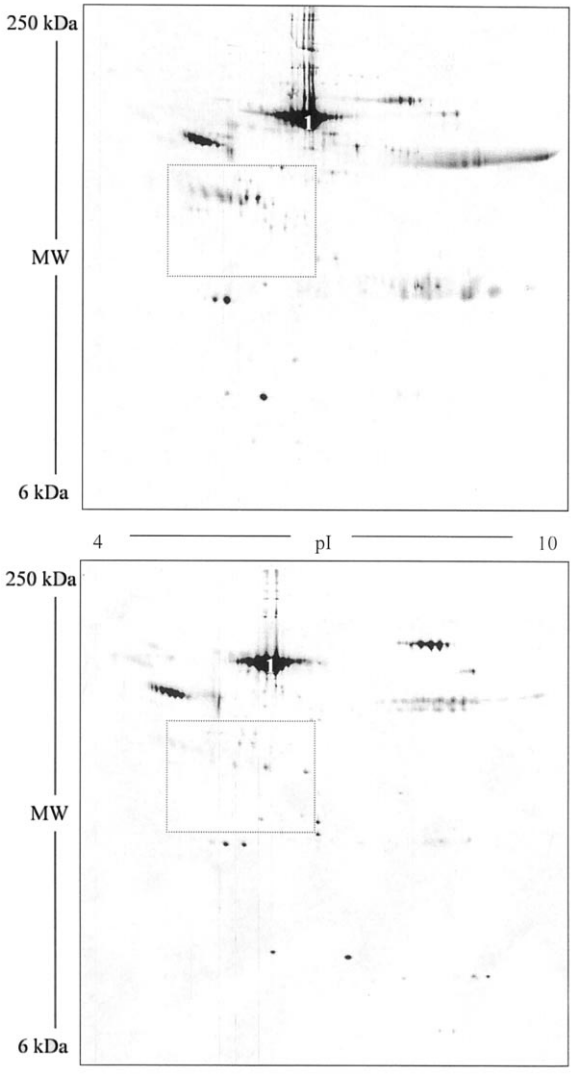

Fig. 1. 2-DE gels of the incubation media, upper: human serum, lower: mouse serum (1) albumin. Areas of different patterns are indicated.

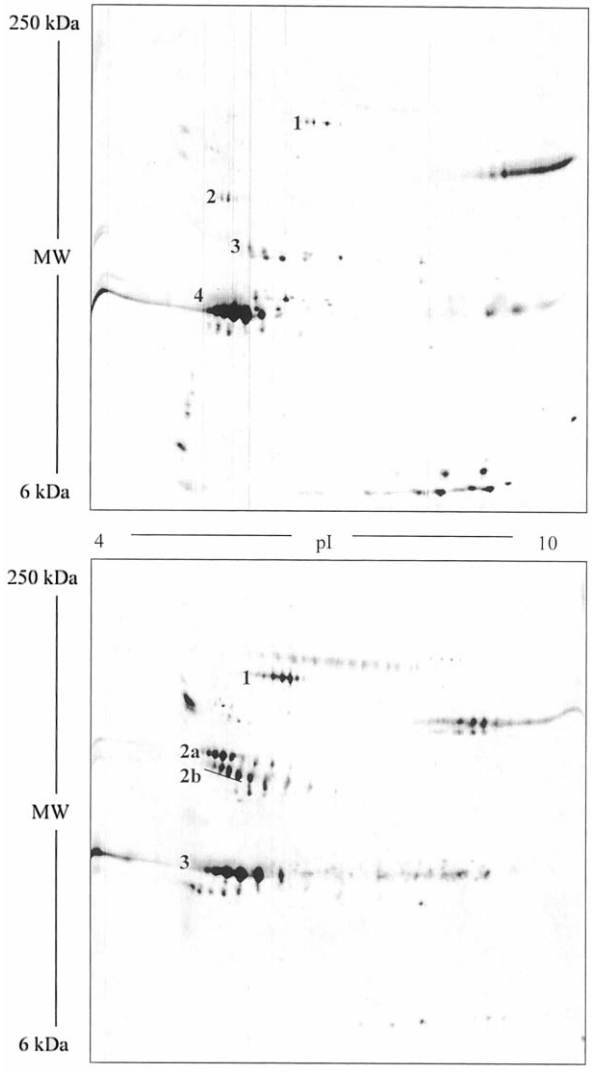

Fig. 2. 2-DE gels of serum proteins adsorbed on Diminazene-LDC nanoparticles, upper: human serum (1) albumin, (2) ApoA-IV, (3) ApoJ, (4) ApoA-I, lower: mouse serum (1) albumin, (2a) Apo-AIV, (2b) ApoA-IV, (3) ApoA-I.

Fig. 1 shows 2-DE gels of the two sera (upper: human, lower: mouse serum). Albumin is the most abundant protein in the two sera, it acounts to about 40% of the entire protein patterns. The most part of the human plasma proteins are identified on a reference map (Golaz et al., 1993), but there is no reference map of mouse plasma or serum. The gels of sera from the different species are comparable only to a limited extend, as demonstrated in the indicated areas of the two gels. Therefore N-terminal microsequencing was used for identification of mouse serum proteins of interest.

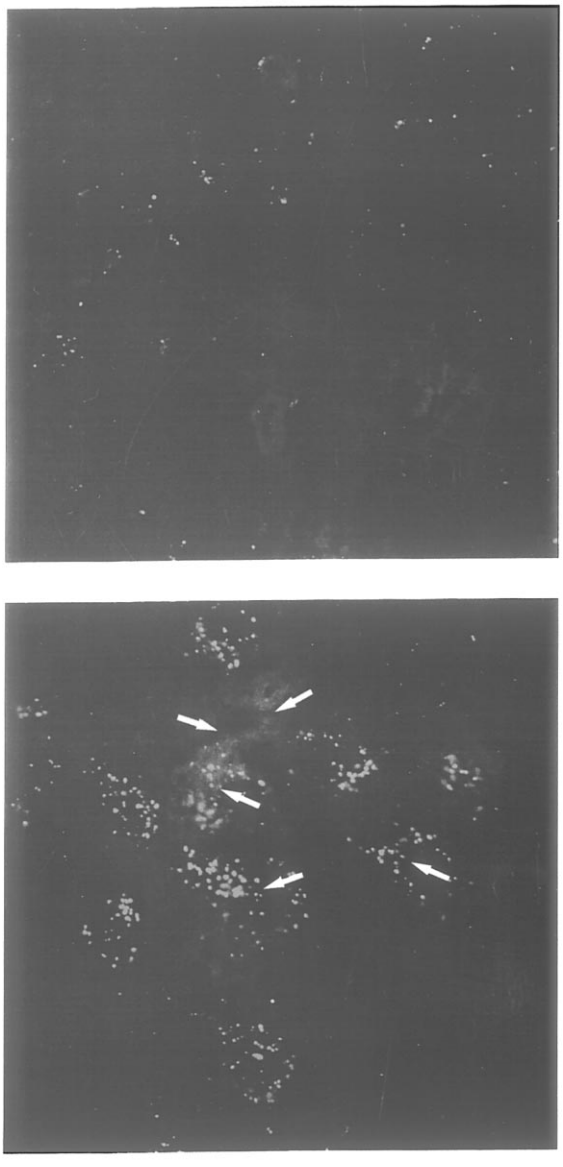

Fig. 3. CLSM of mouse brain tissue, upper: control, lower: after i.v. injection of Nile Red-labeled LDC nanoparticles. Arrows indicate the nanoparticles adhering to the endothelial cells of the brain vessels and the diffusion of the dye into the brain tissue.

Fig. 2 shows the protein adsorption patterns on Diminazene-LDC nanoparticles after incubation in human (upper) and mouse serum (lower). After incubation in human serum, ApoA-I accounts to about 50% of the entire adsorption pattern. There is also an enrichment of ApoE (about 4%) and ApoA-IV (about 1%) on the particles surface, both proteins are not visible on the gel of bulk serum.

After incubation in mouse serum, ApoA-I accounts to over 30% of the entire adsorption pattern. In this case, the identity of this protein was verified by N-terminal micosequencing. The other protein strongly enriched on the particles surface is ApoA-IV. The chain of spots located directly under ApoA-IV (2a), has also been identified by protein sequencing as Apo-AIV (2b), they account together for about 20%. ApoE could not be identified reliable yet. This might be due to the fact, that the protein concentration is too low for unambiguous protein sequencing and other protein spots overlap ApoE. Similar results were obtained with SIPI- and Nile Red-labeled LDC nanoparticles.

Strong adsorption of the Apolipoproteins A-I and A-IV after incubation in human plasma, was also reported for the protein adsorption on the Tween®80 modified Dalargin-loaded PBCA nanoparticles penetrating the BBB (Lück, 1997). The question arises about the role of both proteins on the transport across the BBB. A former in vitro study by Bisgaier et al. (1989) showed that pre-adsorbed ApoE facilitated the uptake of liposomes by hepatocytes. An additional adsorption of ApoA-I or A-IV reduced the hepatocyte uptake. Therefore, a modulation by ApoA-I and A-IV, enabling the contact of large amounts of the nanoparticles with ApoE receptors at the BBB prior to hepatic uptake in vivo, is conceivable.

First results of in vivo studies with mice showed Nile Red-labeled LDC nanoparticles adhering to

the endothelial cells of the brain vessels. Fig. 3 shows pictures obtained by confocal laser-scanning microscopy (CLSM) of the mouse brain tissue. The particles adhering to the endothelium of the brain vessels are clearly visible, as well as the dye diffusing into the brain tissue. These findings support the theory of reduced hepatic uptake of the nanoparticles prior to their contact with the BBB.

In conclusion, this study showed that the incubation of nanoparticles in sera of different species results in different adsorption patterns. These differences are difficult to estimate, especially when there is no reference as in the case of mouse plasma. This has to be taken into account, when the data is to be correlated with in vivo experiments. Therefore, the adsorption studies should be carried out in the appropriate medium, in addition to the standard studies in human serum or plasma.

The ability of BBB passage by a sufficient amount of drug loaded nanoparticles is not only due to adsorption of ApoE. It is probably the 'team-work' of apolipoproteins like A-I and A-IV that prevent the nanoparticles from hepatic uptake prior to their contact with the ApoE receptors at the BBB. Further in vivo studies with mice suffering trypanosomiasis will have to show the efficacy of the LDC nanoparticles.

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